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CONSTRUCTION AND EVALUATION OF HIGH-CAPACITY ADENOVIRAL VECTORS FOR GENE THERAPY APPLICATIONS

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To my brother Luca,
my best friend

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I. ABSTRACT

Gene therapy present a great therapeutic potential for a diversity of liver disorders including inherited metabolic conditions (phenylketonuria, tyrosinemia) and acquired diseases (chronic infections, primary and metastatic tumors). For many of these applications, vectors allowing prolonged, regulable and tissue-specific expression of the transgene would be required.

Adenovirus is the most widely used vector in human clinical assay. To avoid the cellular immune response induced by first and second generation Ad, the third-generation vectors was generated, also called gutless or helper-dependent. To produce these vectors requires three basic elements: a gutless adenovirus with a therapeutic or marker gene of interest, a helper adenovirus which provide viral proteins *in trans* and, a cell line permissive for Ad production. Gutless adenovirus does not contain any viral region, not cellular immune response generated and can accommodate up to 36 Kbp and was demonstrated that the expression of genes that can incorporate lifelong body.

In this study, two distinct gutless adenoviruses were produced, with hepatospecific and regulated expression by the RU inducible system, containing a combination of OSM and IFN genes (HCA-RUIO) and the other the hIL-12 gene (HCA-RUhIL12). After *in vitro* testing the correct functionality of the vectors were carried out *in vivo* tests in mice and hamsters. It showed that *in vivo* expression of gene of interest changes with the species of animal used and the transgene present in the vector. In contrast to previous data that showed in mice infected with HCA-RUmIL12 that mIL-12 can be expressed even after more than a year, in Hamsters, the same vector expressed the transgene only after the first induction with RU and then it could not be detected. The vector HCA-RUIO gave a similar pattern of expression of the transgene in both hamsters, but also in mice. This may be due to the fact that if the protein products by the vector are exogenous to the organism is activated immunostimolatory activity in animals

that leads to the elimination of the transfected cells and thus an inability to reinduce the expression of the transgene.

II. INTRODUCTION

1. Gene Therapy

Recent advances in biochemistry, molecular biology fields (recombinant DNA technology) and the sequencing of human genome permitted to know the causes and the molecular basis of many human pathologies (1, 2). This has driven the development, in the last decades, of the biomedicine, a discipline that has specialized in the development of different techniques and therapies, both cellular and gene, for the treatment of different diseases.

Actually, life expectancy of human population has increased significantly, which is more feasible the development of cancer and neurodegenerative diseases. Despite the impressive progress in biomedical sciences during the last decades, the therapy of many human diseases remains unsatisfactory. To treat a genetic disease is important to correct the deficiency or the gene dysfunction in those cells or tissue in an efficient way, and for this reason, which has only been possible to treat the symptoms of these disease. With stem cells, one of the solution that has raised more hope for genetic diseases is gene therapy. Gene therapy is not limited to hereditary diseases but can be used for a broad variety of different acquired diseases like infections, degenerative disorders and cancer.

1.1 Concept and application of gene therapy

Gene therapy is a discipline of biochemistry that permitted to develop techniques and protocols for the treatments of human diseases. Gene therapy consist to introduce nucleic acid (DNA or RNA), with therapeutic purpose, in a cell or a tissue target to increase or decrease the gene expression of one or more genes. Also the gene, in this definition is included all the genetic units that can normalize the gene expression of the ill as shRNAi (short hairpins RNA interference), the RNAi, the antisense sequences. In

this way, knowing the cause of a genetic disease, it can be possible think an appropriate therapy, introducing nucleic acids that permit to correct the alteration and deal the pathology successfully (3-6). Gene therapy is not limited to hereditary diseases but can be used for a broad variety of different acquired diseases, such as infections, degenerative disorders and cancer (7).
The recent availability of effective recombinant vectors makes this approach even more realistic. Generally, gene therapy for most genetic diseases requires expression of therapeutic protein for the whole life of patient. In order to be efficient for the treatment of genetic disorders, a gene therapy vector has to meet several condition (8):

a. safety, which can be better achieved with a non-integrative vector, as it avoids the risk for insertional mutagenesis,
b. ability to be easily and inexpensively produced at a large-scale in the laboratory
c. stability in target cells, which is favored with low-immunogenic vectors
d. high-capacity allowing the possibility of introducing full-length cDNAs, endogenous promoters or additional regulatory sequences such as enhancers or insulators, which can provide a tightly regulated expression of the therapeutic gene, similar to physiologic conditions.

Until now, have performed over than 1644 human clinical trials, of which 13 are in phase II-III and 57 in phase III (Fig. 1).

1.2 Techniques of gene administration

Gene therapy is a highly plastic procedure and diverse strategies can be contemplated to treat diseases. Gene transfer can be accomplished by (Fig. 2):

- *ex vivo* approaches with posterior infusion of the transduced cells into the patient
- direct *in vivo* administration of the vector to the subject (9).

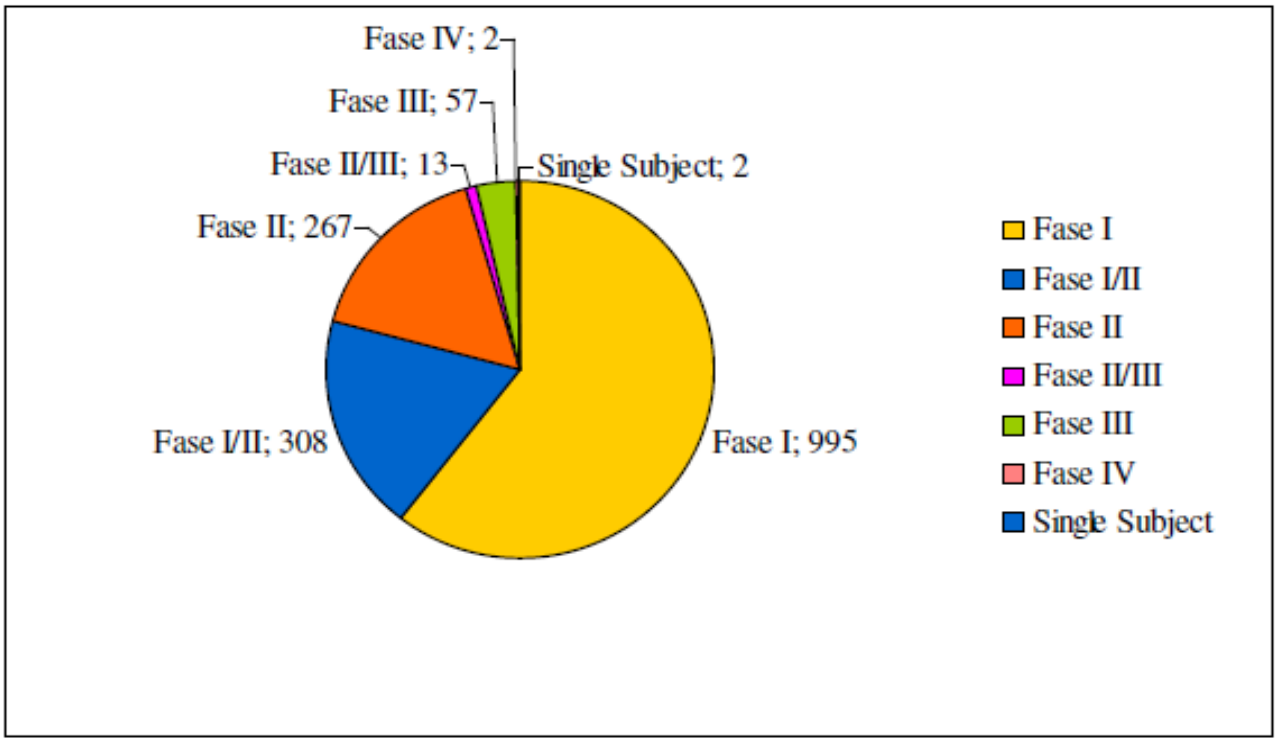

Figure 1. Phases of clinical trials for gene therapy protocols

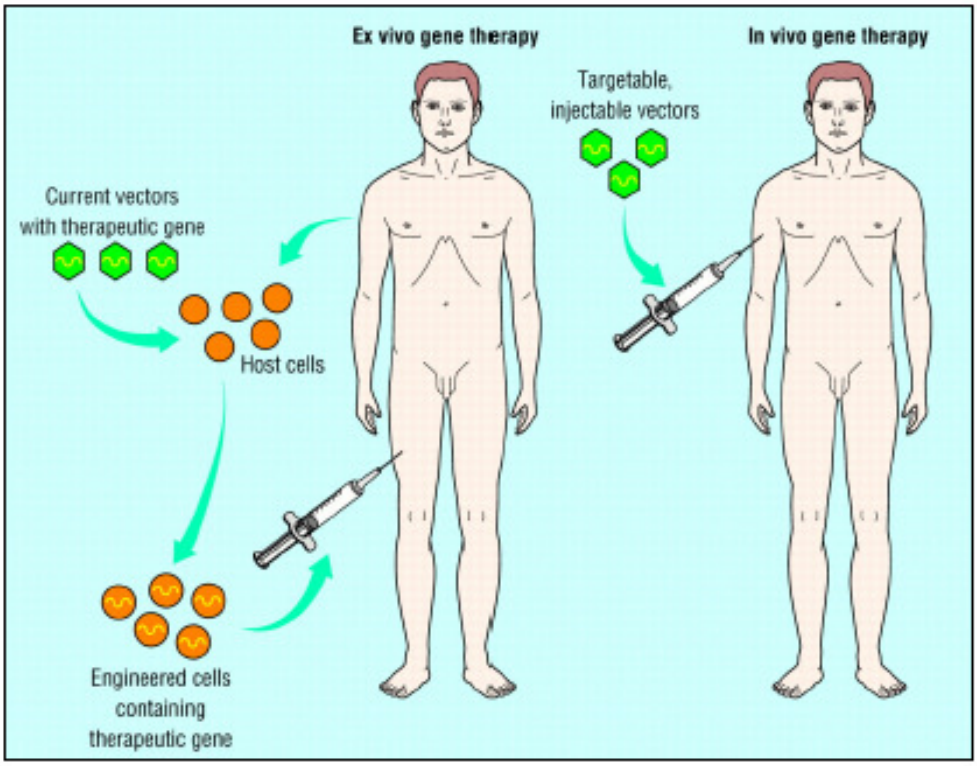

Figure 2. Differences between *ex vivo* and *in vivo* gene therapy.

1.2.1*Ex vivo* gene therapy

Ex vivo gene therapy involves the extraction of target cells from the patient for further *in vitro* transduction and selection. Once cells are transformed with genetic material of interest, these are implanted into the patient (10,11). This therapy has great advantages because the cells tend to be transduced in a high percentage, and besides, being the own patient, are not rejected by the body. In this case, the vectors used for this therapy are often integrative (MLV, Lentiviruses, etc.), since the cells tend to go through numerous cycles of cell division. However, if transient expression is desired, using integrative vectores (Adenovirus, AAV, etc.). *Ex vivo* gene therapy, is being widely studied since the appearance of pluripotent stem cells, which allow the regeneration of tissues or organs.

1.2.2*In vivo* gene therapy

Unlike the ex vivo gene therapy, in vivo gene therapy involves the transduction of target cells by vectors (viral or non-viral) directly into the patient (Fig. 3). Its success will depend on the specificity of the vector by the target cells and their efficiency of transduction.

1.2.3 Types of vectors used in gene therapy

The vectors used in gene therapy belong to two groups:

- the non-viral vectors
- the viral vector

1.2.3.1 Non-viral gene therapy

Non-viral vectors bear some advantages with respect to viral vectors: handling is easier, capacity for DNA sequences is very

high, toxicity is low, they can be specifically targeted to a tissue, and they are not immunogenic thus permitting repeated vector administration (12). These advantages however are compounded by a relatively low transduction efficiency. Non-viral vectors include liposomes, DNA-protein complexes, and naked DNA. The latter can be injected into tissue directly or as DNA coated gold particles using the gene gun (7).

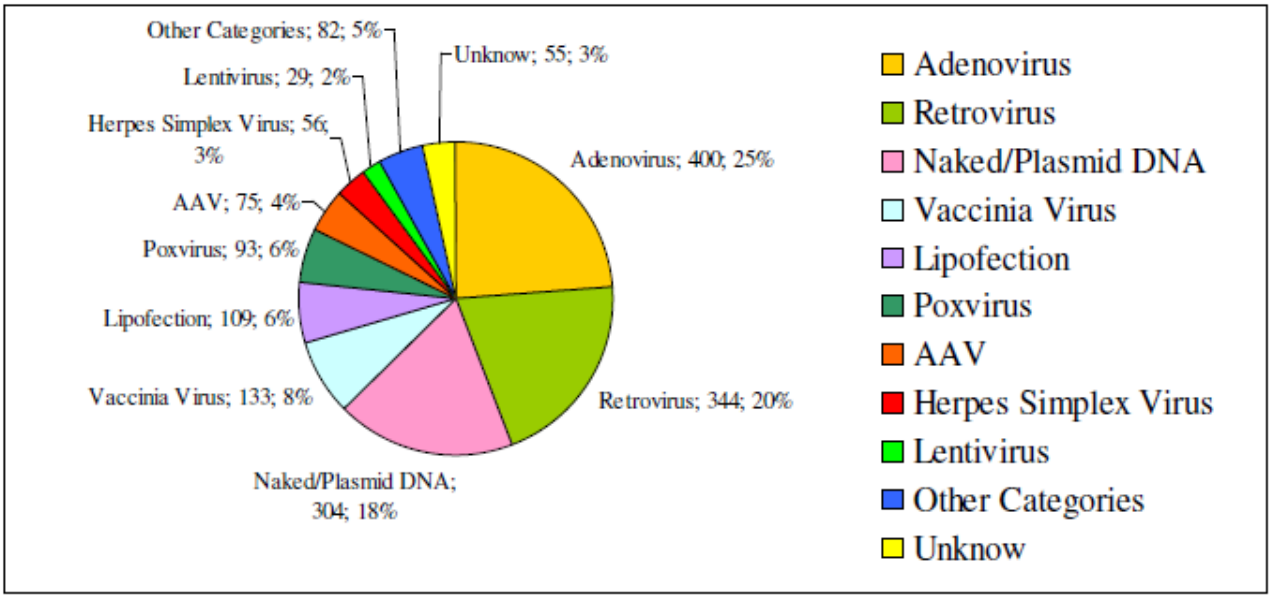

Figure 3. Vectros used in gene therapy (http://www.wiley.co.uk/genmed/clinical/).

1.2.3.1.1 Liposomes

This gene transfer system consists of DNA surrounded by a liposomal coat that allows adsorption to the cell membrane and penetration inside the cell by endocytosis. The transduction efficiency of this system is determined by liposome size and lipid formulation (13). The affinity of liposomes for the target cells can be modified by incorporating monoclonal antibodies into the lipid complexes. These vectors are known as immunoliposomes (14). As DNA can be degraded by lysosomes, viral proteins can be incorporated into the liposome to elude the lysosome pathway (virosomes) (15).

1.2.3.1.2 Gene gun

DNA coated microparticles (tungsten or gold) are delivered into cells by devices using gas pressure as the propelling force (16). Penetration of the microbullets is restricted to superficial cells layers of the skin or of the treated organ (17).

1.2.3.1.3 DNA-protein complexes

Specific membrane receptors can be used for receptor mediated endocytosis of DNA complexed with the ligand of the receptor (18). Trunsduction efficacy of this system is limited partly by DNA degradation due to lysosomal activity.

1.2.3.1.4 Naked DNA

Plasmid DNA directly injected into tissue has been has been shown to be incorporated by different cell types, such as muscle, skin cells and liver cells (19,20). Despite degradation of DNA within a few days after administration, this technique can be used successfully to generate efficient immune responses against the protein encoded by the injected DNA.

1.2.3.2 Viral gene therapy

For millions of years, viruses have co-evolved with its host, which is why they are the most efficient vectors currently exist for the transfer of genetic material. However, they present some complications due to high immune response, high production cost and, in some cases, very difficult technique for its production and its application to industrial level, in addition to potential problems of biosafety.
Viral gene therapy involves the introduction of genetic material through the use of viral vectors, which have a specific tropism

for different cell types, thus allowing more efficient transfer to the target organ (21). Among the most commonly used viral vectors are the MMLV retrovirus (retrovirus Moloney murine leukemia), Adenovirus (Ad), adeno-associated virus (AAV), the lentiviruses and herpes viruses. The retrovirus vector MMLV has been the classic elccion in most clinical trials. However, at present the Ad vectors are the most choice in clinic, increasing the number of protocols 3.6 times since 1999 (86 protocols) until 2010 (400 protocols).
(http://www.wiley.co.uk/genmed/clinical).

1.2.4 Viral vectors

The basic concept of viral vectors is to harness the innate ability of viruses to deliver genetic material into the infected cells. In general, the major preoccupation of viruses is to replicate and produce copious amounts of progeny. Most viruses gain little by killing the host, but unfortunately many viral infections lead to deleterious effects on the host, accompanied by destruction of infected host cells. Damaging effects can be caused by induction of genes whose products are hazardous to the host or by acquiring host genomic material that can lead to pathogenesis.
The first step of viral vector design is to identify the viral sequences required for replication, assembly of viral particles, packaging of the viral genome, and delivery of the transgene to the target cells. Next, dispensable genes are deleted from the viral genome to reduce replication and pathogenicity, as well as expression of immunogenic viral antigens(22).
The gene of interest together with the transcriptional regulatory elements are inserted into the vector constructs, and a recombinant virus is generated by supplying the missing gene products required for replication and virion production. The more genes that are removed from the virus, the more replication defective the vector

will be, and there is less chance of recombination to generate the infectious parental vius.
The list of viral vector is still expanding and modification of already existing system will widen the list of potential applications of gene therapy.

1.2.4.1 Retroviruses

The most commonly used RNA virus vectors are derived from retroviruses, and these were among the first viral delivery systems to be developed for gene therapy applications.
Retroviruses are lipid-enveloped particles carrying a positive-sense ssRNA genome of 7 to 11 Kb. The viral RNA contains three essential gene, *gag*, *pol*, and *env*, and is flanked by long terminal repeats (LTR). The *gag* gene encodes for the core proteins capsid, matrix, and nucleocapsid, which are generated by proteolytic cleavage of the *gag* precursors protein. The *pol* gene encodes for the viral enzymes protease, reverse transcriptase, and integrase, which are usually derived from the *gag-pol* precursor. The *env* gene encodes for the envelope glycoproteins, which mediate virus entry (22).
After binding to its receptor, the viral capsid containing the RNA genome enters the cell through membrane fusion. Following entry into target cells, the RNA genome is retro-transcribed into linear double-stranded DNA and integrate into the cell chromatin.
Lentiviruses belong to the retroviral family and have the important property of efficiently transducing non-dividing cells(23,24).
In addition to gag, pol, and env, lentiviruses encode three to six additional viral proteins, which contribute to virus replication and persistence of infection (25). Two of the accessory proteins, tat and rev, are present in all lentiviruses and mediate

transactivation of viral transcription and nuclear export of unspliced viral RNA, respectively.
The genetic information required to package a functional lentivirus core in the vector was then found to be only a fraction of the parental genome (26). As the non-required genes are critical for viral pathogenesis, new generation of "minimal" packaging constructs have been adopted to increase vector biosafety (27). An important approach to alleviate such concerns is the use of self-inactivating transfer vectors (28,29). These vectors contain a deletion in the downstream LTR that when transduced into target cells, results in the transcriptional inactivation of the upstream LTR and diminishes substantially the risk of vector mobilization and recombination (30).
This type of vectors contains the HIV-1 *env* glycoprotein, that has a highly restricted host range in that it infects cells containing CD4 and coreceptors (31). Vectors are harvested from the supernatant, and those pseudotyped with VSV-G (G protein of vesicular stomatitis virus) can be concentrated to produce high-titer preps. Titers can be determined using assays that measure the amount or activity of proteins incorporated in the vector particles, such as the p24gag ELISA assay. Stable packaging cell lines have now been developed, in which the producer cells express the structural protein from minimal packaging constructs and expression is driven by an inducible promoter to minimize the toxicity of the VSV-G envelope protein (32,33). Other viral glycoprotein have also been used to pseudotype lentiviral vectors and provide altered cell tropism (34,35).

1.2.4.2 Adeno-associated virus (AAV)

AAV are non-pathogenic human parvoviruses which, after deletion of all viral genes except ITR, have been used with notorious success as gene therapy vectors. They were initially

discovered as a contaminant in an adenovirus preparation. Productive AAV infection requires helper functions that can be supplied by coinfection with helper viruses, such as Ad and herpesvirus (36). AAV can also replicate in cells that have been put under stress, such as irradiation or treatment with genotoxic agents. In the absence of a permissive environment that will support AAV replication, the viral DNA can become integrated into the host chromosomal genome to establish a latent infection (37).

Many different serotypes of AAV have been isolated, and the list continues to increase (38,39). They have in common a similar size and genomic configurations of replication and structural genes. AAV serotype 2 (AAV2) is the best characterized (40,41) and has been the most frequently employed recombinant AAV (rAAV) vector. AAV virions are small nonenveloped particles (20-25 nm) that carry a linear single-stranded DNA (ssDNA) genome, which is approximately 4.7 kb in size. There are 2 viral ORFs, *rep* and *cap*, flanked by T-shaped ITRs. The ITRs are important for replication, packaging, and integration, and these are only genetic elements from the virus that are retained in rAAV vectors.

The different serotypes of AAV utilize a variety of approaches for cell entry, and this results in different host ranges. The primary attachment site for AAV2 is the ubiquitous heparin sulphate proteglycan (42). The fibroblast growth factor receptor 1 and integrin $\alpha_v\beta_5$ have both been implicated as coreceptors that facilitate internalization by endocytosis (43,44). AAV4 and AAV5 use sialic acid, although different carbohydrate linkages determine specificity (45) and the PDGF receptors are also involved in AAV5 infection (46). After binding to its receptor, the virus enters the cell through receptor-mediated endocytosis and is subsequently transported to the nucleus (47,48). Viral uncoating in the nucleus releases the single-stranded genome

that then needs to be converted to a double-stranded form to enable gene expression.

AAV vectors based on the serotypes 2 capsid have been the most commonly used foe gene therapy studies and have demonstrated transduction in a large number of cell types and experimental model systems (49). The vector can transduce nondividing cell types and has been used in muscle, retina, brain, liver, and lungs. There is initially a slow rise in gene expression levels over the first few weeks after *in vivo* administration, and then a stable plateau is reached (50). The exact reason for this delay in gene expression is not exactly clear. It may reflect requirements for cytoplasmic trafficking, vector uncoating, and conversion of the incoming ssDNA genome into a dsDNA form capable of gene expression. This step is mediated by the host cell machinery and probably occurs by second-strand synthesis. AAV transduction can occur independently of the cell cycle; however, transduction efficiency is markedly improved in cells during S phase (51). Furthermore, activation of the cellular DNA repair machinery also supports second-strand synthesis, thus improving AAV transduction (52) Some of the alternative serotypes capsids give quicker transduction than AAV2 vectors (53). Transduction can also be obtained with faster kinetics using vectors with genomes half the size of wilde type, which are thought to reanneal through self-complementation, independently of DNA synthesis (54).

One of the major limitations for the use of AAV as a gene delivery vehicle is the relatively small packaging capacity. The unique ability of AAV vectors to become joined into concatamers by head-to-tail recombination of the ITRs has been exploited as a means to increase the coding capacity (55). In this approach, either the gene itself or the different elements of the transgeneexpression cassette are split over two AAV vectors that are administered simultaneously (56). Transgene

expression is obtained only after recombination between the two viral genomes, but the efficiency is often reduced as compared to single vector transduction.
The AAV vectors do not contain any viral coding regions, and therefore, there is no toxicity associated with gene expression. However, a single injection of AAV vector elicits a strong humoral immune response against the viral capsid, which will interfere with re-administration of the vector (57,58). Furthermore, natural infections have resulted in a high prevalence of circulating neutralizing antibodies against AAV in the majority of the population, which may inhibit transduction.

1.2.4.3 Herpesvirus

Herpesviruses have promise as vehicle for transfer of genes to cell in vivo based on their ability to persist after primary infection in humans in a state of latency where disease is absent in human host with normal immune status. Herpesvirus gene vectors should not reactive and produce infectious virus or cause disease even in an immuno-compromised host (59).
Human herpesviruses are a class of large DNA viruses with double-stranded genomes capable of accommodating a large amount of foreign DNA (60). Herpes simplex virus type 1 (HSV-1) has been developed as a vector for gene delivery (61). The HSV-1 virion is about 20 nm in diameter and consists of four components: envelope, tegument, capsid, and viral genome. The envelope is derived from the cellular membrane and contains approximately 12 viral glycoprotein essential for viral entry. The tegument is the protein layer between the capsid and the envelope, and this layer contains at least 10 viral protein, which are involved in the shutoff of host protein synthesis as well as in the activation of immediate early viral gene expression and assembly functions. The icosahedral capsid

consists of seven viral protein and contains the linear dsDNA genome, which is 152 kb in size and is divided into unique long (U_L) and unique short (U_S) region that are flanked by terminal repeats. The virus encodes at least 80 viral proteins with very little splicing of genes, approximately half of which are non-essential for virus replication in cell culture. These features provide for multiple sites of foreign gene insertion, making HSV a large capacity vector capable of harbouring at least 30 kb of non-HSV sequences representing large single genes or multiple transgenes that may be coordinately or simultaneously expressed (62). Highly defective mutants deleted for the five immediate early (IE) genes do not express the remaining lytic viral functions and are essentially silent except for transgene expression. These vectors can be grown to high titer in complementing cell lines without the production of detectable replication competent virus (63). The IE gene deletion vectors are non-cytotoxic (64) yet are capable of persisting in a state similar to latency in neurons and other cell types within non-neuronal tissue (65). A most attractive feature is the efficient infectivity of HSV for a large number of cell types, which results in efficient gene transduction. Efficient infectivity and transductyion has made possible repeat vector administration even in immune host. Limitations of these vectors are include the lack of experience with recombinant herpesviruses in patients, difficulties related to a long-term transgene expression in certain tissues including brain and difficulties related to vector targeting, since the mechanism of HSV attachment and entry in complex, involving multiple viral envelope glycoproteins.

HSV amplicon vectors represent an alternative to replication defective, recombinant genomic vectors (66). Amplicon plasmids are based on defective interfering virus genome that arise on high passage of virus stocks (67). They are generally approximately 15 kb in length and minimally possess a viral

origin of replication and packaging sequences. The standard amplicon system requires the functions of helper HSV for particle production and packaging of genome length concatamerized vector DNA. Amplicon vector production has been improved through use of helper virus genome plasmids deleted for packaging signals: the helper genomes are propagated in bacteria as bacterial artificial chromosomes (68).
Transduction with HSV vectors has been demonstrated in a large number of cell types, and these vectors have been applied to multiple gene therapy strategies, including neurological diseases, spinal nerve injury, glioblastoma and even pain therapy (61). Sensory neurons can be infected by direct interdermal injection of the vector, and the DNA can persist in the nerve cell body. Maintaining high gene expression levels over long periods of time is a problem in certain cell types, such as the brain. The major limitations for recombinant HSV-1 vectors are their cytopathic effect and the induction of an immune response by viral gene expression. The development of amplicon vectors and helper virus-free packaging system has overcome this problem (66). However, additional deletion of nonessential genes from the bac packaging system may also be necessary to prevent cytotoxicity and recombination within this vector system. The large packaging capacity of HSV-1 amplicon (up to a theoretical 152 kb) may be very useful for delivering complex genes and regulatory sequences or multiple copies of the transgene.

2. Adenoviral vectors

2.1 Biology of Adenovirus

The adenovirus virion is a nonenveloped icosahedral particle about 70-90 nm in size with an outer protein shell surrounding an inner nucleoprotein core (Fig.4).The facets of the virus capsid are composed primarily of trimers of the hexon protein, as well as a number of other minor components including protein IIIa (pIIa), pVI, pVIII and pIX. The capsid vertices consist of the penton base, which acts to anchor the fiber protein, the moiety responsible for primary attachment of virions to the cell surface. Adenovirus cores contein the viral DNA as well as pV, mu, and the histone-like protein pVII.

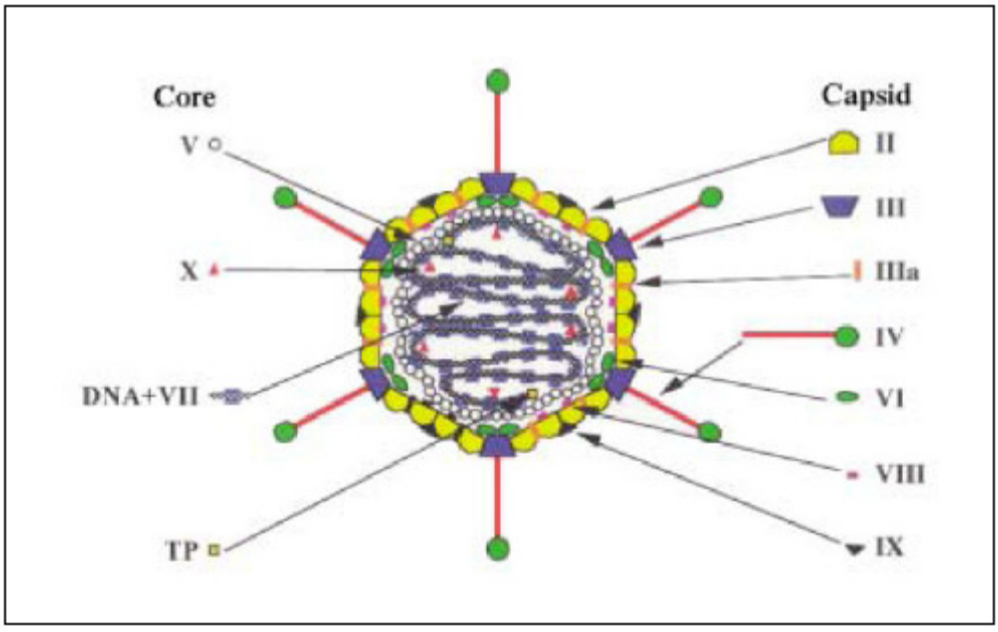

Figure 4. Adenovirus structure, and positions of the peptides of virion in the viral capsid.

The genome itself is linear, double stranded DNA (dsDNA) that is approximately 36 kb long. Each end of the genome has an inverted terminal repeat (ITR) of 100-140 bp to which the terminal protein is covalently linked, at 200 nucleotides of the 5' extreme is located packaging signal (Ψ), sequence that directs the packaging of the viral genome through its interaction with various viral and cellular

proteins. Gene are encoded on both strands of the DNA in a series of overlapping transcription units (Fig.5). Adenoviral genome is divided into *early gene* (E1-E4), which are transcribed in the first phase of the cycle, just before viral DNA replication, and *late genes* (L1-L5), transcribed in a later phase (protein production methods and packaging). In addition, the adenoviral genome codes for one or two sequences calls VA-RNA (virus-associated RNA) transcribed by RNA polymerase III. The VA-RNA, of approximately 160 nucleotides, allows control of the translation efficiency of viral genome and inhibit cell protein synthesis.
Virions also contain approximately 10 copies of the adenovirus protease, a cysteine endopeptidase that cleaves many of the structural proteins into their mature form at the final stage of viral assembly (67).

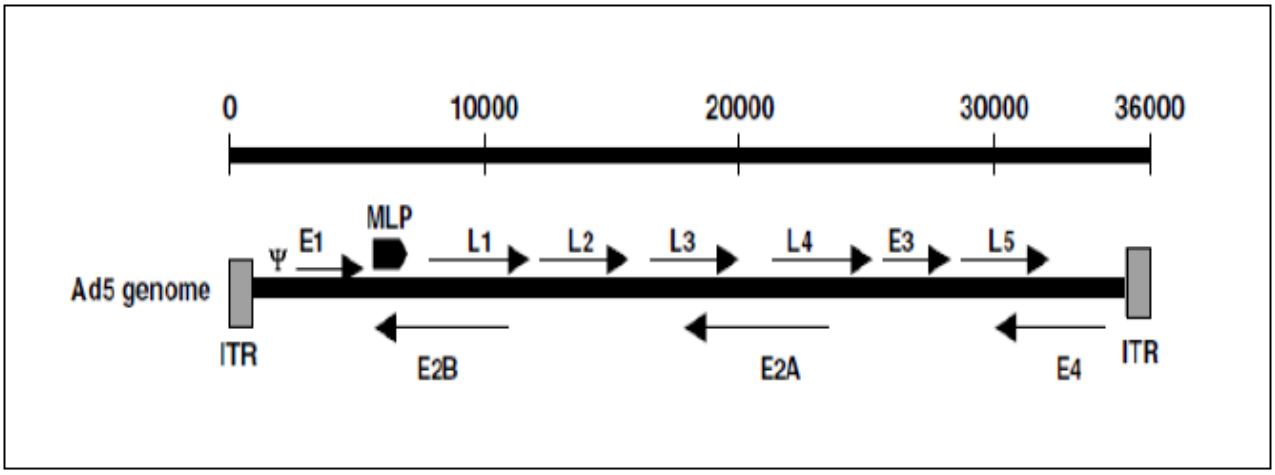

Figure 5. Adenovirus genome map of serotype 5. Regions "early" are described as arrows E1-E4 and regions "late " are described as an arrow L1-L5.

The 51 distinct serotypes of human adenovirus have been classified into six groups (A-F) based on sequence homology and their ability to agglutinate red blood cells (68). Most studies have been carried out on adenovirus serotype 2 (Ad2) and Ad5 and, unless otherwise stated, it should be assumed that the information below refers to work done on these serotypes. For all groups, except group B adenoviruses, initial attachment of virion particles to the cell surface occurs through binding of the fiber knob to the

coxsackievirus B and andenovirus receptor (CAR). CAR is a type 1 transmembrane protein in the immunoglobulin superfamily and is present in many human tissue including heart, lung, liver and brain (69). The CD46 molecule, a complement-regulatory protein, has been identified as a cellular receptor for group B adenoviruses (70). Group B adenoviruses have therefore received considerable attention because of their ability to transduce cells, such as hematopoietic stem cells, dendritic cells, and malignant tumor cells, which can be resistant to infection by adenovirus groups that use CAR as the primary attachment receptor (71).

After initial attachment to the cell surface, the penton base iteracts with member of the integrin family, triggering virus internalization by clathrin-dependent, receptor mediated endocytosis (72). For Ad2 and Ad5, the acid environment of endosome induces escape of virions into the cytoplasm. Once in the cytoplasm, dynein mediates trafficking of virions along microtubles toward the nucleus, where the subsequently dock with the nuclear pore complex (NPC) (73). Disassembly of the capsid at the NPC allows for import the viral genome and commencement of the viral transcriptional program.

Once the adenoviral genome has reached the nucleus, the virus prepares the cell to replicate its DNA safely and then produce all proteins necessary for the formation of new virions. Firstable, the Ad needs to simulate the entry into S phase cell cycle to provide a good environment replication. Secondly, the adenoviral genome needs to express replication proteins, which bind to proteins for initiation of replication of the DNA chain. Finally, adenovirus protects the cell of the cellular immune response may facilitate the dissemination and replication of adenovirus in the body.

Prior to viral replication, of the adenoviral genome transcribes around 25 early genes, which act sequentially in different stages of viral cycle to allow the replication of the viral genome efficiently. E1A genes activate the cascade of all other viral genes because only this gene needs the presence of cellular factors for its

transcription. Furthermore, E1A proteins inhibit cell replication (74), which contributes to viral genome replication more efficient. The region E1B codifies for proteins that inhibit apoptosis and prepare the intranuclear environment for adenoviral replication (E1B 55K and E1B 19K) (75). The E2 gene encodes proteins replication of Ad (AdPol, TP and DBP) (76). The E3 region encodes proteins that prevent the cellular immune response and thus, the adenovirus remains the time required to complete the infection cycle (E3gp-19K and ADP among others) (77). This region is the adenoviral death protein (ADP) to help lyse the cell at the end of the cycle (78). This protein is expressed from the onset of the viral cycle but acts at the very end of the process (79). Finally, the E4 region encodes 7 open reading frames (ORFs) with clearly different functions. These functions include participation in the viral genome replication (80), splicing (81), mRNA transport (82), inhibition of cellular protein synthesis (83), regulation of apoptosis (84) and cell lysis (85).

The synthesis of the viral genome requires the participation of 3 viral proteins: the AdPol (adenoviral polymerase), pTP (pre-terminal protein) (86) and DBP (DNA binding protein) all encoded by the E2 region of adenovirus (87). BPD acts in different levels for help the viral genome replication and the binding of cellular factors that increase replication (88). In addition, the DBP binds to single-stranded chain, which protects the viral genome against cellular nucleases (89). On the other hand, the pTP protein binds to the protein AdPol stably, which is essential for efficient replication initiation and subsequent elongation of the chain (90). Also, besides the viral replication proteins, cellular factors NFI / CTF (nuclear factor I / Nuclear transcription factor), and NFIII/Oct-1 NFII increased to 200 times the replication of Ad in the cell (91), making them essential for a rapid and efficient infection. The conjunction of these proteins initiates chain elongation of DNA from the ITR at both ends of the

viral genome, organized and symmetrically replicating both the chains an organized (90).

The late genes are transcribed by the action of the promoter MLP (Major Late Promoter), from which it generates a transcript of approximately 30000 nucleotides that are polyadenylated and by an alternative splicing, generates up to 18 transcripts divided into 5 families of traditional late mRNAs (L1 -L5) (92). Basically, proteins transcribed in the late regions participates to the formation of the Ad capsid, packaging of the adenoviral genome, final assembly of infectious viral particles and lysis of the infected cell.

The packaging of the adenovirus is a multistage process where different proteins both viral and cellular are involved. Ad packaging is produced in polar way because in 5' end of Ad there is the packaging signal (Ψ), where the process starts. Specifically, the signal Ψ is located between nucleotides 198 and 358 of the genome in the case of Ad5 (93). It has been reported that the Ad5 packaging signal is composed of 7 repetitive regions called "A-repeats" (94). These regions have a sequence motif characteristics: 5'-TTTGN_8CG-3', which is conserved among different Ad serotypes (95,96). Among the 7 "A-repeats", the most important for the Ad serotype 5 are the A1, A2, A5 and A6, as described that their location and sequence are most critical in terms of packaging (97). It has been shown that every A-repeat is independently from the others and therefore, artificial packaging sequences are generated with some of the existing A-repeats with packaging efficiencies similar to wild type adenovirus (98). Also, the position packaging signal is flexible in the number of A-repeats containing as its location, that it can be found in both the 3' end as in the 5' genome. What is absolutely necessary for packaging process is that the signal is in the first 600 nucleotides, approximately, from the end of the Ad. If the packaging signal is located at a greater distance from the ITR end, the adenoviral genome loses its packaging ability(93).

Is postulated that the entry of the viral genome within the capsid is a process that needs energy which could intervene an ATPase localized in the IVa2 protein (99). Once the adenoviral genome is located within the capsid, it is being accommodating by adenovirus proteins themselves which serve as anchor (100), as is the case of precursor of protein VII (pVII) that acts at this level (101). Once the Ad genome is packaged and anchored within the viral capsid, the viral particle is closed and mature through different stages (102).

Once the adenoviral genome is located within the capsid, the DNA has to accommodate and condensate efficiently, by binding to different proteins involved as an anchor. Significantly, the adenoviral genome is anchored on protein V and VII as shown in Figure 5. The adenoviral particle has to mature from a young or immature particle into an infectious particle. For this, the adenovirus synthesizes its protease, a protein that directly involve the development of infectivity of the adenoviral particle (103,104). Protease, also called Adenaina (105), is transported into the capsid, with approximately 10-50 copies per particle (106), and acts in a specific, degrading protein 6 precursor (IIa, PVI, pVII, pVIII, X (mu) and PTP) (107-109). The existence of degraded products into the adenoviral capsid is consistent with the idea of transport of the protease in the Ad capsid.

The adenovirus protease is synthesized from L3 gene and has a molecular weight of 23KDa. It is hypothesized that the Adenaina is synthesized in an inactive form and requires cofactors for activation (110). The adenaina acts in the presence of viral DNA, degrading a viral peptide of 11 aa of the protein pVI (pVIc), which is attached to the protease and finally actives this to initiate proteolysis of adenoviral peptide precursors (111). It has been reported that this protease digested cytokeratin 18, thus reorganizing the cell cytoskeleton. This action on the cytoskeleton undermines the integrity of the cell and promotes virus release by lysing the cell (112,113). Another of the proteins that act in the

latter stages of the viral cycle is ADP protein or E3-11.6K. This protein is required for efficient cell lysis and subsequent release of viral particles outside the cell. In adenovirus mutant protein ADP (Adadp$^-$), the virus remains in the nucleus of the cell and not released to the outside efficiently (78). In the late stages of viral infection (30-40 hours post-infection), the ADP protein is localized in the membrane of the nucleus and the Golgi apparatus, which would locate its function of cell death in the nuclear membrane.

2.2 First-generation Adenoviral vectors

As described above, genes in the E1 region are necessary for activation of viral promoters and expression of both early and late genes. Thus, removal of the E1 coding sequence results in viruses that are severely impaired in their ability to replicate. Furthermore, the E1 region encodes the oncogenic transforming functions of the virus. For these reasons, replacement of the E1 region with transgenes was the initial strategy used in the construction of adenoviral vectors, giving rise to so-called first generation vectors (Fig.6). The ability to delete the E1 region is made possible by the existence of cell lines that provide these function *in trans*. The classic cell line for this purpose is the 293 cell line, a human embryonic kidney-derived line that has been transformed by the adenovirus E1 region (114).
Many of the first-generation vectors also contain a deletion in the E3 region (Fig.6), mainly for practical reasons. E3 genes are entirely dispensable for virus growth in vitro and their removal, together with deletion of E1 genes, allows up to 8.2 Kb for transgene insertion. Data have suggested that expression of E3 genes from vectors may be beneficial in vivo because of their ability to dampen many host immune processes. It has been reported that expression of the entire E3 region or the E3-gp19K product alone can increase persistence of transgene expression in

some rodent model (115). However, conflicting data have shown that expression of the E3-gp19K proteins has no effect on the length of transgene expression (116). These discrepancies may be due in part to differences in the nature of the transgene or the tissue type that was analyzed. Nevertheless, the inclusion of E3 genes in vectors remains an area of active investigation.

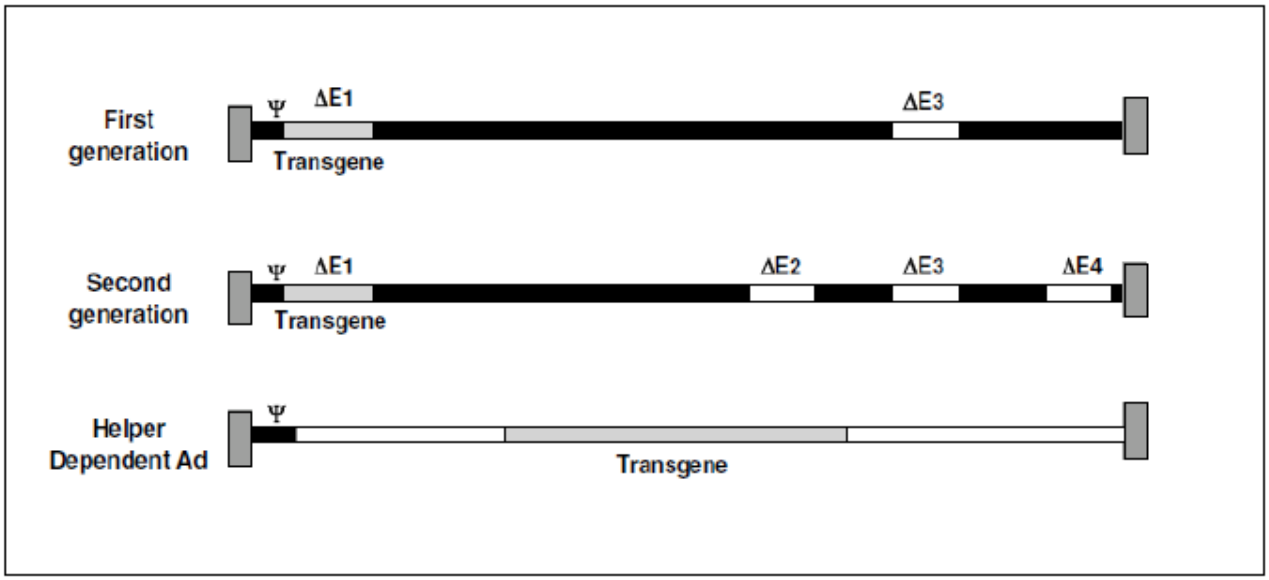

Figure 6. Genome map of the different generations of adenoviral vectors.

Although first-generation vectors have proven to be highly promising as vehicle for gene delivery, problems do exist. The first drawback associated with these vectors becomes apparent during vector production. Recombination between the E1 region sequences in the complementing cell line and recombinant virus can give rise to viral progeny with functional E1 genes that are replication competent (117). Thus, recombinant virus stocks must be assayed for the presence of replication-competent viruses. Helper cell lines such as PER.C6 and 911, in which the overlap between E1 sequences in the cell and those commonly present on recombinant virus chromosomes is reduced, have been constructed in order to minimize this occurrence (118,119). The second and more troublesome problem associated with the use of firs-generation vectors is their stimulation of a cellular immune response, resulting in the destruction of transduced cells that are

expressing therapeutic transgene. Indeed, a number of early studies showed that administration of E1-deleted vectors to immune-competent animals results in only transient transgene expression (120). It is theorized that the immune response is stimulated by low levels of replication that can occur even in the absence of the E1 genes. This idea is supported by findings that genome replication and late gene expression can occur from E1-deleted vectors in vivo (121). Although stimulation of a robust immune response may preclude the use of first-generation vectors in some settings, they still remain promising for applications requiring short-term gene expression such as cancer therapy and vaccination.

2.3 Second-generation Adenoviral vectors

To prevent the immune response generated by low-level replication of E1-deleted viruses, vectors deleted for multiple genes have been created to inhibit viral gene expression more efficiently. These second generation vectors have been constructed primarily by removal of E2 (122,123) and E4 (124,125) coding sequences (Fig.6), also providing the benefit of a larger capacity for transgene insertion. The major drawback encountered during construction of these multiply deleted viruses is the need for isolation of cell lines expressing the missing function *in trans* (126-128). Vectors containing deletions in these genes are incapable of genome replication, and in the case of polymerase-deficient vectors, no replication occurs even in the presence of high levels of E1A (129). Results from experiments in which all part of the E4 region has been deleted are less clear. The E4 region encodes products involved in many aspects of viral replication. It was thus theorized that removal of all or part of the E4 transcription unit would impair viral replication and gene expression such that an immune response would not be triggered. Rodent models have suggested that the deletion of some or all E4

proteins may affect the length and level of transgene expression; however, this regulation appears to be both tissue and promoter specific (130,132).

2.4 The immune response induced by adenovirus

The Ad2/5 is a virus very common in the human population as it has a very important incidence in common colds and conjunctivitis (133). For this reason, a humoral immune response is present in the 90% of the human population again the viral proteins of the capsid (134). Also, the administration of first-generation adenoviral vectors, in gene therapy protocols, caused the elimination of the therapeutic gene 2-3 weeks after the vector administration (135-137). To avoid the pre-existing immune answer, the actual strategies are based on the use of different serotypes of Ad (138) or on the use of no-human Ad (139).
Besides the pre-existing immune response, the immune response, caused by the Ad, is divided in 3 grou:

a) innate immune response (140)
b) humoral immune response
c) cellular immune response (134)

When Ad enters in the organism, the innate immune response in activated (135,140). This system is composed by a set of constitutive and inducible elements (as defenisies, proteases, coagulant factors, cytokines and the complement) including fisical and chemical barriers (140). This response activates inflammatory genes and recruits macrophages, neutrophils and NK cells (Natural Killer), that eliminate the 80-90% of virus in the first 24 hours (141). The inflammatory response is clearly dose-dependent and occurs independently of viral gene transcription. Ad induces the expression of a large number of inflammatory gene (142) as TNF-α, IL-6, IL-1β, IFN-γ and IL-12, among others (143). In addition, the ingestion of Ad by macrophages and / or Kuppfer

cells causes activation of the inflammatory signal of tissue damage and systemic effects (144,145)
One of the most important functions of the innate immune system is pattern recognition through a large number of receptors at both intracellular and extracellular level (146). The most studied receptor family is for *Toll-like* receptors (TLR) (147). Virus binding to these receptors TLR triggers a cascade of events that limit and eradicate the viral infection (148,149). Of the different TLRs (10 types), TLR9 specifically recognizes double-stranded foreign DNA such as adenoviral genome (150).

The cellular immune response appears between 4 and 7 days after Ad entry into the body. This response is activated when the APC (Antigen Presenting Cells) capture particles of Ad, and process into small peptides or proteins of the virion that will be presented later in the cell membrane by MHC-I (Major Histocompatibility Complex I) (134). Subsequently, $CD8^+$ T cells bind to these peptides and activated transforming in CTL (cytotoxic T lymphocytes). These remove cells containing on the surface the Ad peptides presented (151) and thus put an end to the expression of the transgene of interest. The union between MHC-I and $CD8^+$ lymphocytes is mediated by the interaction between the molecule CD28 (T lymphocyte $CD8^+$) and B7 (APC) (152). Together, helper T cells $CD4^+$ (helper 1) are activated, which secrete IL-2 (interleukin 2) and IFN-γ (interferon gamma), that differentiate $CD8^+$ T cells in cytotoxic T lymphocytes (153). Unlike $CD8^+$, these cells recognize epitopes of virion presented by MHC-II on the surface of the APC.
The Ads can fight the cellular immune response through -gp19k gene, which retains MHC antigens in the endoplasmic reticulum avoiding in this way, the recognition process (154). In addition, products of the Ad E4 gene inhibit cytolytic CTL response (155).

The humoral immune response begins when the Ad is attached to the immunoglobulins present on the membrane of the B cell (134). Subsequently, peptides of Ad are presented by MHC-II to T helper cells Th2 $CD4^+$ producing cytokines such as IL-4, IL-5, IL-6 and IL-10, which will make differentiating B cells in a plasma cell to generate antibodies (Ab) specific against the Ad capsid (156). These Ab difficult later the re-administration of vector. Once the adenoviral genome entered in the target cell, antibodies can not eliminate transduced cells and the genome can persist within the cell only if their proteins are not presented in membrane by the MHC-I system (136).
The humoral immune response is specialized to recognize the virus thanks to Ab and remove thanks to the action of macrophages. For this reason, repeated administration of adenoviral vectors requires immunosuppression of the individual to maintain the expression of the transgene (157). This is true as long as the dose of Ad is high enough, since low doses of the vector allow a dose of tolerance, which does not activate the immune response.

Once the Ad vector has passed all physical barriers and the components of humoral and cellular immune system, the adenoviral vector can express the transgene incorporated into its genome for long periods. For this reason, the patient acquieres the desired gene expression and the effects of their illness can be alleviated. However, if this is the first time of expression in the body, the immune system can recognize the new protein as a foreign agent. Thus, the protein can generate antibodies, be neutralized by the immune system and lose the desired therapeutic effect (158,159). To avoid possible side effects due to transgene expression in all tissues infected with Ad, the use of specific promoters is recommend to express the gene of interest only in the target organ or cell. In this way, it has been reported to increase the duration of transgene expression (160,161).

3. Third-generation Adenoviral vectors

To avoid the problems of immunogenicity induced by Ad, 3rd generation adenovirus was generated also called *gutless*, *helper dependent* (HD-Ad), or *high capacity* (HC/Ad High-Capacity/Ad) (162-168). This vector has very different names in function of the properties of the genome or the amplification characteristics. The reason they are called *gutless* is because they have eliminated all viral coding regions, *helper-dependent* because they depend on a helper adenovirus to occur, and High-Capacity because they support up to 36 Kbp inserts.
Gutless adenovirus keep only the ITR ends (5' and 3') of its genome and packaging signal (Ψ), which is essential for the final assembly of the virion (Fig. 6). In addition to the gene (marker or therapeutic), a stuffer DNA is used to complete the 36 Kbp need adenoviral genome (169). No containing any viral coding region, the gutless Ad can not be produced by itself and needs the help of a *helper adenovirus* that contribute with "*in trans*" adenoviral proteins necessary for its production (166). To avoid propagation of the helper Ad polluter the gutless adenovirus preparations different, strategies are used to reduce packaging capacity of Ad helper against Ad gutless. With the absence of viral genes, the gutless Ad avoids the cellular immune response, thereby increasing the levels and timing of expression of the transgene (170-175).

3.1 DNA stuffer

Ad capsid can accommodate a genome size between 75-105% of the wild type genome (176, 138, 162, 167, 177). Due to the elimination of all viral coding region is necessary to add filler DNA with which to replace the 36 Kbp eliminated. Thus, use stuffer DNA, together with the gene of interest, to reach a capacity of between 27.5 and 37.8 Kbp (178). Initially it was thought

important only DNA that was used as stuffer, was the right size for packaging. Therefore, the first stuffer DNA were chosen from DNA sources easy to obtain, as in the case of lambda bacteriophage, yeast and human DNA in general (178).

However, the stuffer DNA plays an important role in the stability of gutless Ad *in vivo* (178). The first administration of gutless Ad *in vivo* with stuffer DNA from lambda bacteriophage resulted in a strong immune response against the transduced cell (138,170). This was because small peptides generated by the stuffer DNA (lambda bacteriophage) were processed and presented on the cell membrane, thus induce a cellular immune response mediated by CTL. Therefore, it began using stuffer DNA sequences with introns of the human genome, which favored the persistence of the transgene *in vivo* (138). For this purpose, gene sequencing was used from the intronic HPRT (hypoxanthine-guanine Phospho Ribosyl Transferase), which contains MAR regions (Matrix Attachment Regions), which provide greater stability to the adenoviral genome in the cell *in vivo*. Different sequences of other loci, such as HSU gene (locus HSU71148), AFO (locus AF011889) and ER (region of estrogen receptor beta) were used with similar or improved results (178).

However, not all the intron sequences are suitable for forming part of gutless Ad stuffer DNA. To choose the stuffer DNA, the DNA candidate should follow the following characteristics: a) do not contain coding regions, b) not to be rich in repetitive regions (such as Alu regions), c) avoid areas where there are, more ease, recombination processes, d) avoiding regions that may interfere with the expression of the transgene, e) avoid immunogenic regions, and f) choosing regions that contain, as far as possible, MAR sequences that stabilize the adenoviral genome in the nucleus and permitted the expression of the transgene for long periods of time (178).

3.2 Production of gutless adenovirus vector

To amplify the Ad gutless was recommend the use of a cell line that can restrict the growth of Ad helper respect to Ad gutless. The separation of the two vectors is essential to obtain a vector without contamination of Ad helper. The strategies used to minimize contamination by helper Ad has evolved in the last 10 years since the physical separation by centrifugation (162), using specific recombinases which permitted elimination of the Ad packaging signal helper (166), to specific mutation of Ad helper packaging signal to reduce its assembly (179-181).

Initially, the CsCl gradient centrifugation was used for the separation of adenoviral vectors (162). The Ad gutless, partially, contained no different coding regions for several genes (L1, L2, VAI+II and pTP) and contained an expression cassette for maker gene. Growth of Ad wild type enabled the spread of both Ad, without restriction of growth for any of them. However, after purifying the two vectors, there were high levels of contamination of wild type Ad and various recombinations between them (162). Although the first proposed system is inefficient for optimum production of Ad gutless, this study led to multiple systems that allowed the production of gutless adenovirus with the idea of using this vector in human clinical trials.

Then, another system was proposed to try to prevent the spread of the helper with the maximum efficiency of production of gutless Ad. In this system, the packaging signal of helper Ad was mutated to avoid their production. Thanks to this system and the separation by CsCl, the helper Ad contamination was 1% (182). The existence of A-repeats of greater importance in the packaging signal was described and its mutation has a direct impact on the efficiency of viral particle assembly (183). Thus, the signal Ψ can be modified by removing specific regions in order to restrict the growth of helper adenovirus during the production of gutless Ad. Similarly, the modification of the signal Ψ has dropped

considerably the helper Ad contamination in canine gutless Ad (139,181).

The packaging signal plays a structural role in which different proteins involved with accompanying DNA strand to the viral capsid (184). The modification of the signal and its location may decrease the ability of Ad to be packaged (185). One of the approaches used to further reduce contamination of Ad helper, was the reversion of the packaging signal, since genomes recombined by Ψ, being over 38 Kbp not be packaged (186).

The system that has brought greater efficiency to reduce contamination of helper Ad, was the specific excision of the packaging signal by recombinases. The signal was flanked by loxP sequences, which were recognized specifically by Cre recombinase, which was expressed by the cell line HEK293/Cre (166) (Fig.7). When both Ad, helper and gutless were co-transfected in this cell line, the Cre recombinase split the signal from the helper adenovirus, which could not packaged this genome (Fig.7). However, if it could provide *in trans* the proteins necessary for the gutless Ad production. The average contamination of helper Ad ranged from 0.1-10% with what still needed to further reduce such more contamination for possible use in clinic.

Although several systems have been used by varying the gutless Ad genome, the Ad helper, the cell line or protocol, has been difficult the amplification of the gutless Ad at high concentration with low contamination of helper Ad. In this way, usually has been possible to obtain small amounts of vector to administer to a small number of animals in experiments *in vivo* or small animal testing, as the case of the mouse.

Another of the proposed system was using an improved helper Ad with packaging signal inverted together with the use of more sophisticated protocols for larger scale production with cells in suspension. The disadvantage of low production titles has been resolved, in part, through the use of cells in suspension systems

where the cell HEK293/Cre was adapted to growth in these conditions. With this method there were more of 10^{13} viral particles from 3 liters of culture in a time of two weeks. The specific productivity was > 10,000 viral particles/cell with a 0.4 to 0.1% contamination of Ad helper. After centrifuging the virus in CsCl gradients, the helper adenovirus contamination decreased to a 0.02 to 0.01% (179).

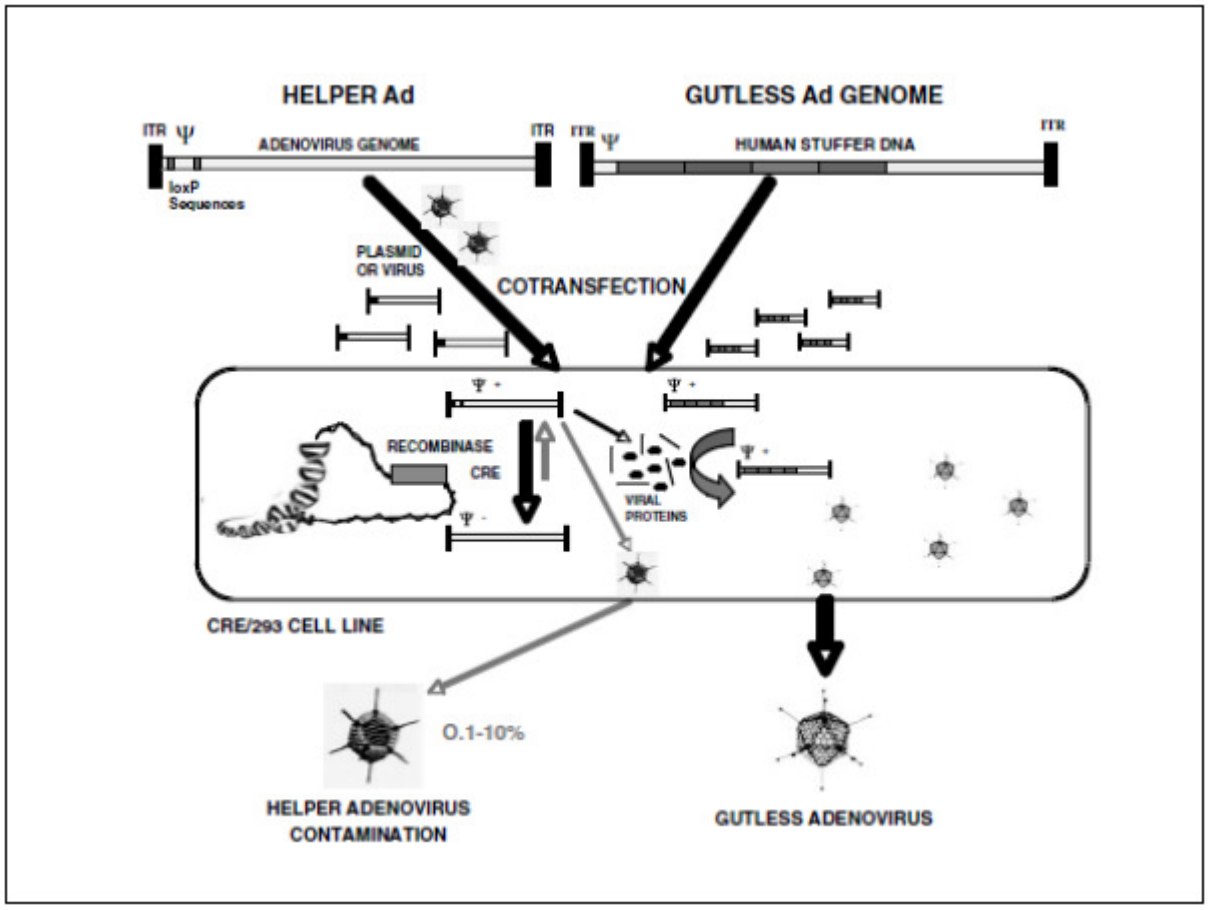

Figure 7. Generation of gutless Ad with Cre-loxP system (166).

With the same purpose, we used the recombinase FLPe, from yeast, which specifically recognizes frt signals. HEK293/FLPe cell lines were generated and HEK/293CreFLPe (186,187) with helper Ad whose signal was flanked by *frt* sequences. The Cre recombinase and FLPe have similar efficiencies in excision. However, the use of double HEK293/CreFLPe cell line did not confer any advantage in increasing the removal of the signal.

Also, alternative virus have been used to provide in trans the proteins necessary for packaging of Ad as is the case under the herpes simplex virus-1 (188) and baculovirus (189). In the case of herpes simplex virus amplicon-1, the advantage given by the authors is the collection of gutless adenovirus herpes virus-free thanks to the elimination of the amplicon by extraction with phenol or by heat inactivation. However, this system has low efficiency of production and its scale is technically difficult. In the case of the baculovirus system, recombinant viruses are generated containing the adenoviral genes. Thus, the baculovirus protein contributes in trans allowing the propagation of gutless Ad. This system increases to one hundred times the title of the vector at each step of amplification. However, the baculovirus system results in high rates of RCA particles, which are still being improved to make the move to large-scale production (189,190).
In addition, also the use of cell lines containing all viral coding region of Ad was proposed. However, the toxicity associated with viral proteins has not permitted the generation of these cell lines, although it generated a cell line that contains all the early genes and vectors with these regions deleted (191).
For a system of gutless Ad production can be used on a large scale is needed that allows to produce high titers of Ad gutless, avoiding the maximum of contaminantion of helper adenovirus particles and the appearance of RCA. Another approach that can also minimize the contamination of Ad helper is the choice of unidirectional recombinases prevent reinstatement of the packaging signals in the adenoviral genome.

3.3 Gutless adenovirus and immune response

Systemic delivery of first-generation adenoviral vectors is known to induce a strong host's immune response, resulting in the rapid elimination of vector-transduced cells and the generation of neutralizing antibodies against the transgene products and the

adenovirus capsid. Both non-specific innate and adaptive immune responses are involved when first and second-generation adenoviral vectors are administered. Thus, the innate immune response is rapidly developed after virus entry by induction of inflammatory gene expression and further recruitment of macrophages, neutrophil and natural killer cells, leading to an 80-90% of first generation vector removal from the liver in 24h (192). Basically, innate immunity is triggered by the adenovirus particle, is Ad-dose dependent and does not require viral gene expression (193,194).

In a second step, adaptive cellular and humoral immune response are developed about 4-7 days after delivery. At this time, a second peak of cytokine and chemokine gene expression and inflammation occurs leading to lymphocytic infiltrates and to the induction of adenovirus-specific CTL (193). Initially, cellular immune response is activated when antigen-presenting cells (APCs) uptake adenovirus particles, process the particles into small oligopeptides and present them through the MHC-I molecules at the cell surface. Further binding of $CD8^+$ T cells to the MHC-I/peptide complex induces formation of Ad-or transgene-product-specific CTLs. Therefore, the novo synthesis does not seem to be required to initiate the process (195). However, for late inflammation, the expression of viral genes still encoded within Ad vectors plays a significant role. In immunocompetent hosts, this response limits the duration of transgene expression and results in adenovirus vector clearance within a few weeks of administration (192).

On the other hand, adaptive humoral immune response is initiated by the binding of adenovirus particles to the surface immunoglobulins of B cells. After internalization and virus processing, the adenovirus-derived epitopes are presented at the surface of the B cell by MHC-II molecules. Exposure of these cells to cytokines from activated $CD4^+$-Th2 helper cells will result in differentiated plasma cells secreting antibodies towards the

adenoviral capsid (196). High titers of antibodies against capsid proteins, either pre-existing because of previous exposure to natural virus or generated as a result of vector administration, may inhibit subsequent dosing with the same vector.

Different strategies to circumvent innate and adaptive immune responses have been developed. However, most of them present secondary complications and/or their use in human patients is questionable. These strategies include macrophage depletion (197,198), use of immunosuppressive agents (cyclosporine A, cyclophosphamide, dexamethasone, FK506, Interleukin-12 and deoxypergualin) (199-204), use of antibodies to deplete CTLs (205), blockade of costimulatory interaction between APCs, T and B cells (206-208), intrathymic administration of adenovirus (209), use of vector derived from non-crossreacting serotypes (210), use of adenovirus from other species (211) and coating vectors with inert chemicals like polyethylene glycol (PEG) (212).

Diverse *in vivo* studies in mice suggested that, in the absence of an immune response, firs-generation adenoviral vector DNA is maintained as a stable episome in the host cells (213,214). Last generation helper-dependent or gutless adenovirus vectors display reduced long-term toxicity and prolonged transgene expression compared to first-generation vectors after administration to peripheral organs of immunologically naïve animals (210,215-217).

Lack of coding viral genes may account for reduced adaptive cellular immune response after systemic delivery of gutless vectors. Initially, gutless vectors are capable of transducing dendritic cells and stimulating Ad-specific T-cell responses, independent of viral gene transcription (218). However, the expression of viral genes is required for T cells to exert their effector functions in the liver (219), which possibly explains the vector persistence and improved transgene expression following transduction with gutless vectors compared to results with first-generation Ad vectors.

As expected, systemic delivery of gutless vectors still induces adaptive humoral response against the vector capsid as for the firs-generation Ad vectors. Indeed, the development of Ad-specific antibodies does not contribute to the elimination of Ad-transduced cells and therefore does not affect the persistence of transgene expression. However, Ad-specific antibodies will bind the readministered Ad vector and thereby prevent cell entry and promote opsonization by macrophage.

Humoral response can also be developed towards circulating antigen induced by gutless adenoviral transfer (219). Vectors that mediated transgene expression in APCs trigger antibody formation because they increase the probability of neoantigen presentation by APCs (220), and, hence, careful selection of tissue-specific promoters may significantly improve adenovirus-associated toxicity profiles and diminish or abolish APC transduction and transgene expression (221). In addition, systemic administration of gutless vectors in a clinical setting might be inefficient because of the presence of circulating neutralizing antibodies against the same or crossreactive serotypes as a consequence of a natural infection or as a result of previous vector administration. Different successful strategies to circumvent pre-existing immunity have been applied, such as the use of alternative gutless serotype (222) and the use of a non-human gutless adenovirus (211).

As innate immune responses are dependent on the viral capsid or particle, innate responses stimulated by gutless vectors are similar to those stimulated by first-generation adenovirus vectors. Thus, dose-dependent acute inflammation was reported by Brunetti-Pierri and colleagues (223) in non-human primates following administration of high-dose gutless vectors. However, innate response, as these recently reported, may be reduced by PEG modification, probably due to lower vector uptake by Kupffer cells *in vivo* (224,225).

Surprisingly, gutless vectors have been proved to be very efficient in vaccination, due to their longer duration of expression, their

lower antiviral reactivity and their higher levels of transgene protein in dendritic cells compared to the same amount of first-generation Ad-vectors (226).

3.4 *In vivo* application of gutless adenovirus

Gutless adenovirus were administered *in vivo* to different tissues such as liver (174,227), muscle (228), central nervous system (173), retina (2), lung (229) and uterus (230) in different animal models such as mouse, rat, dog and non-human primates (baboons). Initially, the use of gutless Ad in small animals such as the mouse was successful because the transgene expression could be observed during the lifetime of the animal (170,231). Also, in larger animal models, has been reported that the Ad gutless can prolong transgene expression for over a year in baboons (Fig.8), although the expression level decreases with time due to the rate of cell replication and replacement of new cells (138,232).

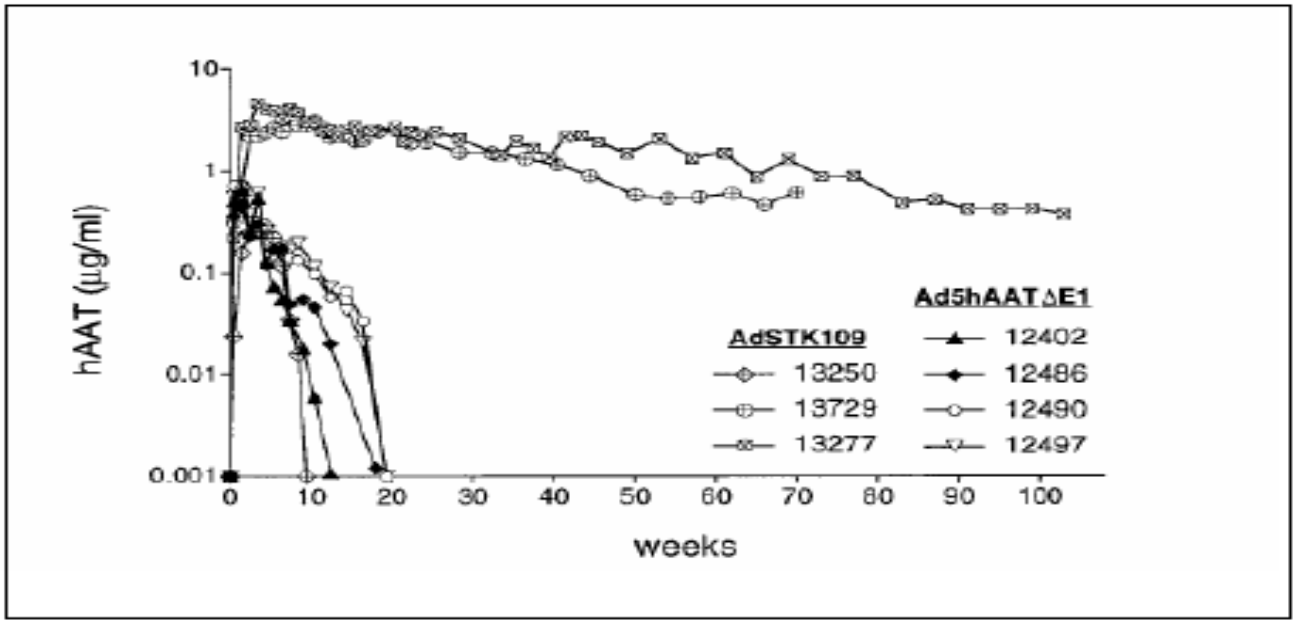

Figure 8. hAAT gene expression after administration of a gutless vector in the liver (138).

As the administration via systemic of Ad2/5 presents a high efficiency of transfection by the liver, most studies have chosen the liver as target organ of gutless Ad, which is, moreover, a very attractive organ for gene therapy due to the structure of fenestrated endothelium, which allows that the Ad can contact the parenchyma cells (233). Although a significant portion of the viral load administered systemically can reach to the liver, adenoviruses can also interact with blood cells, which inhibits the activity of virus infectivity (234-236). In liver, gutless adenovirus have been used to treat diseases such as hemophilia A, diabetes and OTC deficiency among others (171, 237-239). In all cases, levels and duration of transgene expression were always higher compared to first generation Ad.

The central nervous system (CNS) is also a very attractive organ for studies with gutless Ad, as the neurons do not replicate, and for this reason, if neurons are transfected *in vivo*, the expression of the transgene could be maintained for long periods of time, even all the life of the animal. The administration needs to break the blood-brain barrier, so the vector should not be readministered. Despite the immunoprotection of the CNS, first generation adenovirus only maintain transgene expression after 2 months of his administration (173). However, the use of gutless vector improves transgene persistence up to one year (181). It should be emphasized that the canine Ad serotype 2 (CAV-2) have a clearly neuronal tropism and can infect neurons and specifically allow retrograde axonal transport. Moreover, infecting few glial cells and oligodendrocytes (240), for this reason the gutless CAV-2 vectors are good candidates for CNS and for the treatment of neurodegenerative diseases.

The muscle is also one of the most interesting tissues in studies dealing with gutless Ad and comprising 40% of total body mass, is highly vascularized and, in addition, skeletal fibers can be transduced *in vivo*. The myofibers show a slow cell duplication, which ensures a stable expression over time. Specifically, the

disease of choice for studies with gutless Ad is Duchenne muscular dystrophy (DMD) (241,242). This disease is characterized by the deficiency of dystrophin, which induces instability in the membrane of cells and degeneration of muscle fibers. Dystrophin cDNA is 14 Kbp, for this reason for use in first-generation Ad is impractical due to its large size. However, the gutless Ad allowed cloning up to two copies of cDNA of human dystrophin gene into the genome of Ad gutless (228), obtaining prolonged expression up to 6 months. Although transgene expression was prolonged, the levels decreased by 51% at the end of the experiment. It is speculated that the expression of human cDNA results in an immune response against the transgene due to the constitutive expression of the promoter. However, when the vector is administered in immunodeficient SCID mice did not show such a response and prolonged transgene expression (243). In a similar study, Dudley and colleagues were able to obtain expression of the transgene for a year with a significant improvement of the strength of the fibers (241).

In summary, the gutless Ad offers a number of advantages as gene therapy vectors *in vivo* against first and second generation adenovirus. The absence of viral genes greatly reduces the immune response that cause and permit to reduce the administration dose. Its large capacity allows the cloning of genes for up to 36 Kbp. These virus are not integrated, thus avoiding insertional mutagenesis and can remain episomals expressing the transgene for long periods of time. However, while having a high number of advantages, its use in clinical trials with humans is questionable because even has developed an efficient system for large-scale production with low levels of contamination with helper Ad.

4. Two examples of transgenes

In this study we have used three different gutless Ad:
- HCA-RUIO as a transgene that has the combination of hIFNα and hOSM
- HCA-12 RUhIL expressing human IL-12
- HCA-RUmIL12 expressing murine IL-12

The first two vectors are also being studied *in vitro*, because they were produced during the course of this work.

4.1 Oncostatin M and Interferon-α

Oncostatin M (OSM) is a member of the interleukin-6 (IL-6) cytokine family, which includes IL-6, cardiotrophin-1 (CT-1), IL-11, leukemia inhibitory factor (LIF), and ciliary neutrophic factor (244-246). Such IL-6 type cytokines have been demonstrated to be crucial mediators during normal development, hematopoiesis, and immunity as well as in many disease states (247). The OSM signaling pathway in particular has been implicated as an essential regulator for hematopoiesis (248,249) (especially extrathymic, thymus-indipendent T-lymphocyte development) and hepatic (248), neural (250), and reproductive tract (251) organization as well as an important contributor to autoimmune disease (252) and a potential factor in controlling neoplastic cell growth (253).
Signaling by members of the IL-6 cytokine family is mediated by a heterodimeric receptor complex comprising a ligand-binding α subunit and a signal-transducing β subunit (254-257). Each of these cytokines binds its own specific α component (255,257,258). However, multiple cytokines share the common β subunit, including gp130 (259), LIF receptor β (LIFR β) (260), and OSM receptor β (OSMR β) (261,262). When cytokine is bound to the appropriate hetorodimeric complex, the β component is phosphorylated and launches a signal transduction cascade that activates other cytoplasmic proteins, such as Janus kinase (Jak1,

Jak2, and Tyk2) and signal transduction and transcription (STAT1 and STAT3) molecules (263,264). In humans, functional interaction have been demonstrated for OSM with both the gp130/LIFR β (OSM receptor type I) (260) and gp130/OSMR β (OSM receptor type II) (262) complexes.

Of the cytokines in the IL-6 family, OSM shares the greatest structural homology with LIF. Genes encoding these two proteins occupy contiguous loci on human chromosome 22 (265,266). Recently was determined the sequences of the mouse and human OSMR β genes and demonstrated that their signaling subunits also exhibited good homology with that of LIFR β (261,262).

OSM is produced by activated moncytes and macrophages (267,268), and it also secreted by dendritic cells in response to pathogen-associated molecular patterns (269). It also been shown that neutrophils produce and release OSM upon stimulation with lipopolysaccharide (LPS) or granulocyte-monocyte colony stimulator factor (270-272). OSM, as IL-6 is known to enhance the synthesis of acute-phase proteins hepatocytes (273). Altogether, these findings indicate that OSM might be a player of innate immunity. However, its role in the defense against pathogens and in the orchestration of immune response has not yet been defined.

Type I interferons (IFN-α/β) constitute a group of closely related molecules fulfilling essential functions in the early reaction against infectious agents. IFN-α/β are rapidly produced in response to viral infections and are also induced by bacteria (274). IFN-α/β interacts with a single receptor composed of two subunits, IFNAR1 and IFNAR2 (274). Signal transduction is mediated by Jak1 and Tyk2, which phosphorylate and activate STAT1, STAT2 and STAT3 proteins (274,275). STAT1 and STAT2 dimerize and together with ISGF3G form ISGF3 transcription factor complex. In addition, activated STAT1 and STAT3 can form homodimers or STAT1-STAT3 heterodimers, which also drive gene transcription (276). Binding of IFN-α/β to its receptor activates the

expression of a variety of genes that interfere with viral replication and induce an antiviral state neighboring noninfected cells. This effect, together with the enhancement of the cytotoxic activity of NK cells and macrophages (277), makes IFN-α/β a master player in innate immunity.

Type I IFNs are instrumental in linking natural and adaptive immune response (277). In particular, IFN-α is an efficient Th1-biasing cytokine which is necessary for priming $CD8^+$ T cells by antigen-presenting cells (278) and for generation and activity of citotoxic T lymphocytes (CTL) (279). Since both OSM and IFN-α activate Jak/STAT pathways after binding to their specific receptors and the two cytokines are induced in response to infection, is demonstrated the existence of functional interactions between them (280).

Larrea and colleagues characterized OSM as a new cytokine involved in the defence of the liver against infection. This idea is based on the following facts:

- in liver epithelial cells OSM increases the antiviral properties of type I IFN and induces key players of innate immunity;
- in these cells OSM synergizes with IFN-α to anhance antigen processing and presentation;
- OSM augments the immunostimulatory properties of cells of hepatocellular lineage.

Taken together these data suggest an important role for this cytokine in the activation of both innate and adaptive immunity and in linking together these two biological response to pathogens.

In conclusion, OSM has an important role in the orchestration of the defense of the liver against infection. OSM activates natural immunity and reinforces the antiviral effects of IFN-α. On the other hand, OSM may behave as a trigger of adaptive immune responses to hepatotropic viruses by stimulating antigen processing and presentation and by boosting the immunostimulatory properties of hepatic epithelial cells. These

findings open new avenues for more efficient antiviral and antitumoral therapies.

4.2 Interleukin-12

Interleukin-12 (IL-12) is recognized as a master regulator of adaptive type 1, cell-mediated immunity, the critical pathway involved in protection against neoplasia and many viruses. Since the initial preclinical and clinical studies of IL-12, done over a decade ago, basic and translational science studies have contributed to the greater understanding of IL-12 immunobiology. In addition to its noted effects in the priming of T helper 1 (Th1) cell responses and IFN-γ production by T and natural killer (NK) cells, more recent studies support its critical role as a third signal for $CD8^+$ T cells differentiation (281) and its ability to serve an important factor in the reactivation and survival of memory $CD4^+$ T cells (282). This is particular relevant in the repolarization of $CD4^+$ T cells from dysfunctional antitumor TH2 into TH1 cells in the cancer (283).

IL-12 was discovered in 1989 as “natural killer-stimulating factor” and as “cytotoxic lymphocyte maturation factor” (284,285). It was identified as a heterodimeric cytokine composed of two covalently linked p35 and p40 subunits. Initial, characterization of its biological activities revealed that IL-12, when added to human peripheral blood lymphocytes, induced IFN-γ production, increased NK cells cytotoxicity as well as T cell proliferation in response to mitogenic lectins and phorbol diesters. Subsequent studies indicated that IL-12 could boost the generation of cytotoxic T cells by promoting the transcription of genes encoding cytolytic factors including perforin and granzymes (286). In 1993, was discovered that IL-12, produced by macrophages in response to microbial pathogens, was a key cytokine in TH1 T cell differentiation. This finding established the central role of IL-12 in a pathway in which innate immune cells drove the adaptive

immune response, polarizing naïve $CD4^+$ cells towards the TH1 phenotype. The general model on the biological role of IL-12 predicts that this cytokine is required for resistence to bacterial and intracellular parasites, as well as for the establishment of organ-specific autoimmunity (286). The biological functions of IL-12 are mediated by the IL-12 receptor (287) composed of two chains (β1 and β2). Triggering of the receptor activates the JAK-STAT signaling pathway, with STAT4 being the predominant mediator of cellular responses activated by IL-12.

The antitumor and antimetastatic activities of IL-12 have been extensively shown in murine models including melanomas, mammary carcinomas, colon carcinoma, renal carcinoma, and sarcoma (288). Some of these studies have addressed the issue of local IL-12 production versus systemic delivery (i.e., intraperitoneally). Production of IL-12 at the tumor site (by neoplastic cells engineered to release IL-12 by appropriate expression vectors) induces the rejection of neoplastic cells by $CD8^+$ T cells associated with macrophage infiltration, vessel damage, and necrosis (289). Improved antitumor effects have been shown when IL-12 was administered with other cytokines such as IL-2 and IL-18 (290) or with neoplastic cells expressing costimulatory molecules (291).

The activity of IL-12 has been investigated in patients with advanced solid tumors and hematologic malignancies (292-295), as either monotherapy, or in combination with other therapies. With the exception of the results obtained in cutaneous T cell lymphoma variants (296), in AIDS-related Kaposi sarcoma (297) and non-Hodgkin's lymphoma (298), efficacy was minimal, with an objective response rate ranging between 0% and 11%.

Systemic administration of IL-12 in patients is limitated by toxicity. Based on the promising results obtained in a large series of preclinical IL-12 gene therapy studies (299), clinical trials have been designed with the aim of achieving production of the

cytokine at the tumor site, whereas maintaining low serum concentrations to reduce systemic toxicity.

III. OBJECTIVES

In this work two distinct plasmids were cloned, used for the subsequent production of two third-generation adenoviral vectors. The objectives of this work are:

- Producing the third generation of adenoviral vectors with low contamination by helper virus

- Testing *in vitro* the functionality of these vectors

- To test *in vivo*, in healthy animals, and check for any differences in the behavior of the expression of the transgene

IV. MATERIALS AND METHODS

1. Materials

1.1 Bacterial strains

DH10B™ Competent Cells obtained from Invitrogen used for general amplification of plasmids
Stbl2™ Competent Cells obtained from Invitrogen used for the amplification of big plasmids, like the plasmid used for the construction of gutless adenovirus (36 kpb)

1.2 Plasmid vectors

The plasmids used to generate all plasmids of this work were: *pGOSMIFN* (transgene hOSM and hIFNα), pRS21 (shuttle plasmid that contains the hIL-12 gene) *pRS17* (RU inducible system), *pD28E4* (backbone of the gutless vector).
Instead, the plasmids constructed for this work are: *pRUIFNOSM* (shuttle plasmid), *pHCA-RUIO* (gutless plasmid) and *pHCA-RUhIL12* (gutless plasmid).

1.3 Viral vectors

In this study we have used the following viral vectors: *HCA-RUIO* (gutless adenovirus expressing hOSM and hIFNα), *HCA-RUhIL12* (gutless adenovirus expressing hIL-12), *HCA-RUmIL12* (gutless adenovirus expressing mIL-12) and *AdTetCre* (helper adenovirus used to produce the gutless adenovirus).

1.4 Cell lines

HEK 293: ***Human Embryonic Kidney cells 293.*** Immortalized cell line from human embryonic kidney cells, obtained from American Type Cell Culture Collection (Rockville, MD).
HEK 293Cre4: HEK 293 cell line that has incorporated the Cre recombinase in the genome in a stable form.
Huh7: is a well differentiated hepatocyte derived cellular carcinoma cell line that was originally taken from a liver tumor.

1.5 Primers

The primers of IRES (sequence that divide p35 reagion and p40 in the pHCA-RUmIL12) used for the quantification of viral genome in the liver are:

- Sense: 5'-AGAAGACGCACTCCCTCTCCT-3'
- Antisense: 5'-TGGCAGATGGAGATTGAGAGC-3'

1.6 Animals

C57BL/6J mice, 5 to 8 weeks old, were purchased from Harlan (Barcelona, Spain)
Golden Syrian Hamsters, 6 to 9 weeks old, were purchased from Charles River.
The animals were kept under standard pathogen-free conditions and received care according to criteria outlined in the "Guide for the Care and Use of Laboratory Animals" by the National Academy of Sciences.
The experiments were performed in accordance with the local animal commission.

2. Methods

2.1 Construction of pHCA-RUIO and pHCA-RUhIL12

The pHCA-RUIO was constructed as follows: an ClaI fragment from the pGOSMIFN, that contains the hOSM and hIFNα genes, was cloned into pRS17, that contains the liver specific inducible system, cut with SwaI, to generate pRUIFNOSM.
In the NotI fragment from the pRUIFNOSM, but with blunt ends, was created by the insertion of an oligonucleotide sequence. The same blunt ends were done in the pD28E4 cut with AscI. The binding between the fragment from pRUIFNOSM and pD28E4 creates pHCA-RUIO.

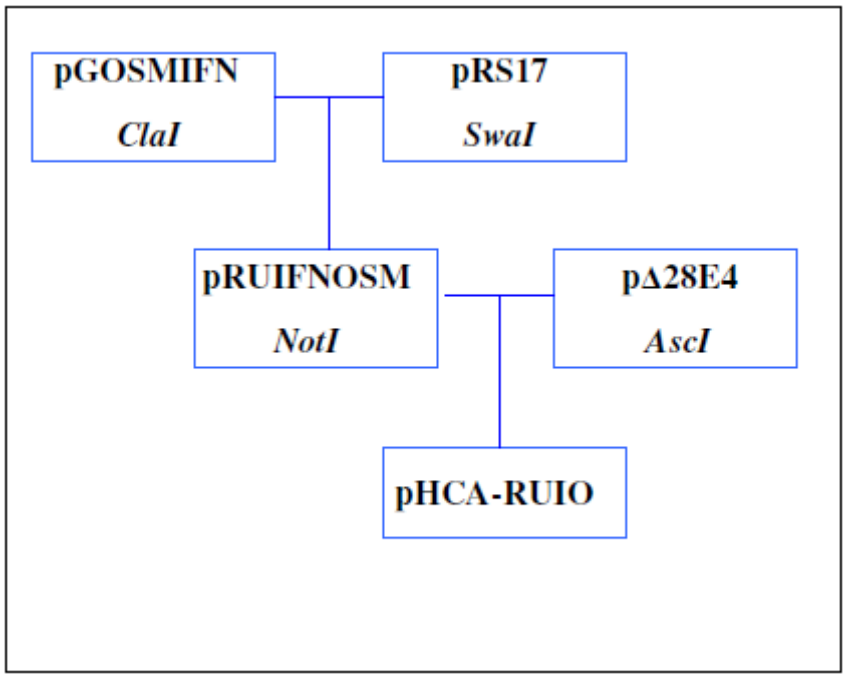

Figure 9. Scheme of cloning to obtain pHCA-RUIO

The binding between the NotI fragment from the pRS21, with blunt ends, and pD28E4 cut with AscI, creates pHCA-RUhIL12.

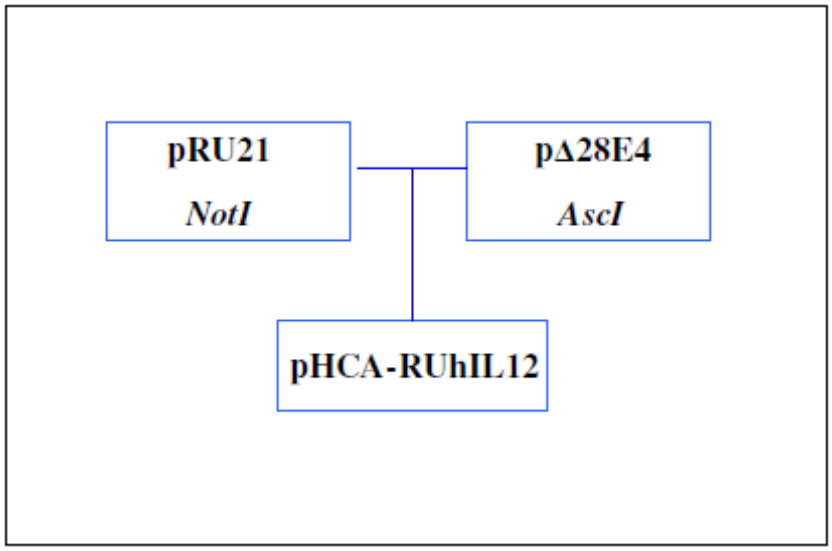

Figure 10. Scheme of cloning to obtain pHCA-RUhIL12

2.2 Production of Gutless Adenovirus Vectors

After PmeI digestion, and ethanol precipitation, 15µg of pHCA-RUIO or pHCA-RUhIL12, were transfected into 10^6 293Cre4 cells with 20 µg of lipofectamine. After 6 hours from the transfection, AdTetCre (helper virus) was added at MOI=1. After 2 days of incubation with the helper Adenovirus, the cells were harvested, and frozen at -80°C. For another three steps, 10^6 293Cre4 cells are infected with a mixture containing the cell lysate of the previous step and helper adenovirus (MOI = 1). From the fifth step begins an exponential amplification of the vector. The fifth step 2 x 10^7 293Cre4 cells must be infected, in the follow step the infected 293Cre4 cells are 2 x 10^8, and the last step needs 6 x 10^8 293Cre4 cells.

Finished the last step of amplification, the cells are harvested, lysed (subjecting them to three cycles of rapid freezing with dry ice) and purified twice by CsCl equilibrium density centrifugation. The first purification takes place through a discontinuous CsCl gradient centrifugation. The discontinuous gradient was formed, starting from the bottom, from a first phase with CsCl to a concentration of 1.5 g/ml, a second phase to a concentration of 1.35 g/ml and a third at 1, 25 g/ml. Above this gradient of CsCl trying to settle the cell lysate. After centrifugation at 25000 rpm

over night, you get a band of the first virus that would be in a position between 1.5 and 1.35 g/ml. This band is subjected to a second overnight centrifuge at 30000rpm in a continuous CsCl gradient (only 1.35 g/ml).
After this second centrifugation gives you a new band of virus, which will be collected and subjected to purification by a saphadex G50 column. This step is used to purify the virus from CsCl that was used previously. Having balanced the Sephadex column with 20ml of 0.1M Tris pH 8.1, the band containing the virus was loaded. The virus passes slowly through the column and eluted. The eluate obtained is collected in eppendorf and subjected to spectrophotometric analysis to determine in which fractions of the eluate the virus is present. The fractions of eluate that have an optical absorbance at 260 nm greater than 0.04, are retained because they contain the virus. The formula for calculating concentration of the virus is:

$$\text{Concentration (v.p.)} = OD_{260} \times \text{dilution} \times 10^{12}$$

Finally, the fractions of eluate containing the virus are merged and stored at -80°C.
Titration of the helper adenovirus is made to infection of 293 cells with serial dilutions of the vector. The cells after incubation for 45 hours are fixed with methanol and is then carried out an immunocytochemical against exon VI of the adenoviral capsid (Sigma Aldrich) according with to the manufacturer's instructions.

2.3 Test of expression in cells infected with HCA-RUIO and HCA-RUhIL12 *in vitro*

Huh7 cells were cultured on 24-well plates for 24 hours before the infection. The cells were infected with HCA-RUIO or HCA-RUhIL12 with 0,1 µl of virus, or with different volumes of the lysate of the last step of production of vector, in medium containing 2% FBS for 24 hours at 37°C. The vector containing medium was replaced with culture media containing 10^{-8} mol/L of

RU486. The supernatants were collected at different times, from 10 hours to 7 days after the administration of mifepristone. The hIL-12 or hOSM protein levels were determined in an enzyme-linked immunosorbent assay (ELISA; Pharmigen; R&D Systems) according with to the manufacturer's instructions.
The same experiment was done with the plasmids pHCA-RUIO and pHCA-RUhIL12. In these cases, the Huh7 cells were transfected with the plasmids by the use of lipofectamine.

2.4 Animal studies by HCA-RUIO, HCA-RUhIL12 and HCA-RUmIL12

The gutless adenoviruses were administered into Golden Syrian Hamsters and C57BL/6J mice by intrahepatic injection at different doses per animals.
Injection of the virus was performed to laparotomy. After making the abdominal incision, was carried in the identification of the left hepatic lobe. Performing a liver lobe prick and make the injection slowly. After entering all the volume, remove the needle and stop the bleeding. At the time the bleeding stops, replace the hepatic lobe. If necessary, the vectors were diluted in physiological saline in a total volume of 100µl.
Depending on the type of experiment, different inductions were made by intraperitoneal injections of RU486 at different concentration, and serum was collected at different time points after induction. RU486 was dissolved in sesame oil (Sigma Aldrich).
Blood samples were collected from retro-orbital plexus of the animals. The hIL-12, hOSM and mIL-12 protein levels were determined by using ELISA assay (Pharmigen; R&D Systems) according with to the manufacturer's instructions.

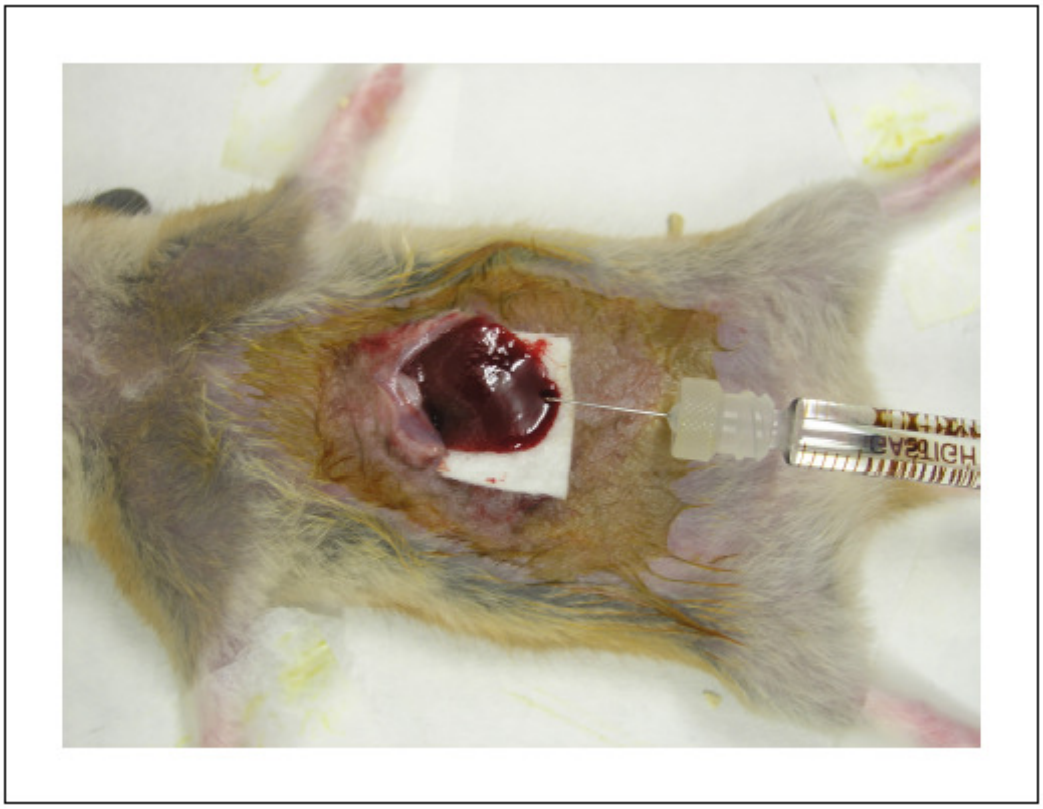

Figure 11. Intrahepatic injection of the vector in hamsters

2.5 Isolation of DNA

The hamsters receiving HCA-RUmIL12 were killed at the indicate time, and samples of left hepatic lobe were collected. Total DNA from the liver were isolated with TRI Reagent (Sigma Aldrich) according to the instruction of manufacturer.

2.6 Polymerase chain reaction analysis

For detection of vector in tissue, polymerase chain reaction (PCR) Quantification was used with primers corresponding to the region IRES. This region is the part of genome that divides the p35 region, and the p40 region in the pHCA-RUmIL12 gene. These primers included IRES sense:
5'-AGAAGACGCACTCCCTCTCCT-3'
and IRES antisense:
5'-TGGCAGATGGAGATTGAGAGC-3'

DNA, 2µl, from the tissue was used for amplification in 20µl reaction volumes including 6pmol of each primer and 10µl of SYBR Green. PCR amplification was carried out for 1 cycle of 3 minutes at 95°C followed by 40 cycles of 10 seconds at 95°C and 30 seconds at 61,4°C.
The quantification of DNA obtained from this PCR were normalized according to the weight of the piece of tissue used for DNA extraction.

V. RESULTS

For the realization of this work, two of the three Third Generation Adenoviral vectors were produced *de novo*: HCA-RUIO and HCA-RUhIL12.
After the production of these we proceeded with the characterization of the expression *in vitro* and *in vivo*.

1. Construction of Adenoviral vector genome HCA-RUIO

For the construction of HCA-RUIO, the expression cassette was introduced into the plasmid **pΔ28E4**, which contains the ITRs, the packaging signal (Ψ) and the stuffer DNA of human origin. All the elements of the expression cassette were subcloned in a shuttle vector named **pRUIFNOSM**. It contains the liver-specific TTR promoter controlling the transactivator GLP65, together with the coding sequence for OSM and IFN-α linked by a internal ribosomal entry site sequence (IRES), and controlled by the inducible promoter that responds to the transactivator. For the construction of pRUIFNOSM plasmid, the OSM-IRES-IFN fragment present in the **pGOSMIFN** plasmid was inserted into the SwaI site of the previously described pRS17 plasmid, which contains all the elements of the inducible system (300).
To check if the insert has the right orientation in the plasmid, it was necessary to digest of **pRUIFNOSM** with restriction enzyme *KpnI*. Using a free database of restriction enzyme, the previous reaction must give piece of plasmid with sizes reported in the table:

Right Orientation	**Opposite orientation**
6798	6798
1177	1416
1009	722
416	416

Table1. pRUIFNOSM size fragments after digestion with *Kpn*I, in the case of the right or the opposite orientation of the insert

After the digestion with the restriction enzyme, with an electrophoresis in agarose gel 1%, it is possible to see that the insert has a right orientation in the shuttle plasmid (Fig. 12)

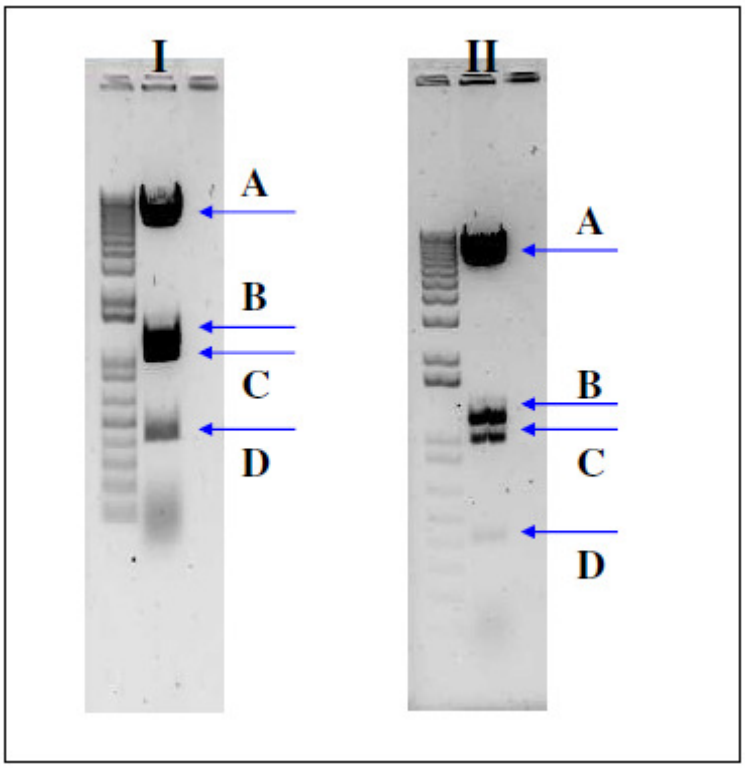

Figure 12. electrophoresis in Agarose ._gel 1% of the digestion of pRUIFNOSM with *KpnI*, after 20 minutes (I) and after 60 minutes (II)

With the confirmation of the right orientation of the insert, now it is possible to do the final ligation between the insert of the plasmid pRUIFNOSM and the vector pΔ28E4.
The final result is the plasmid pHCA-RUIO, it was checked with three different digestions with the restriction enzymes HindIII, XbaI and PmeI (Fig.13). The plasmid pΔ28E4 was included as a control.

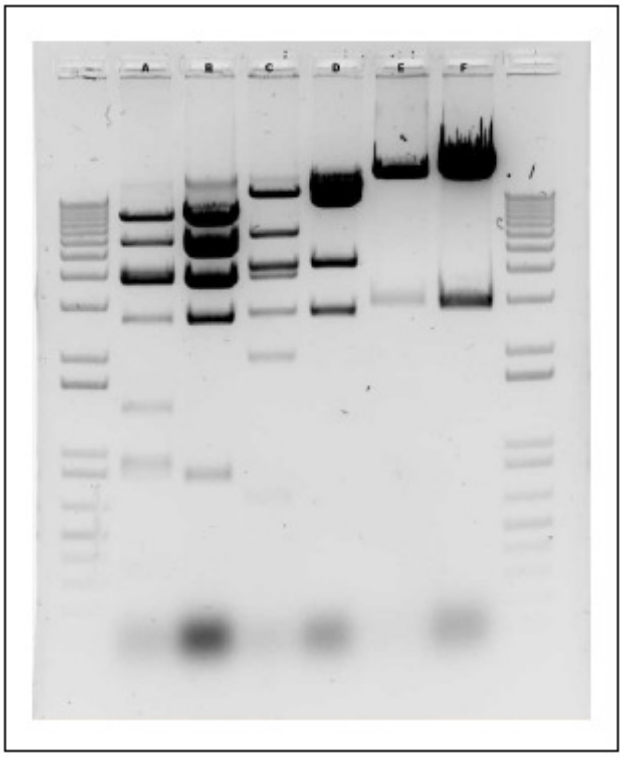

Figure 13. The restriction enzymes HindIII and XbaI (Fig.3A-3D) were used to evaluate the transgene orientation of pHCA-RUIO. PmeI (Fig.3E-3F) flanked the resistance Kanamycine gene, so its digestion verified the pΔ28E4 backbone.

2. Expression of the Adenoviral vector genome pHCA-RUIO

Before the production of the adenoviral vector HCA-RUIO, it is necessary test the expression *in vitro* of the plasmid. To this end, we carried out quantification of OSM by a commercial ELISA kit. We verified that the expression of hOSM is more intense than hIFNα in this construct, which was predicted based on the localization of the genes relative to the IRES. Although we confirmed the expression of hIFNα, detection of hOSM was routinely used as a sensitive marker of the function of the expression cassette. The hOSM was detected, in the supernatants of the human hepatocellular carcinoma cells Huh7 tranfected with pHCA-RUIO. The expression of the transgene was induced with RU486 after 24 hours from transfection of the cells. The hOSM was detected after 120 hours (5 days) of incubation with RU486

and 10 hours (5days + 10 hours) after the changing of the medium with a new dose of inductor.

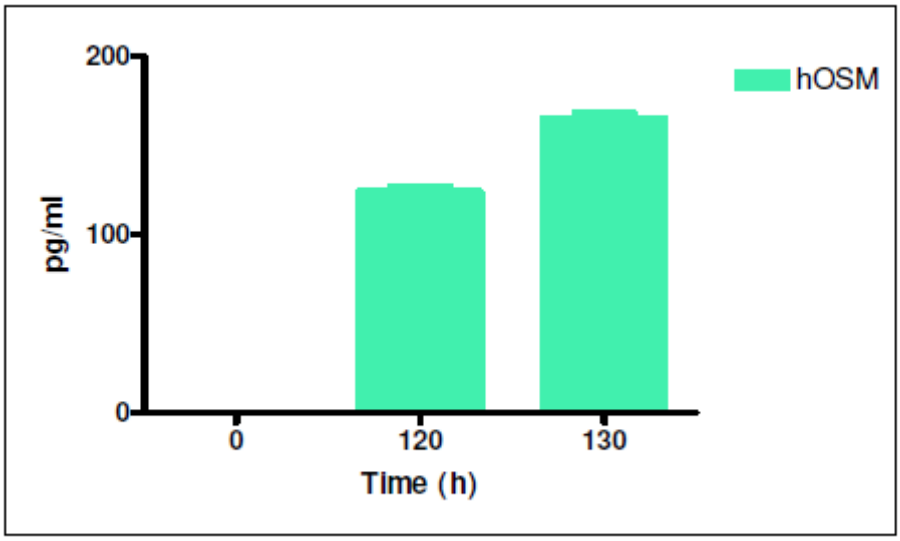

Figure 14. Characterization of pHCA-RUIO in vitro. Huh7 cells were transfected with pHCA-RUIO for 24 hours. The media were removed and the cellswere incubated in presence of RU486 10^{-8}mol/lfor 120 and 130 hours. The supernatants were collected for determination of hOSM by ELISA.

As reported in Fig.14, the Huh7 cells transfected with the plasmid pHCA-RUIO, after induction with RU486, start to express the trangene and after 5 days of incubation of RU486 and 10 hours with a new dose of inductor we have the top of expression.

3. Construction of Adenoviral vector genome HCA-RUhIL12

To create the genome of the adenoviral vector HCA-RUhIL12, the shuttle plasmid pRS21 containing the transgene for human IL12 was previously created. The gene of interest was released by cutting this plasmid with the restriction enzyme NotI, and generating blunt ends by T4 DNA polymerase treatment.
The final plasmid pHCA-RUhIL12 was obtained with the ligation between the plasmid pΔ28E4, cut with the restriction enzyme AscI and with a artificial blunt ends, and the insert from pRS21. To

confirm that the insert is well oriented it is necessary a digestion with two restriction enzymes: BglII and XhoI.
If the insert is oriented correctly, the expected fragments are indicated in the table.

Bands with BglII	Bands with XhoI
10150	12713
7714	8206
6500	7208
6192	6203
4673	3721
2694	
128	

Table 2. pHCA-RUhIL12 size fragments after digestion with *Bgl*II and *Xho*I

The backbone plasmid pΔ28E4 and the shuttle plasmid pRS21 were used as controls in the digestion, and the result is indicated in Figure 15.

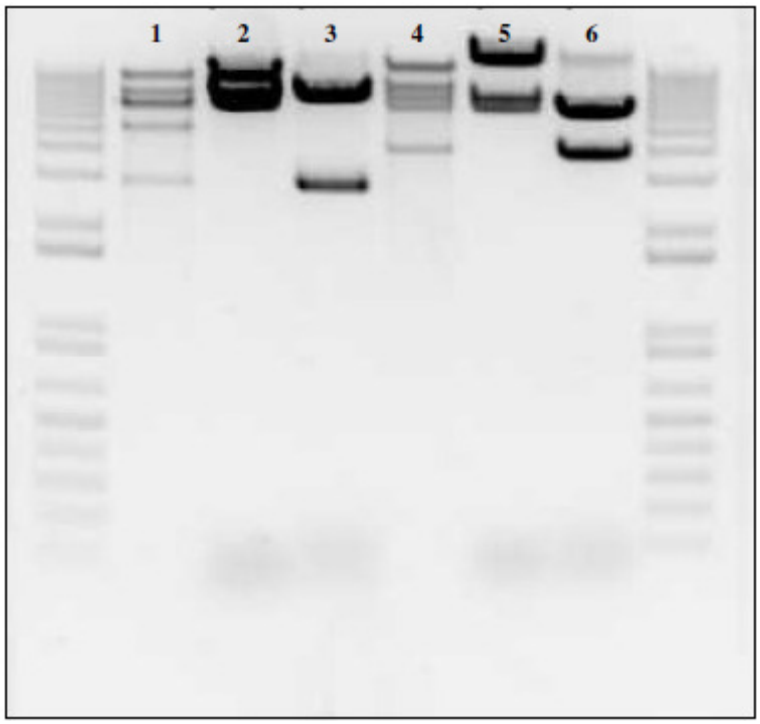

Figure 15. The restriction enzymes *Bgl*II and *Xho*I (1,4) were used to evaluate the transgene orientation of pHCA-RUhIL12, pΔ28E4 (2,5) and pRS21 (3,6) were used as control.

4. Production of the two Adenoviral vector gutless HCA-RUIO and HCA-RUhIL12 and titration of the contamination with the Helper Virus

Both adenoviral vectors were produced with the system developed in the department of gene therapy and hepatology, at the University of Navarra (patent WO2009/138544), using the 293Cre4 cells and the Adenovirus Helper AdTetCre.
Using this system the third generation adenoviral vectors were generated with a low percentage of contamination with the Adenovirus Helper.
After the amplification and purification with a double gradient of CsCl (Fig.16), quantification of viral particles of the Adenoviral vector was performed by absorbance at 260 nm using a spectrophotometer.

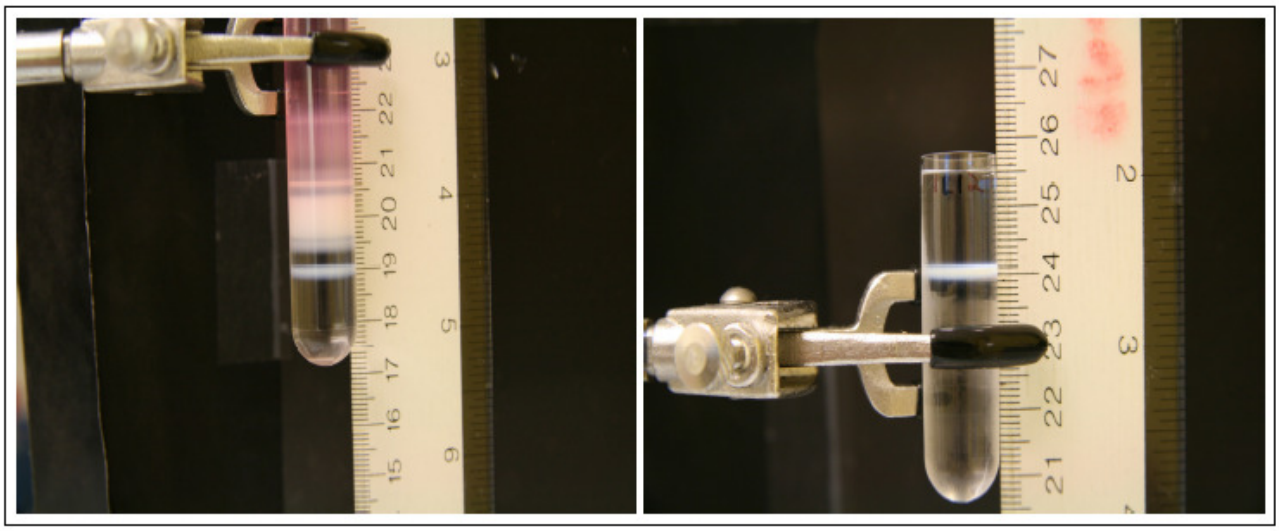

Figure 16. Result after the two centrifuges with CsCl, there is clearly a thin white stripe of virus. The first is after the discontinuous CsCl gradient, the second is after the continuous gradient of CsC

The titration for the contamination of the Adenovirus Helper was done by immunocitochemistry using antibodies against adenoviral late proteins. The titration of the Helper virus was verified in all steps of the production of the viral vector in order to monitor the adequate progress of the process. The percentage of contamination in the last step is shown in the table 3.

HCA-RUIO			
	Titer of the virus (v.p/ml)	Titer of the Helper virus (i.u./ml)	%
Stock 1	$1{,}14 \times 10^{12}$	$3{,}92 \times 10^{6}$	$3{,}44 \times 10^{-4}$
Stock 2	$1{,}13 \times 10^{12}$	$1{,}68 \times 10^{6}$	$1{,}49 \times 10^{-4}$

HCA-RUhIL12			
	Titre of the virus (f.p/ml)	Titre of the Helper virus (i.u./ml)	%
Stock 1	$2{,}11 \times 10^{12}$	$4{,}09 \times 10^{8}$	$1{,}94 \times 10^{-2}$

Table 3. Titration of the two gutless Ad produced in this work, contamination of helper Ad and its percentage.

4.1 Test of expression of HCA-RUIO before the purification of the virus

The function of the High Capacity Adenovirus vector was tested before the final step of amplification and purification, infecting Huh7 cells with different volumes of lysate of 293Cre4 cells from the fifth passage of production.
Huh7 cells were infected with the virus and RU486 was added to the culture medium 24 hours later. The treatment was maintained for 5 days, and then the medium plus RU486 was changed. Ten hours later the supernatants was collected and the presence of hOSM was measured by ELISA (Fig.17).

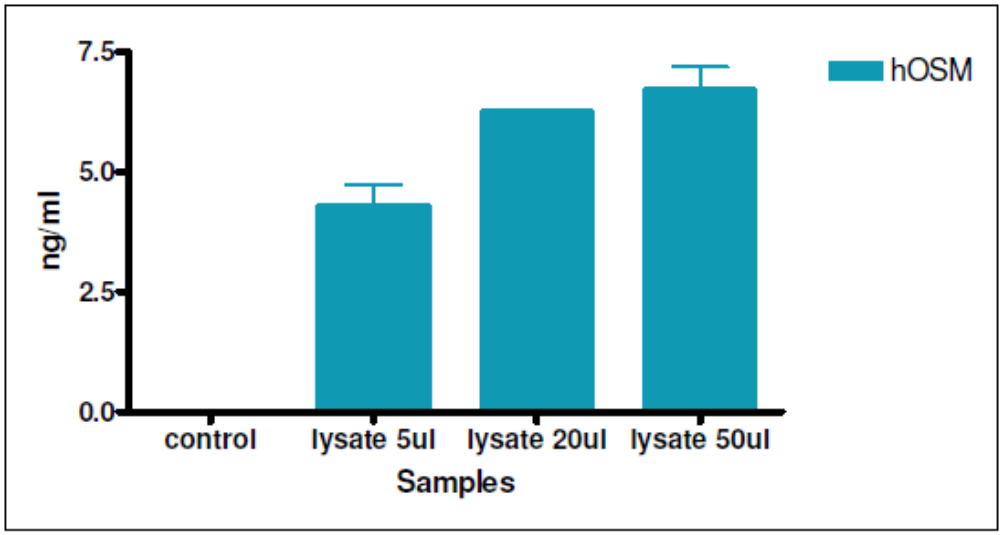

Figure 17. Expression of hOSM in Huh7 cells infected with the lysate of the fifth passage of the production of HCA-RUIO

Once the function of the vector was verified, the final steps of production were carried out .

4.2 Test of expression of HCA-RUhIL12 before the purification of the virus

The same experiment was done to testing the activity of the HCA-RUhIL12, but infecting Huh7 cell with the last passage of production.

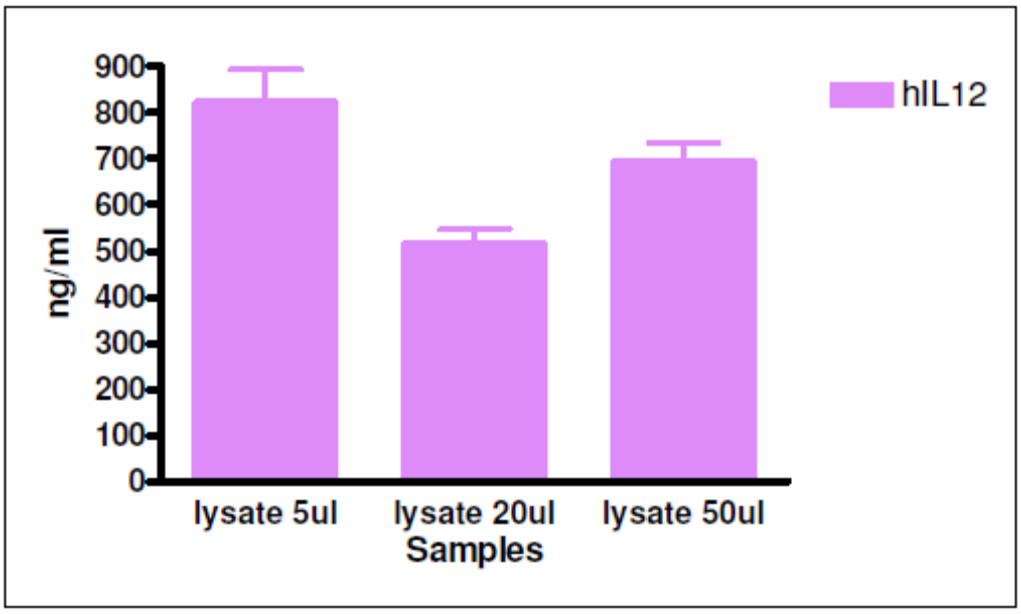

Figure 18. Expression of hOSM in Huh7 cells infected with the lysate of the last passage of the production of HCA-RUhIL12

This result demonstrates the function of the HCA-RUhIL12 vector (Fig.18).

4.3 Test of expression of purified HCA-RUIO and HCA-RUhIL12

After the purification of the two adenoviral vectors, and before the performance of *in vivo* tests, the expression of the transgenes was verified again on Huh7 cells.

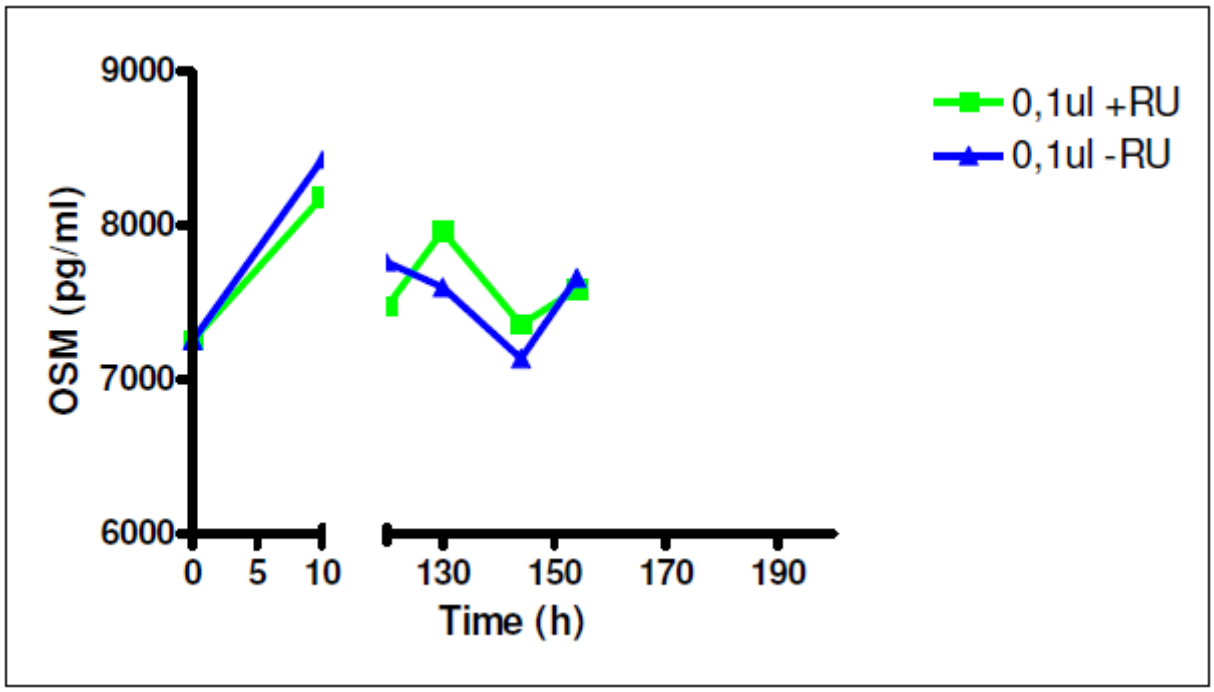

Figure 19. Expression of hOSM in Huh7 cells infected with 0,1µl of HCA-RUIO

The graphic represents the amount of OSM detected in the supernatant of cells infected with 0.1 ul of HCA-RUIO vector. The induction protocol was the same as described above (treatment with RU486 for 5 days and then renovation of medium with inducer). A peak of OSM is observed 10 hours after initiation or renovation of treatment. Surprisingly, production of OSM was also observed in infected cells in the absence of RU486. This effect may be due to the experimental conditions in cell culture, because the same phenomenon was observed with other HC-Ad vectors such as the HC-Ad/RUmIL-12 (Fig.20), in which no basal activity was detected in vivo. The expression of transgene in the absence of inducer may be due to high MOI obtained in cell culture and the accumulation of transactivator in these circumstances.

As for the previous vector, was tested if Huh7 cells transfected with HCA-RUhIL12, after an incubation with RU486, express the molecule hIL-12.

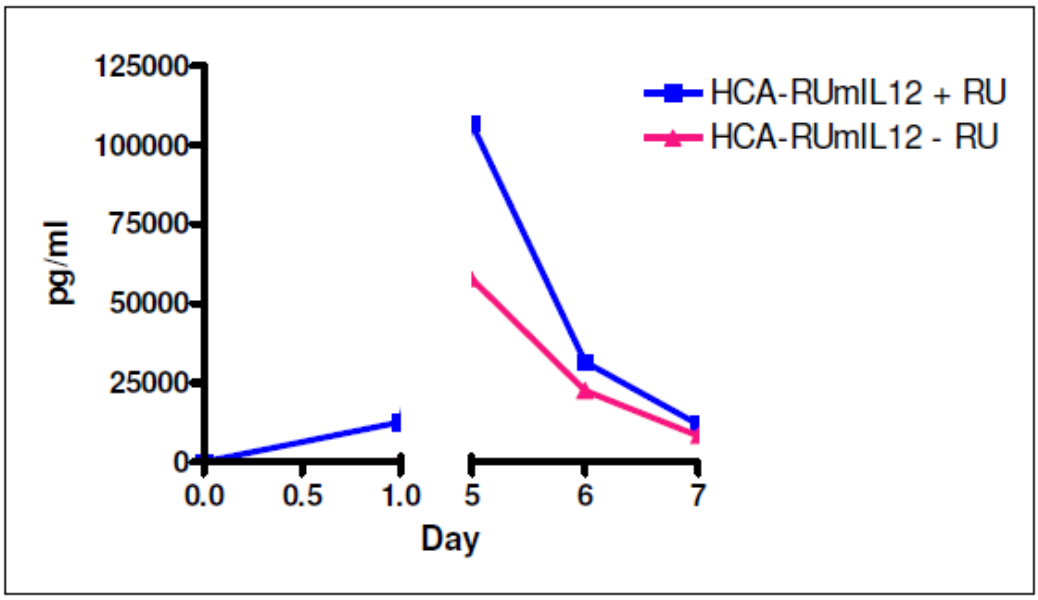

Figure 20. Expression of mIL-12 in Huh7 cells infected with HCA-RUmIL12 and incubate in the absence or presence of RU486 10^{-8} mol/L

The result reported in the Fig.21, highlights that after 5 days of incubation with RU486 the Huh7 cells express hIL12 and it was detected 8 ng/ml, but if you change the medium at day 5 and wait 10 hours, the cells express more or less the double of hIL12. It is also clear that cells tranfected with the virus, but not induced with RU486, do not express hIL12.

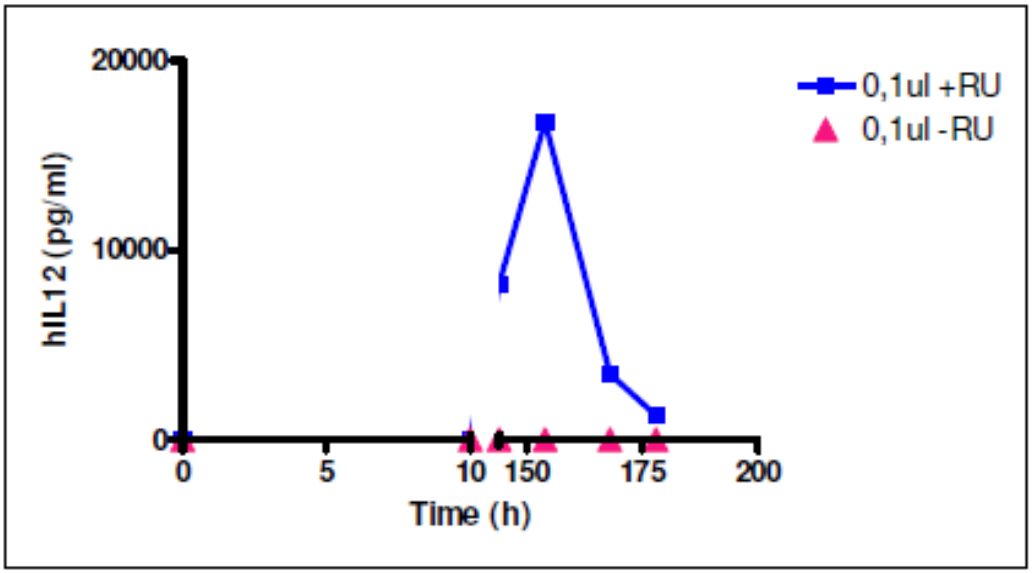

Figure 21. Expression of mIL-12 in Huh7 cells infected with HCA-RUhIL12 and incubate in the absence or presence of RU486 10^{-8} mol/L

5. Test in vivo with HCA-RUIO in Hamsters

In order to characterize the function of the vectors in vivo in an animal model in which hIFNa and hOSM are active, we started experiments in Syrian hamsters. In an initial experiment, 5,7 x 10^{10} v.p. of HCA-RUIO was administered by intrahepatic injection following laparotomy . Since this was the first time that a HC-Ad vector was used in these animals, a control vector (HC-Ad/RUmIL-12) was administered in parallel in a second group of syrian hamsters. The performance of the HC-Ad/RUmIL-12 vector has been extensively studied in mice (300). Using this vector, intense and controlled expression of mIL-12 can be maintained for long periods of time using different induction regimes with RU486. After 10 days of the injection of the HCA-RUIO virus, 125µg/Kg of RU486 was administrated and 10 and 24 hours later blood samples were taken from the retro-orbital plexus of the animals. Another administration of RU486 was done after 21 days from the injection of virus, using a higher dose of inducer (see scheme of the protocol below).

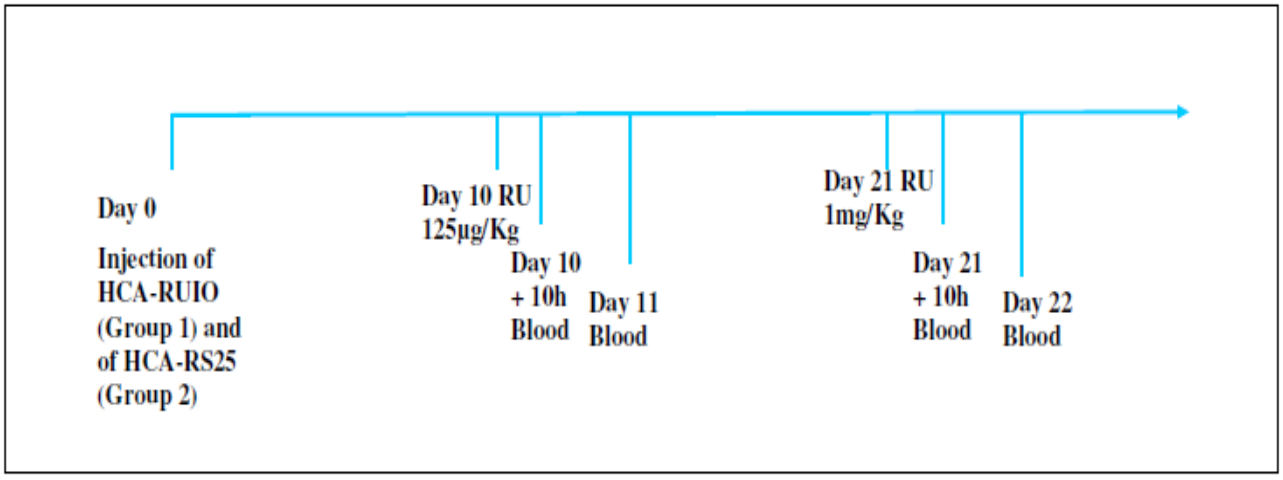

Figure 21. Scheme of the experiments

It was not possible to detect neither OSM nor mIL-12 in the serum of these animals, even with 1 mg/Kg of RU486. One possible explanation is that the dose of vector was too low to sustain detectable transgene expression in these animals.

After this result other two groups of Hamsters were done with two different doses of HCA-RUIO, the first group of four Hamsters with 2,28 x 10^{11} v.p. of HCA-RUIO and the second group of one Hamster with 5,7 x 10^{11} v.p. of HCA-RUIO.

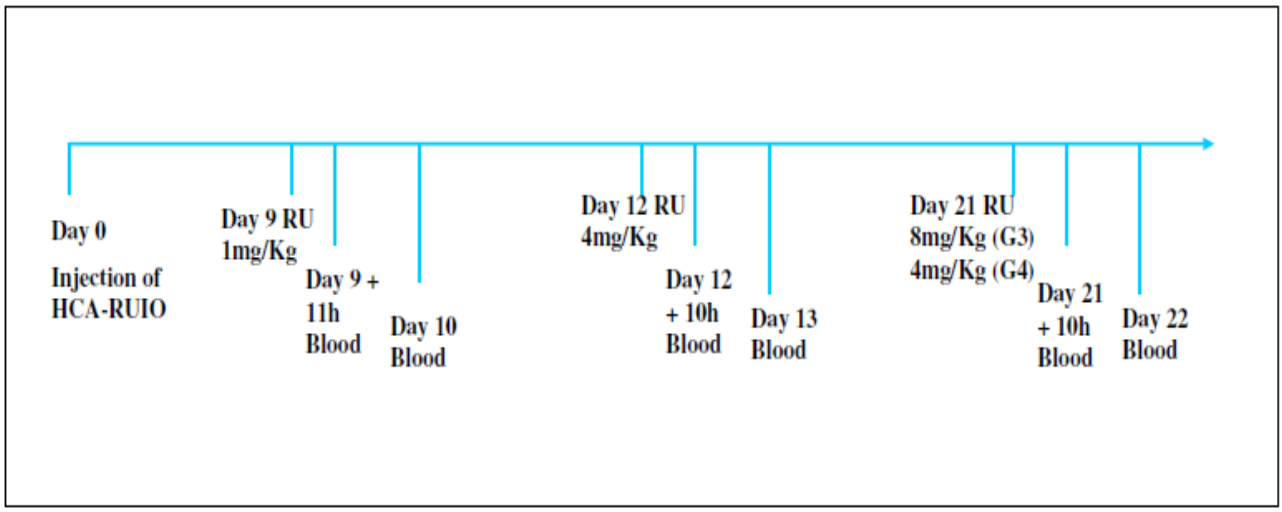

Figure 22. Scheme of the experiment

Following the scheme reported in the previous table, hOSM was not detect in the Hamsters with 2,28 x 10^{11} v.p. of HCA-RUIO. Only in the Hamster with 5,7 x 10^{11} v.p. of HCA-RUIO and with the administration of 4 mg/kg RU486 at day 12 a very low level of hOSM (60 pg/ml) could be detected. Expression of hIFNa was verified in this animal. The kinetics of expression was identical for both transgenes, although hIFNa production was lower than hOSM, as expected (Fig.23). When the same amount of RU486 was used at day 21, no expression of OSM could be detected. These results suggest that high doses of HC-Ad are needed to achieve expression of transgenes in hamsters. In addition, problems with the re-induction of OSM production were detected.

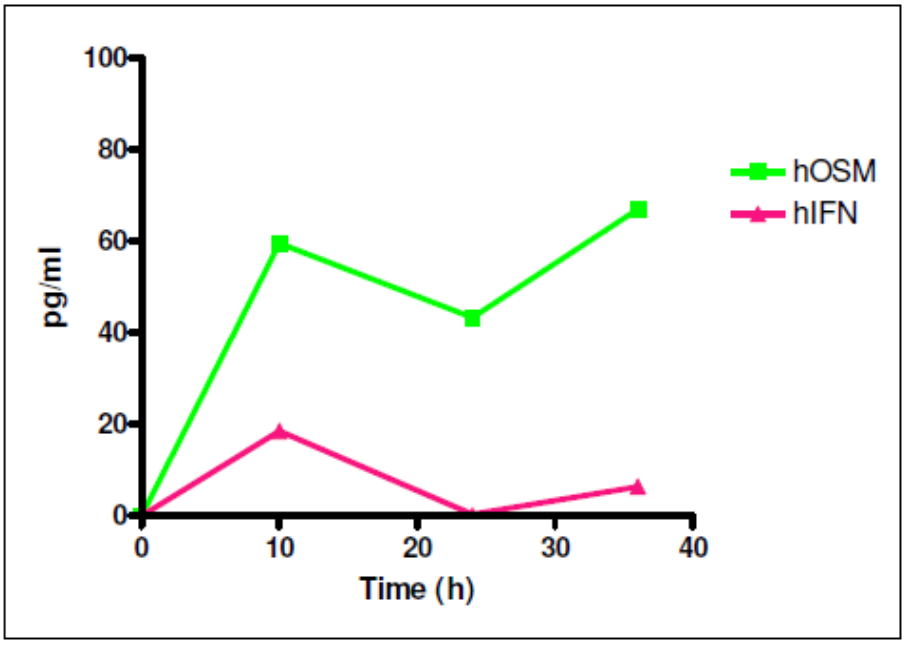

Figure 23. Expression of hOSM and hIFNα in Hamster with 5,7 x 10^{11} v.p. of HCA-RUIO

6. First approach of HCA-RUIO in mouse

After the first approaches with the vector HCA-RUIO in Hamster, 1,14 x 10^{11} v.p. were injected in a mouse, in order to determine if the difficulties observed were due to the different species.

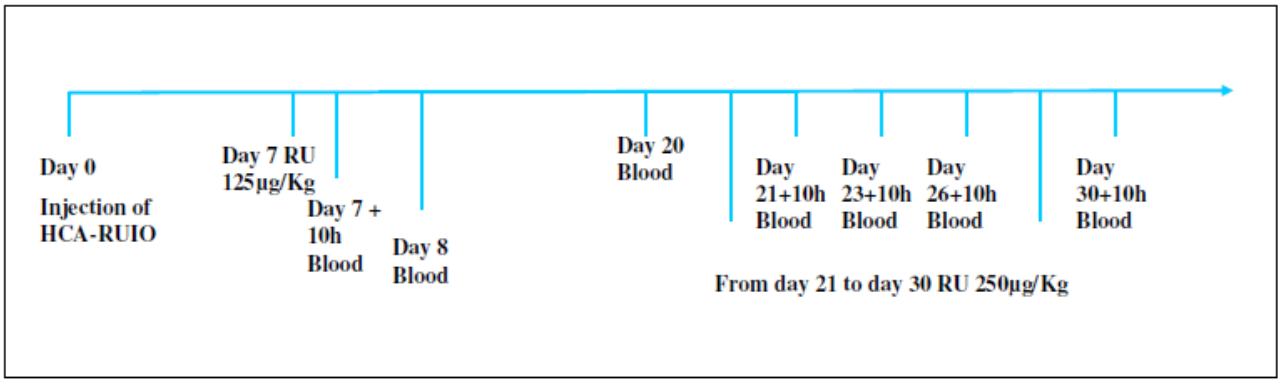

Figure.24 Scheme of the experiment

Following the timetable reported in the previous table, the first dose of RU486 was administrated after 7 days from the injection of the vector. Blood samples were collected after 10 and 24 hours and hOSM could be detected. As reported in fig. after 10 hours from the induction there is a pick of expression of hOSM. With

this result after 21 days from the injection of the virus, 250 µg/Kg of RU486 was administrated for 10 days. During this second induction blood samples was collected for four times, but expression hOSM was not detected.

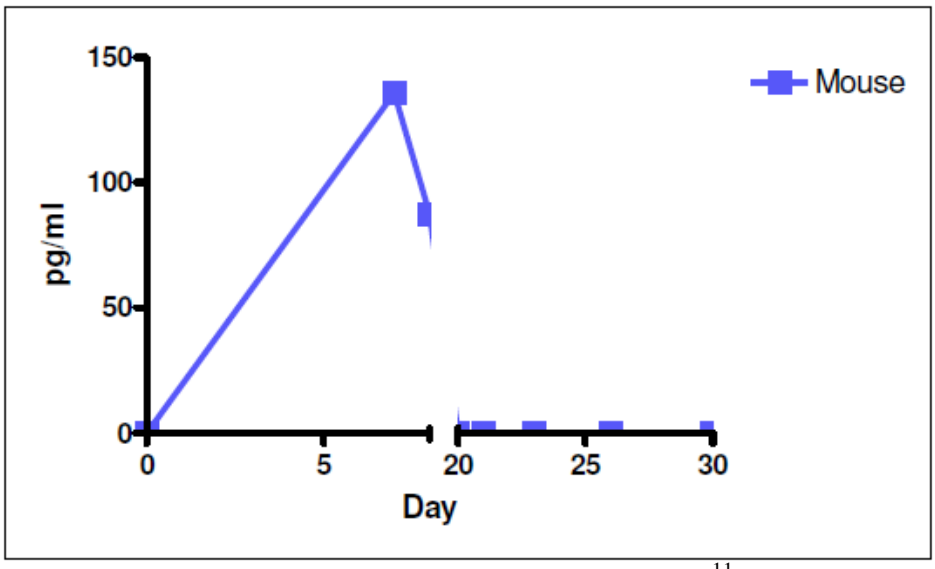

Figure 25. Expression of hOSM in a mouse with 1,14 x 10^{11} v.p of HCA-RUIO

As can be seen from the figure 25, after the first induction there is a peak of expression at 10 hours from the induction, that is diminishing at 24 hours. This is consistent with the kinetics of transgene expression already described for mIL-12 in mice. However, in the case of HCA-RUIO it was not possible to detect OSM if induction is repeated two weeks later, in contrast with the results observed with vectors that express murine or human IL-12 (Ref).

7. Comparison of the expression of the vectors HCA-RUIO, HCAd/RUmIL12 and HCA-RUhIL12 in mice

To further clarify these differences, HC-Ad vectors expressing hOSM plus hIFNa, murine IL-12 or human IL-12 were administered in mice and transgene expression was studied in parallel. The following experimental groups were established:

- 5 mice with 1,13 x 10^{11} v.p. of HCA-RUIO
- 5 mice with 2,26 x 10^{10} v.p. of HCA-RUIO

- 4 mice with 6,65 x 10^{10} v.p. of HCA-RUIO
- 5 mice with 1,05 x 10^{11} v.p. of HCA-RUhIL12
- 4 mice with 2,11 x 10^{11} v.p. of HCA-RUhIL12
- 5 mice with 1,23 x 10^{11} v.p. of HCAd/RUmIL-12

The same protocol of administration of RU486 was performed for all the groups. As reported from in the figure, the first induction was done after 9 days from the injection of the virus, with a concentration of RU486 of 250µg/Kg, and samples of blood was collected after 10, 18, 24 hours from the induction. A second induction, with the same concentration of RU486, was done after 40 days from the injection of the virus.

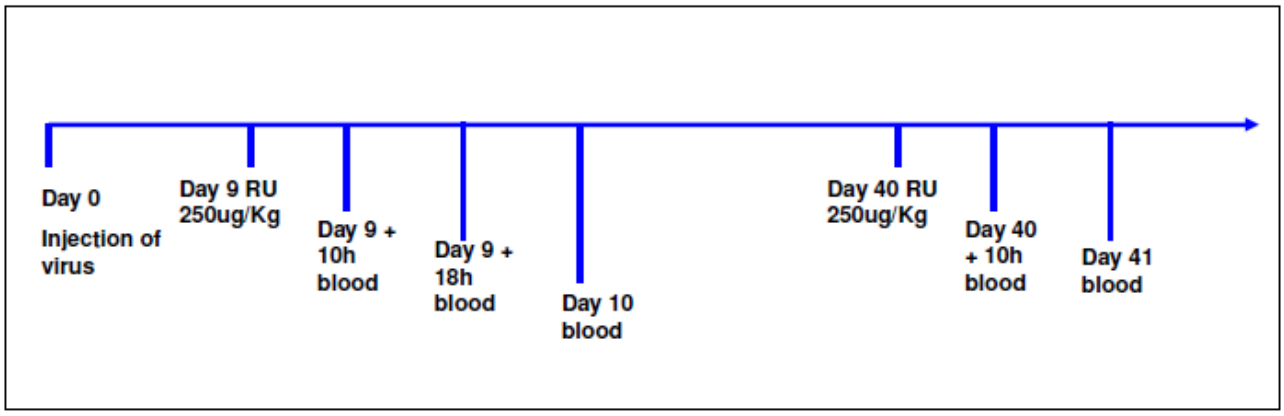

Figure 26. Scheme of the experiment

For the group of mice with the vector HCAd/RUmIL-12, the expression of mIL12 can be detected at the first induction as well as at the second induction, as expected based on previous results (Fig.27).

In the group of mice with 1,13 x 10^{11} v.p. of HCA-RUIO, as reported in figure 28 , there is a high expression of hOSM, but the consequence of this high expression of the transgene is a high mortality rate, with 4 out of 5 mice dead after the first induction. The alive mouse did not express the transegene, probably because of an inefficient vector inoculation in the liver.

In the group of mice with 2,26 x 10^{10} v.p. of HCA-RUIO, the expression of the transgene was not detected, probably due to a low dose of virus

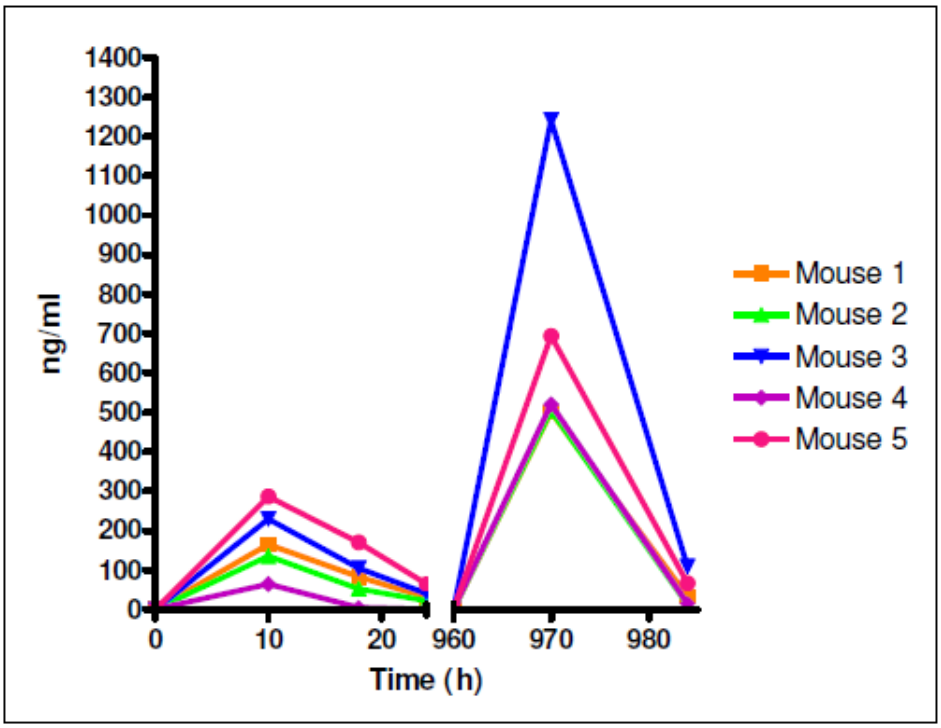

Figure 27. Expression of mIL-12 in mice with HCA-RUmIL12

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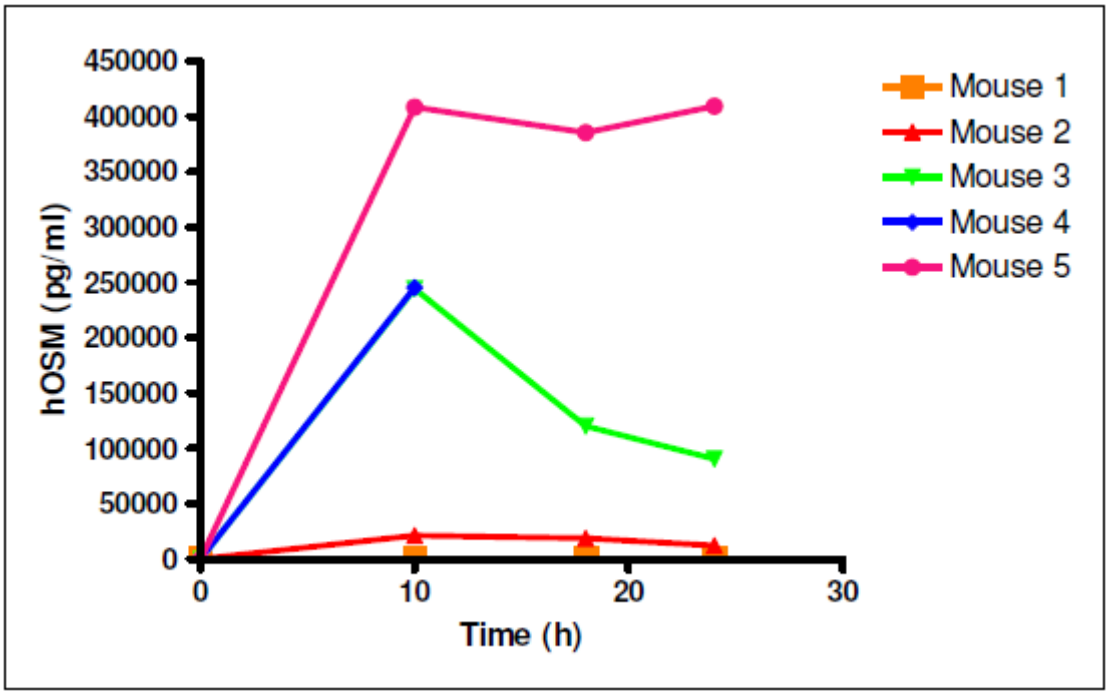

Figure 28. Expression of hOSM in mice, with a high dose of HCA-RUIO

The third group of mice with 6,65 x 10^{10} v.p. of HCA-RUIO, as reported in the figure 29, it could be possible to detect hOSM only in the 50% of the mice. One of these animals showed moderate levels of hOSM (3000-5000 pg/ml), and died one week after RU486 administration. The other mice, with very low levels of

hOSM, remained alive. When the surviving animals received a second induction with RU486, no expression of hOSM could be detected.

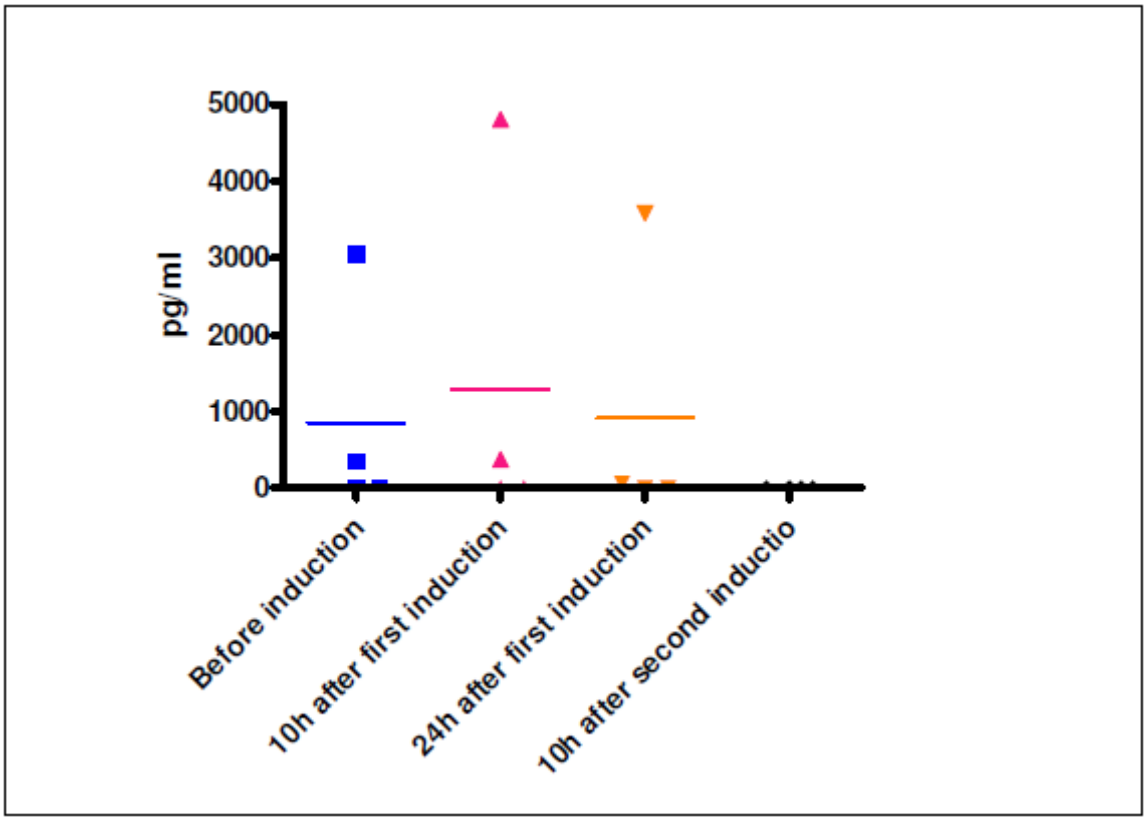

Figure 29. Expression of hOSM in mice with 6,65 x 10^{10} v.p. of HCA-RUIO

In the group of mice with 1,05 x 10^{11} v.p. of HCA-RUhIL12, the expression of hIL12 was not detected, but in the group with a double dose of the vector the concentration of the transgene could be readily measured in most animals, as shown in figure 30. In addition, re-induction of these animals was possible, with an average intensity of transgene expression similar to the first induction.

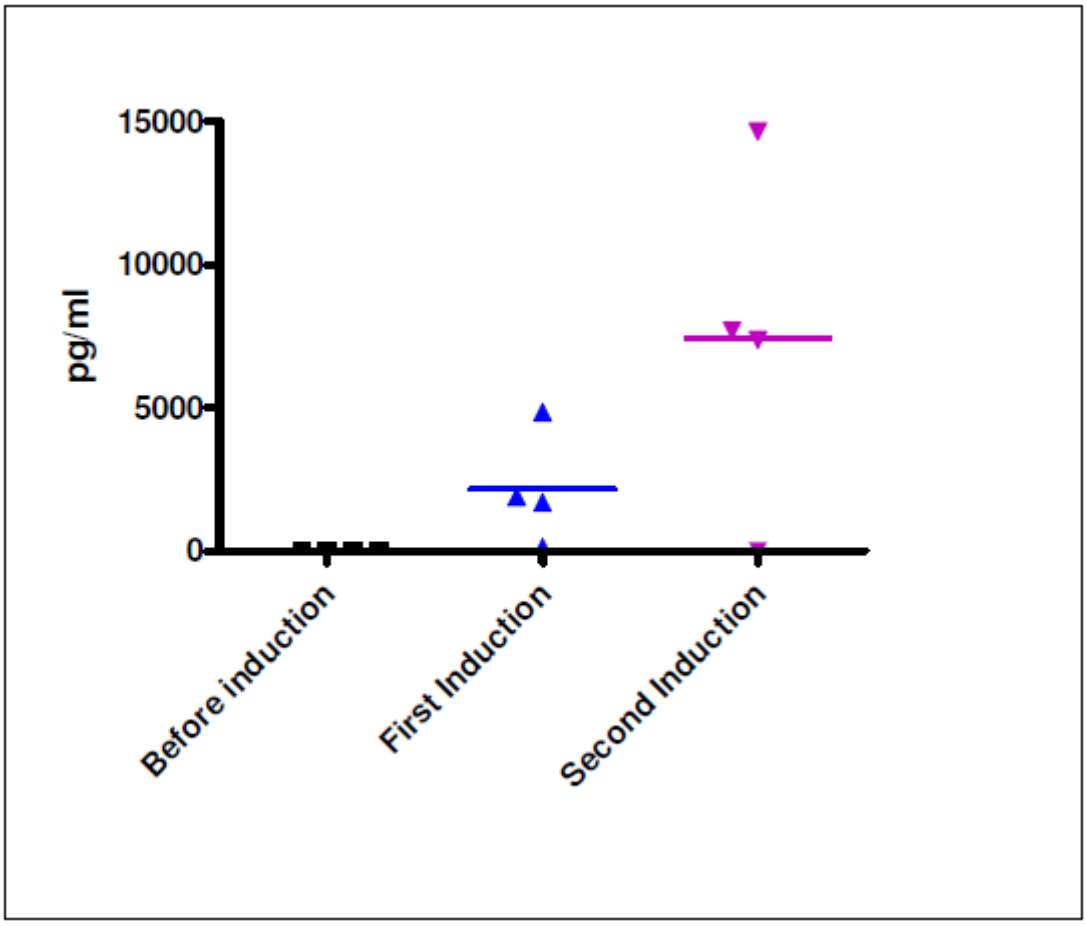

Figure 30. Expression of hIL-12 in mice with 1,05 x 10^{11} v.p. of HCA-RUhIL12

8. Transgene expression from HCA-RUmIL12 in Hamsters

The behaviour of the HC-Ad/RUmIL-12 vector was studied in more detail in Syrian hamsters. A group of 4 animals received 6,13 x 10^{11} v.p of the vector by intrahepatic injection. The first induction was performed 9 days after the injection of vector, with a concentration of 1mg/kg of RU486. The second induction took place one month later, with daily administrations of 1mg/Kg of RU486 for 10 days. Finally, a third induction was attempted at day 60 with 4mg/Kg of RU486.

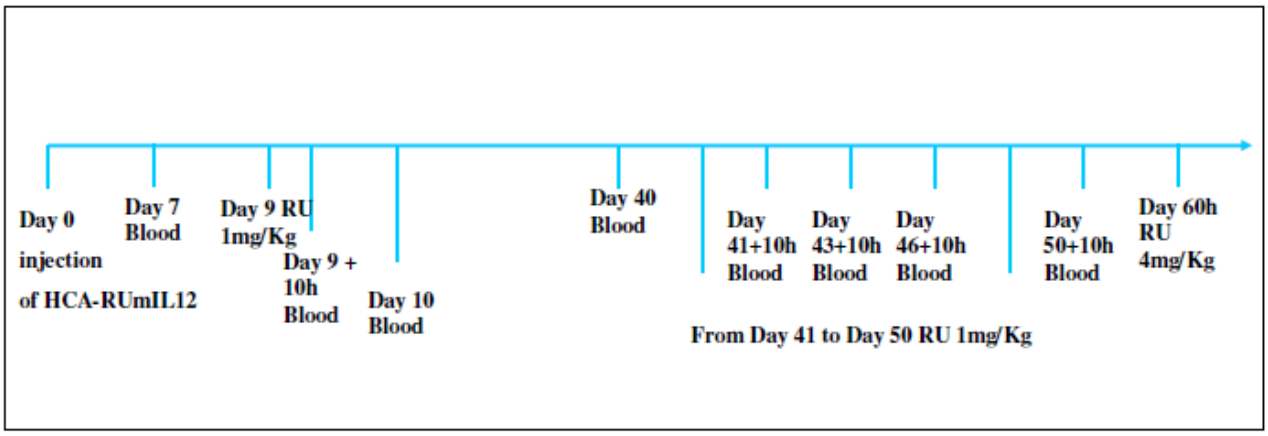

Figure 30. Scheme of exeriment

After the first induction, the presence of mIL12 could be detected in the serum of the Hamsters, with a peak of expression after 10 hours from the administration of RU486 and a decrease almost to 0 at 24 hours, as expected based on the previous data in mice. Only one of the animals failed to express mIL-12, probably due to inefficient intrahepatic inoculation. During the second induction samples of blood were collected at day 41, 43, 46 and 50, always after 10 hours from the administration of RU486. As seen from the graph, the second and third rounds of induction were unable to stimulate expression of mIL-12.

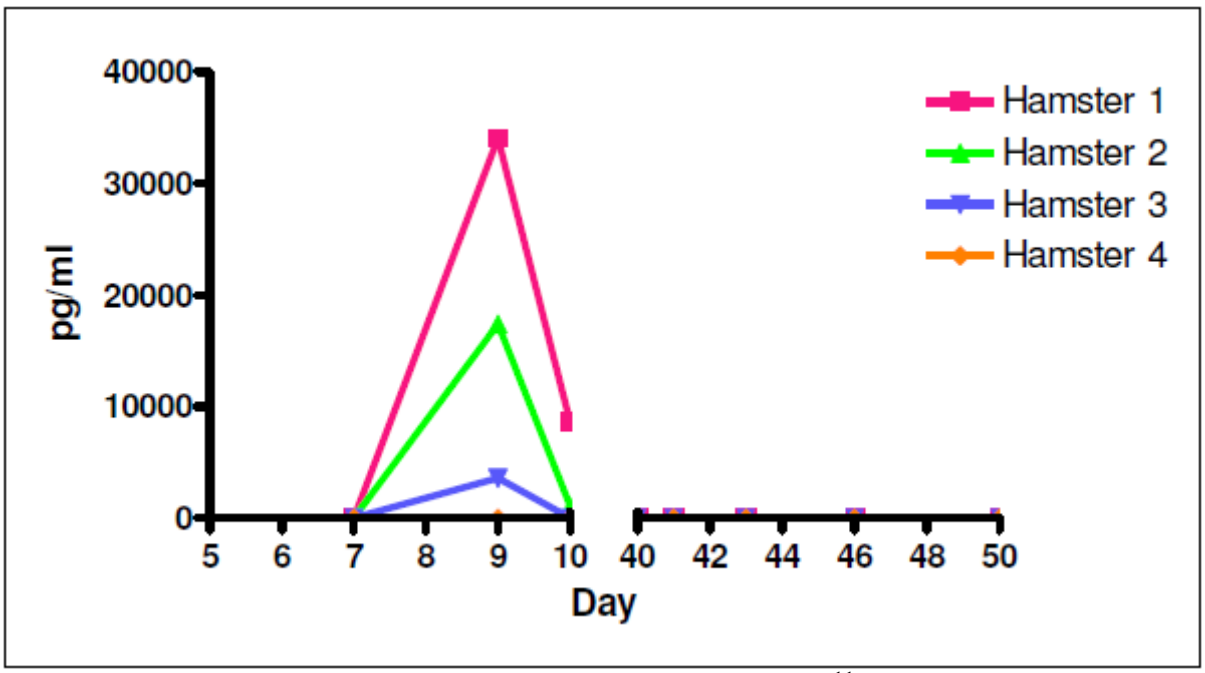

Figure 31. Expression of mIL-12 in Hamsters with 6,13 x 10^{11} v.p of HCA-RUmIL12

After the day 50 the Hamsters were sacrificed and samples of liver were collected to study the presence of vector genomes.

9. Potential methods to allow multiple rounds of transgene expression with the vector HCAd-RUmIL12 in hamsters.

Next, we investigated if the use of higher doses of the vector, or immunosuppression with dexamethasone could allow the expression of the transgene in different rounds of induction. The following experimental groups were established:

- 4 Hamsters with 6,13 x 10^{11} v.p. of HCA-RS25
- 4 Hamsters with 6,13 x 10^{11} v.p. of HCA-RS25 and administration of Dexamethasone
- 4 Hamsetrs with 1,84 x 10^{12} v.p. of HCA-RS25

The first group will be sacrificed at day 9, just before the induction, in order to collect liver samples and determine the amount of vector genomes. In this way the loss of vector genomes before and after mIL12 induction can be calculated (comparison with the group of hamsters from the previous experiment that received the same dose of vector and underwent 3 cycles of induction).

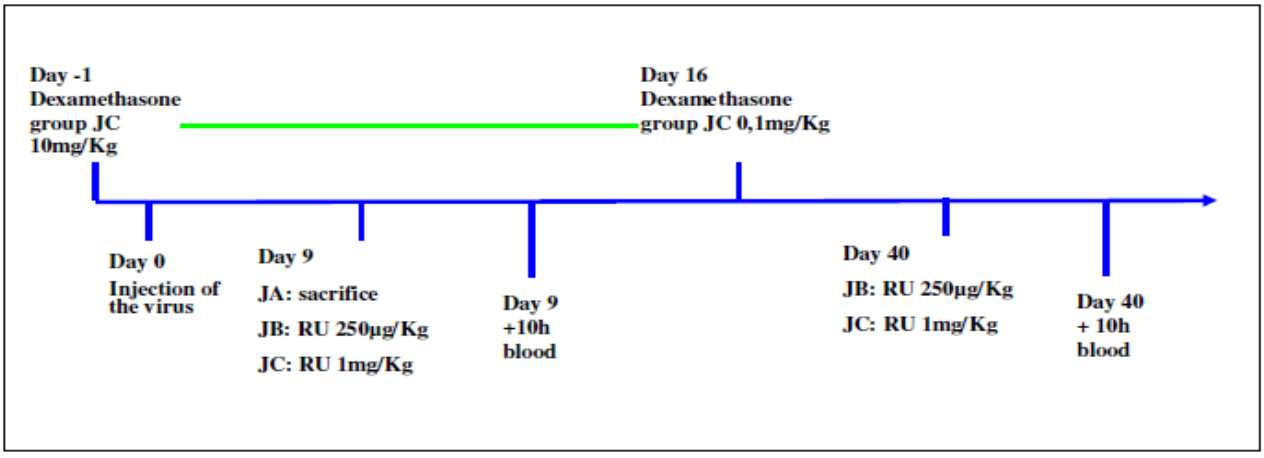

Figure 35. Scheme of the experiment

The second group received the same amount of virus and followed a treatment with Dexamethasone, from the day before the injection of the virus to the day 16 of the study, with a dose that decreased from 10mg/kg at the day -1, to a dose of 0,1 mg/kg at the day 16, according to the schedule indicated in table 4. Induction was carried out with 1 mg/Kg RU486.

Day	Dose of Dexamethasone
-1 - 0	10 mg/kg
1 - 2	5 mg/kg
3 – 9	1 mg/Kg
10 – 12	0,5 mg/Kg
13 – 16	0,1 mg Kg

Table 4. Scheme of somministration of dexamethasone

The group with the higher vector received 250µg/Kg RU486. Both groups of Hamsters were induced the first time 9 days after the injection of virus, and then with the same doses of inducer one month later. The quantification of mIL-12 in the serum of animals is represented in figure 36. Interestingly, the addition of dexamethasone caused an increase in the production of mIL-12 in the first induction. As expected, animals that received the high dose of vector (3-fold relative to the other groups) showed a dramatic enhancement of mIL-12 concentration in serum. However, the induction performed one month later was only productive at low levels in one of the hamsters with the high dose

of vector. A subsequent induction with 4 mg/kg RU486 obtained only a small restoration of mIL-12 expression in these animals.

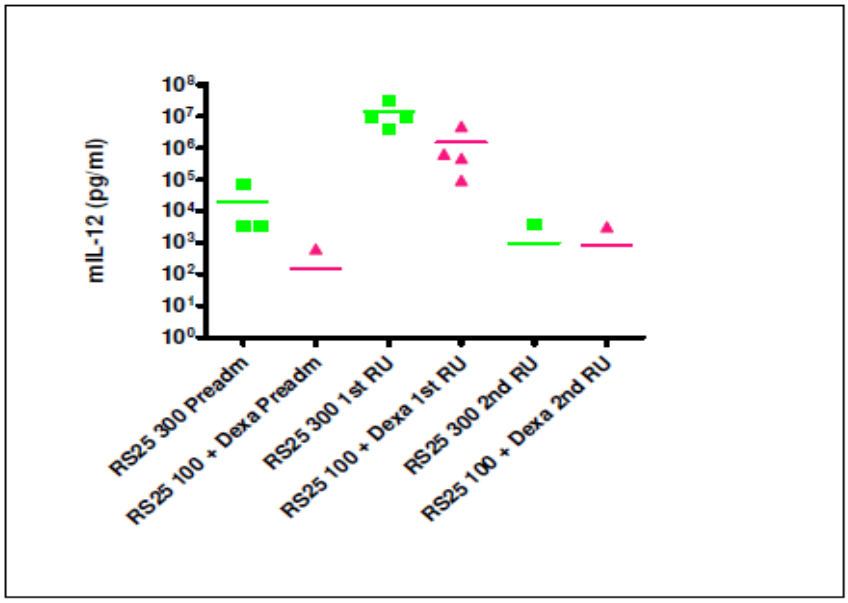

Figure 36. Expression of mIL-12 in Hamsters with and without treatment with dexamethasone

10. Quantification of viral genomes in the livers of hamsters treated with HCA-RUmIL12.

The objective of this experiment is to determine if the lack of efficient re-induction of the transgene in hamsters is due to the loss of transduced cells in the liver. To this end, we extracted DNA from liver samples obtained from two groups of hamsters described in previous sections, and quantitative PCR was performed to analyze the number of vector genomes per gram of tissue. Both groups received the same dose of HCAd/RUmIL-12 vector (6,13 x 10^{11} v.p), but one of them was sacrificed 9 days after vector administration, without induction with RU486, whereas the second group received 3 rounds of induction over a period of 40 days, and was sacrificed 50 days after vector inoculation. The results presented in figure X indicate that 90% of viral genomes were eliminated during this period. The implications of this reduction in copy numbers on the ability to obtain repeated cycles of induction will be discussed in the next section.

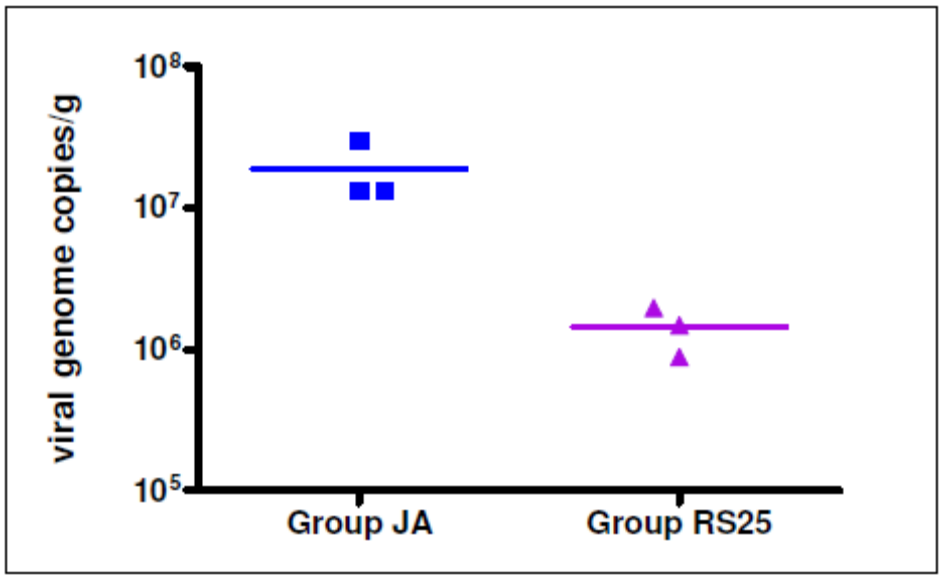

Figure 37. Quantification of viral genome in the liver of hamster

VI. DISCUSSION AND CONCLUSION

1. Generation of gutless adenoviral vectors

Gene therapy offers considerable promise to treat a great diversity of conditions involving the liver, including metabolic, infectious, and neoplastic diseases. In many cases, sustained expression of the transgene for long periods of time is required. Long-term expression can be achieved by using vector with the ability to integrate into the host genome such as retroviruses or adeno-associated viruses. Problem with these vectors include low transduction efficiency and the risk of insertional mutagenesis.
The adenovirus have the advantage of possessing marked hepatotropism, high transduction efficiency, and persistence in an episomal form. The first adenoviral vectors generated (first generation) offered the chance to test a large number of concepts through the expression of various proteins and markers or therapeutic genes in different tissues or cell types. However, its effectiveness as a vector for gene therapy has been inadequate and that the expression of therapeutic genes used have a limited duration of around 2-3 weeks (124). To try to prolong transgene expression, were used second generation vectors, but these still offer the same problems associated with the first generation vectors (125). Therefore, to decrease the cellular immune response and increase the time of expression of the vectors of first and second generation, have been generated third generation, gutless or helper-dependent adenoviral vectors (232). These vectors are devoid of all adenoviral genes (2 inverted terminal repeats and packaging signals are the only conserved sequences), and therefore transduced cells do not express any adenoviral product and do not elicit cellular immune response against the vector, thus offer the possibility to express a therapeutic gene until 2 years in baboons (138), even during the lifetime of the organism (231). However, while the gutless Ad offers great advantages *in vivo*, its production has always presented two major drawbacks: the helper adenovirus contamination and obtaining high titers (179).

For many gene therapy applications, it is necessary to use long-term expression vectors encoding molecules capable of inducing powerful biological effects but also having the risk of significant potential toxicity. The clinical use of such therapeutic genes makes mandatory using regulatory system, allowing a strict control of transgene expression.
This study employed three different gutless adenoviral vectors, two of which were built ex novo. In all these vectors, the mifepristone (RU486) regulatory system was incorporated to mediate long-lasting, regulable and liver-specific expression of the transgene. The system is based on two expression cassettes: one encoding a mifepristone-inducible transactivator under the control of a liver-specific promoter (transthyretin) and the other encoding the gene of interest (IL-12 or OSM-IFN) under the control of a minimal promoter, which is operative only in the presence of the active transactivator.
HCA-mIL12, previously characterized *in vivo* in mice, was originally used as a control. For the other two vectors, HCA-RUIO and HCA -RUhIL12 first to test and characterize the expression *in vivo*, were subjected to a characterization of expression *in vitro*. In addition, before proceeding with the production of viral vectors, all viral genomes were digested by restriction enzymes to control potential gene rearrangements might have taken place in the process of homologous recombination in bacteria.

2. Amplification of the Adenovirus gutless

Current systems of gutless Ad production contains 3 key elements. First, a gutless adenovirus that does not contain any viral gene and that provides a marker or therapeutic gene; secondly, a helper adenovirus which provide viral proteins *in trans* and some system of restriction; and third, a cell line that produces gutless Ad with the minimal contamination of helper Ad.

Both adenoviral vectors were produced with the system developed in the department of gene therapy and hepatology, at the University of Navarra (patent WO2009/138544), using the 293Cre4 cells and the Adenovirus Helper AdTetCre. This system allowed amplification of adenovirus vectors with low helper contamination, which is crucial in the production of adenoviral vectors.
From the results it is clear that the system used in this work reflects the nature of the request, the helper adenovirus contamination in the three batches produced is very low and ranges from 0.00034% to a maximum of 0,019%. These concentrations are far more than acceptable for hypothetical clinical application, which should not exceed 0.1%.

3. Characterization of the expression of the gutless Adenovirus vectors

3.1 Characterization *in vitro*

The in vitro data show that the expression of hIL-12 and and hOSM with hIFNα, in Huh7 cells, is completely different. In the case of hIL-12 , the expression occurred only in a dose-dependent manner, in the absence of RU486, leakage was absent and the expression level mediated by HCA-RUhIL12 in the presence of mifepristone was similar, as reported by Wang and colleagues, to that induced by first generation adenovirus carrying hIL-12 driven by CMV promoter.
As mentioned above, with regard to OSM-IFN, the expression pattern is not similar to that of hIL12. A peak of OSM is observed 10 hours after initiation or renovation of treatment. Surprisingly, production of OSM was also observed in infected cells in the absence of RU486. This effect may be due to the experimental conditions in cell culture, because the same phenomenon was observed with other HC-Ad vectors such as the HC-Ad/RUmIL-

12 in which no basal activity was detected *in vivo*. The expression of transgene in the absence of inducer may be due to high MOI obtained in cell culture and the accumulation of transactivator in these circumstances.

3.2 Characterization *in vivo*

The *in vivo* studies in hamsters that received HCA-RUIO by intrahepatic injection have not yielded the expected results, since only one hamster, the one with extremely high doses of viruses (5,7 x 10^{11} v.p.) and of mifepristone (4mg/kg), has been able to verify a minimum expression of the transgene. In the first test *in vivo*, where we tried to compare the expression of OSM-IFN and mIL12 in hamsters, thinking of getting similar results with those obtained from the HCA-RUmIL12 in mice, expression of both transgenes was not detected.
After this negative result, it was decided to see how is the expression of HCA-RUhIL12 and HCA-RUIO vectors in mice, because this model had been used for the characterization of HCA- RUmIL12 vector. Again the results differed from those expected, mIL-12 and hIL-12 can be re-induced in mice, but not OSM and IFN. There are some possible explications. Technically, it is difficult to obtain expression of OSM in mice without high toxicity. We do not have many examples of mice that clearly express OSM in the first induction and then they do not express after the second induction, because they died after the first induction. Therefore, we should mention that we are not sure that OSM cannot be re-induced in mice. But if we assume that the lack of re-induction is true, we should considered that there is a difference between mIL-12, hIL-12 and hOSM: **hIL-12** is an exogenous protein for mice (so they could react against it), but they do not do it, probably because hIL-12 is not functional in mice and therefore does not stimulate the immune system. On the other hand, **mIL-12** can activate the immune system of mice, but

it is not an estrange protein for them. Only **hOSM** is estrange for mice and also has biological activity. This could be a difference that explains why re-induction is more difficult.

Having observed this difference in expression caused by different types of transgenes used, we proceeded with an analysis of the expression of the vector HCA-RUmIL12 in hamsters. As in all *in vivo* studies of this work, the virus was administered by intrahepatic injection, and as a work of Sergin and collaborators (301) has been described that transient glucocorticoid pretreatment also significantly reduced Ad-induced adaptive immune responses, including a decreased induction of Ad-neutralizing antibodies, one group of hamsters was subjected to a transient treatment with dexamethasone. In this experiment, high doses of vector were need to obtain expression of transgene in hamsters compared to mice, but once a threshold is achieved, the intensity of expression increases sharply. The same happens in mice. This is demonstrated comparing the expression of mIL-12 in hamsters inoculated with 6,13 x 10^{11} v.p versus 1,84 x 10^{12} v.p. of vector. This can be caused by the uptake of virus by macrophages in the liver (Kupffer cells), which are efficiently infected but normally do not contribute to transgene expression. Only when these cells are saturated the virus enters the hepatocyte in a dose-dependent manner. The fact that dexamethasone increases the expression of mIL-12 supports the concept that Kupffer cells are responsible for the low expression of transgenes at low doses of virus. This is because corticoids could block the function of macrophages, although we have not demonstrated this in our hamsters.

What has been demonstrated in the group of hamsters submitted to a transient treatment with dexamethasone is that, even in these, like the other groups, we can not re-induce transgene expression. Probably, maintaining a constant dose of dexamethasone, thus blocking the function of macrophages in the liver (Kupffer cells) you could get a successful re-induction of mIL-12.

Finally, we wanted to compare the amount of genomic copies present in the liver between the group of hamsters subjected to three inductions with different doses of mifepristone, within 60 days after vector administration, and the group of hamsters that received the vector, but were not subjected to induction, as were sacrificed 9 days after injection of the virus. This value is also compared with a previous study in which they analyzed the genome loss of the same vector, but in mice. Mice that receive the HCA-RumIL12 vector lose 90% of copies in one year, but still they can express some IL-12. In hamsters, the same loss of copies occurs in less than 2 months, and they cannot express IL-12. This means that the loss of transduced cell is faster, and the amount of copies per gram that is needed for expression is higher.

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ABBREVIATIONS

Ψ	packaging signal
AAV	adeno-associated virus
Ad	Adenovirus
AdPol	Adenoviral Polymerase
APC	Antigen Presenting Cells
CAR	Coxsackievirus B and Andenovirus Receptor
DBP	DNA Binding Protein
DMD	Duchenne Muscular Dystrophy
dsDNA	double stranded DNA
HPRT	Hypoxanthine-guanine Phospho Ribosyl Transferase
IFN-α	Interferon- α
IL-12	Interleukin-12
ITR	Inverted Terminal Repeats
LTR	long terminal repeats
MAR	Matrix Attachment Regions
MLP	Major Late Promoter
MMLV	Moloney murine leukaemia Virus
NK	Natural Killer cells
NPC	Nuclear Pore Complex
ORF	Open Reading Frame
OSM	Oncostatin M
pTP	pre-Terminal Protein
shRNAi	short hairpins RNA interference
ssDNA	single stranded DNA

TLR	Toll-like receptors
VA-RNA	virus-associated RNA
VSV-G	G protein of vesicular stomatitis virus

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RIASSUNTO

La terapia genica presenta un grande potenziale terapeutico per una varietà di disturbi epatici tra cui patologie metaboliche ereditarie (fenilchetonuria, tirosinemia) e malattie acquisite (infezioni croniche, tumori primari e metastatici). Per molte di queste applicazioni sarebbe necessario l'utilizzo di vettori ad espressione prolungata, regolabile e tessuto-specifica del transgene.
Adenovirus è il vettore più utilizzato nei trials clinici umani. Per evitare la risposta immunitaria cellulare indotta da adenovirus di prima e seconda generazione, sono stati generati i vettori di terza generazione, chiamati anche gutless o helper-dipendenti. Per produrre questi vettori sono richiesti tre elementi fondamentali: un adenovirus gutless con un gene terapeutico o marker di interesse, un adenovirus helper che fornisca proteine virali *in trans* e, una linea cellulare permissiva per la produzione di Ad. Gli Adenovirus Gutless, il cui genoma non contiene alcun gene virale, non genera risposta immunitaria cellulare e può ospitare fino a 36 Kbp. Hanno dimostrato che l'espressione di geni che possono incorporare può permanere per molto tempo.
In questo studio, sono stati prodotti due distinti adenovirus gutless, con espressione epatospecifica e regolata dal sistema inducibile RU, contenenti una combinazione dei geni OSM e IFN (HCA-RUIO) e l'altro il gene hIL-12 (HCA-RUhIL12). Dopo la sperimentazione *in vitro* la corretta funzionalità dei vettori sono stati effettuati test *in vivo* nei topi e criceti. E' stato dimostrato che *in vivo* l'espressione del gene di interesse cambia con la specie animale utilizzata e il transgene presente nel vettore. In contrasto con i dati precedenti che hanno dimostrato nei topi infettati con HCA-RUmIL12, mIL-12 può essere espresso anche dopo più di un anno, nei criceti, lo stesso vettore esprime il transgene solo dopo la prima induzione con RU e quindi non poteva essere rilevato successivamente. Il vettore HCA-RUIO ha dato un

modello simile di espressione del transgene in criceti, ma anche nei topi. Ciò può essere dovuto al fatto che se le proteine prodotte dai vettori sono esogene per l'organismo si attiva l'attività immunostimolatoria negli animali che porta alla eliminazione delle cellule transfettate e quindi l'incapacità di reindurre l'espressione del transgene.