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Adenoviral Vectors: Potential and Challenges as a Gene Delivery System

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1. Introduction

Adenoviruses (AdV) have been well delineated and made into suitable vectors for gene transfer by their somewhat benign nature, broad tissue tropism, and the relative ease of manipulation of their genome. These viruses readily transduce both quiescent and dividing cells, are adaptable for large scale production and have certified cell lines that have been developed for purification. Nevertheless, these vectors are not recommended for all purposes. Currently, adenoviral vectors are more widely evaluated in applications where a short-term transgene expression is sought after (i.e., cancer and vaccines) (Jolly et al., 2008; Sharma et al., 2009b).

Rowe and colleagues in 1953 were the first to isolate AdV from adenoidal tissue and to describe the spontaneous degeneration of primary cell lines from whence they were derived (Rowe WP et al., 1953). A similar virus was isolated from an epidemic of respiratory illness in U.S. military recruits in 1954 (Hilleman MR & Werner JH, 1954). In 1956, the viruses were named adenoviruses after the tissue from which they were isolated (Enders et al., 1956). Their anti-tumor potential was explored in the clinical treatment of cervical carcinoma following AdV inoculation not long after 1956 (Huebner et al., 1956). Despite this initial use, it took several years following the advent of recombinant technology before the therapeutic potential of AdV was truly realized. Since then, adenoviral vectors have received much attention as gene transfer agents and currently are being tested in a wide variety of gene therapy applications.

There are more than 120 AdV that infect a wide host range including mammalian, avian, and reptile species. AdV are divided into four genera: Atadenovirus, Aviadenovirus, Mastadenovirus, and Siadenovirus. Mastadenovirus serotypes which infect humans are placed in groups (A-F) or subgenera. Initial parameters for grouping were based on patterns of hemagglutination from various animal species; now the grouping is based on DNA homology. Some of these genera are being engineered for use as AdV vectors.

The *Adenoviridae* family is characterized by a linear, double-stranded DNA genome that is encapsulated in an icosohedral shell of protein that measures 70 to 90 nm in diameter. Virions of AdV are comprised of 13% DNA and 87% protein (Rux & Burnett, 2004; San & Burnett, 2003). Their genome is approximately 35-38 kilobases in size. The majority of the viral capsid encompasses three proteins: fiber, penton base and hexon. Fiber and penton base proteins are necessary for receptor binding and cell internalization, whereas hexon

proteins form most of the viral capsid. These three proteins are also crucial targets of virus neutralizing antibodies.

AdV infections are common in humans and have relatively benign sequelae. Primarily, they are the etiologic agents that cause upper and lower respiratory infections. However, they do infect other tissues such as the eyes, gastrointestinal and urinary tracts, brain, heart, kidney, and liver where they pose minor health risks. Individuals with a normal immune system have self-limiting infections without notable clinical sequelae. Yet immunocompromised individuals may develop conditions more frequently associated with uncommon variants (i.e., group B, serotype 35). During infection, adenoviral DNA is not usually integrated into host cell chromosomes thereby minimizing concerns regarding insertional mutagenesis or potential germ line risks. The clinical mildness of a natural AdV infection is probably attributed to their immunogenicity which has made these viruses popular as gene transfer vectors. In spite of this positive side of their immunogenicity, these viruses without considerable modifications may not be useful for applications that necessitate long-term expression since they do not incorporate into the host genome.

1.1 Advantages of AdV vectors

There are several factors that contribute to the advantages of AdV-based vectors. The molecular mechanisms underlying the AdV genome and its life cycle have been extensively studied which has facilitated molecular manipulations of the viral genome in plasmid form. Only the packaging signal and the inverted terminal repeat (ITR) (approximately 300bp) are essential to package nearly 105% of the total AdV genome. This facilitates a decrease in the occurrence of replication-competent recombinants while also permitting introduction of a large transgene cassette (up to approx. 35kb) into the AdV virion. Additional benefits include relatively simple and reliable manufacturing methods, high levels of expression in various replicating and non-replicating tissues even at low temperatures, delineation of its toxicity profile, minimized risk of genomic insertional mutagenesis due to its episomal persistence in the nucleus, and extensive knowledge of the effects pertaining to vector genome uptake following a particular route of administration. Also, AdV vectors have demonstrated the ability to prime and boost T- and B-cell responses (Pinto et al., 2003; Vemula & Mittal, 2010). Most importantly, safety has been demonstrated in the therapeutic application of AdV vectors in a number of clinical trials (Sharma et al., 2009b).

AdV vectors can be generated to express multiple proteins from various expression cassettes located within the genome of the AdV. Vaccine formulations that exploit the advantages of these AdV vectors increase the potency and reduce the prospective costs. Also the use of multi-gene expression technology permits flexibility in vaccine design regarding rapid antigen swapping after AdV vector development. A prerequisite for such an AdV vector is that it must adequately express multiple antigens (Jolly et al., 2008). Finally, while some may view the transient nature of AdV vectors to be a disadvantage, it does have beneficial medical applications. AdV vectors have demonstrated therapeutic potential in treating various forms of cancer, infectious disease, and tissue remodeling.

1.2 Disadvantages of AdV vectors

Transient expression of the transgene by AdV vectors can be a disadvantage for gene therapy applications where continous expression of the trangene is necessary for a desired

therapeutic effect. Due to the promiscuous nature of AdV biodistribution following systemic administration, targeting of the AdV vector to only a specific cell type is a considerable challenge.

AdV vectors at a very high dose could lead to significant toxicity. Expression of viral proteins by the AdV vector and activation of innate immunity are partially responsible for this *in vivo* toxicity. The deadly potential of AdV *in vivo* toxicity was highlighted after the intravascular delivery of an AdV to a patient enrolled in a clinical trial in 1999. A large dose of vector was administered into the hepatic artery of the patient recruited in a partial ornithine transcarbamylase (OTC) deficiency clinical trial. This large dose resulted in liver dysfunction and death due to multiple organ failure (Raper et al., 2003). The investigators concluded that the toxicity was due to the 'extremely high dose' (3.8×10^{13} virus particles) of AdV causing a saturation of the available AdV receptors on hepatocytes and the subsequent spread of the vector to other organs. The patient's death was thought to be due to strong activation of an innate immune response. This unfortunate event has led to new guidelines for the validation of new technologies.

AdV are a common human pathogen, especially human adenovirus 5 (HAdV5); therefore, *in vivo* delivery of AdV may be hampered due to pre-existing vector immunity in the majority of human population. Although low levels of vector immunity can be quelled by increasing the dose of administered AdV vector without increasing toxic side effects (Pratt et al., 2010), the issue of pre-existing vector immunity is a cause for concern. Challenges also exist in correlating the protective outcomes of the quality of T cell responses over the quantity of T cells that are stimulated by different immunization protocols and vector strategies. Priming with some AdV vectors stimulates transgene-specific immune responses that have a low correlation with type I interferon (IFN) production; the reduced levels are associated with reduced transgene expression. Reduced IFN also hampers quality T cell responses and B cell differentiation into effector plasma cells. However, too much type I IFN results in clearance by innate effector systems and poor transgene expression. Thus, type I IFN responses must be rigidly controlled to attain therapeutic efficacy (Draper & Heeney, 2010).

2. AdV induced innate immune response

The AdV vector-mediated acute toxicity subsequent to intravascular inoculation is known to be a direct result of potent activation of the innate immune system - a desirable outcome for the purpose of vaccine development or cancer immunotherapy (Muruve, 2004). The activated immune system in these scenarios also results in the induction of a stronger immune response against the desired antigen or cancer cells. However, this strong immune reaction remains an obstacle for AdV-mediated gene therapy since the danger of severe toxicity prevents administration of the dose necessary to achieve the desirable therapeutic effect. The immune system activation follows a dose-dependent pattern and is independent of viral gene expression. Immune system activation is manifested as severe liver inflammation, thrombocytopenia, and systemic flu-like symptoms such as fever and myalgias (Raper et al., 2002). The severe inflammatory response is also associated with poor target cell tranduction and loss of viral genome and transgene expression within two to three weeks post administration (Yang et al., 1994).

The initial host response to AdV vectors occurs within minutes after systemic administration and may last from several hours to days. It is characterized by elevated serum levels of proinflammatory cytokines such as tumor necrosis factor- α (TNF- α), IL-

6, IL-1β, IFN-γ, and chemokines such as macrophage inhibitory protein (MIP)-2, IFN-γ inducible protein 10 (IP-10), RANTES, MIP-1α, MIP-1β and monocyte chemoattaractant protein 1 ((MCP-1) (Muruve et al., 2004). Induction of inflammatory response to viral pathogens proceeds through opsonization, uptake of opsonized viral particles, antigen processing and presentation, and, finally, the release of inflammatory cytokines. Likewise, high doses of systemically administered AdV vectors result in activation of splenic dendritic cells (DCs) and macrophages inducing the production of inflammatory cytokines such as IL-6, IL-12 and TNF-α. Depletion of tissue macrophages and DCs prevents the production of the aforementioned cytokines, indicating a central role of these innate effectors in the acute inflammatory response (Kuzmin et al., 1997; Lieber et al., 1997; Zhang et al., 2001). Recently, an *in vitro* study using co-culture of epithelial cells and macrophages demonstrated the dependence of AdV-induced inflammatory response on the activation of macrophages through interactions with epithelial cells where the activation was mediated by NF-κB. AdV infection of the co-culture resulted in cytotoxicity, expression of inflammatory cytokines and chemokines, NOS and ROS generation and activation of inflammatory transcription factors (Lee et al., 2010). However, the nature of macrophage and epithelial cell interaction and their implications during AdV infection *in vivo* remain to be elucidated.

Neutrophils also seem to play an important role in AdV-induced acute inflammation. The chemokines, MCP-1, RANTES and MIP-1β, upregulate the neutrophil chemoattaractant chemokine MIP-2 which then recruits neutrophils to the liver. Recruitment of neutrophils and rapid induction of C-C and C-X-C chemokines correlates with acute liver injury and histopathological changes (Muruve et al., 1999). Moreover, AdV vectors are shown to activate endothelium in post-sinusoidal venules and promote P and E selectin-mediated rolling. This is followed by adhesion mediated through interactions between α4-integrin on the neutrophil surface and vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells (Li et al., 2002). Blocking of either leukocyte rolling and adhesion or neutrophil depletion results in lower expression of the proinflammatory gene expression implying a central role of neutrophils in AdV-induced liver inflammation (Liu et al., 2003b). The neutrophils are recruited to the liver where they take up AdV particles in a complement receptor 1 (CR1)- and Fc receptor-dependent manner sequestering the AdV particles away from target cells (Cotter et al., 2005). How the neutrophil sequestration of AdV particles affects the vector-induced innate immune response calls for further exploration. Although other types of leukocytes such as monocytes and macrophages are also recruited to the liver, neutrophils constitute more than 70% of the total leukocytes in the liver following systemic AdV administration.

Natural killer (NK) cells are another cell type activated and populated to the liver following AdV infection and are directly associated with liver injury. Type 1 IFNs, produced in response to viral infection, promote activation of NK cells which mediate clearance of AdV genome and lowers transgene expression. Pre-treatment with anti-NK cells antibodies enhances AdV genome persistence, prolongs transgene expression and dampens the innate immune-mediated liver injury (Liu et al., 2003a; Zhu et al., 2007). A very interesting strategy to mitigate the impact of AdV-induced innate immune response would be to transiently remove the effector cells of the innate immune system, such as neutrophil and NK cells. Alternatively, in the light of evidence of IFN-γ-mediated NK cell activation, transient suppression of type 1 IFN response prior to AdV administration might also be beneficial (Zhu et al., 2007).

Several studies in the recent past have focused on delineating the molecular mechanisms governing AdV-mediated activation of the innate immune system. This system, being the first line of defense against invading pathogens, has evolved a highly conserved repertoire of 'pattern recognition receptors (PRRs)' which specifically identify 'pathogen-associated molecular patterns (PAMPs)' (Kumar et al., 2009). A variety of PRRs, including Toll-like receptors (TLRs), RIG-1-like receptors (RLRs) and Nod-like receptors (NLRs), are employed for the recognition of all types of pathogens ranging from bacteria, fungi, viruses and protozoa.

2.1 Role of TLR signaling in AdV-induced innate immune response

Depending on the cell type, the sensing of AdV by innate immune response is mediated by both TLR-dependent and independent pathways and appears to be very complex (Appledorn et al., 2008b; Cerullo et al., 2007; Nociari et al., 2007; Zhu et al., 2007). Among the members of the TLR family, four are known to recognize viral nucleic acids: TLR3 (dsRNA), TLR7, TLR8 (ssRNA) and TLR9 (dsDNA). The myeloid differentiation primary response gene 88 (MyD88), a TLR Adaptor protein for downstream activation of signaling cascades such as MAPK and NF-κB pathways, is necessary to initiate an AdV-induced proinflammatory cytokine and chemokine response (Hartman et al., 2007a; Hartman et al., 2007b). TLR9 is spatially and temporally co-localized with AdV as it is expressed in endosomes and is specifically upregulated during AdV infection. As a result, it effectively recognizes unmethylated CpG motifs in dsDNA released after proteolytic degradation of AdV particles within endosomes and initiates a signaling cascade through MyD88 in plasmacytoid DCs (pDCs). This culminates with their maturation and the production of high levels of type 1 IFNs as witnessed during AdV infection. The type 1 IFN production in pDCs is TLR9/MyD88-dependent, as opposed to non-pDCs which recognize AdV DNA through a cytosolic sensor different from TLR9. Significant amounts of type 1 IFNs are produced with an AdV infection. In addition to the activation of NK cells, the type 1 IFNs play a crucial role in the AdV-mediated activation of innate immune response by enhancing production of proinflammatory cytokines IL-6 and IL-12 (Zhu et al., 2007). TLR2 also plays a crucial role regulating AdV-induced innate immune response by mediating the rapid activation of MAPK and NF-kB pathways which are linked to induction of inflammatory cytokine and chemokine gene expression. Specifically, TLR2 is required for sustained late phase induction of these pathways; initial activation is TLR2 independent (Appledorn et al., 2008b; Tibbles et al., 2002).

The induction of chemokines MCP-1 and RANTES depends solely on TLR-9 within one hour post-infection (hpi), but relies on both TLR2 and TLR9 at eight hpi. Collectively, TLR2 and TLR9 play crucial roles through the differential induction of cytokines and chemokines. However, the induction of chemokines independent of both TLR2 and TLR9, was discovered to be MyD88-dependent at beth early and late time points suggesting the involvement of different MyD88- dependent sensors in the full spectrum induction of innate immune response (Appledorn et al., 2008b).

As noted above, AdV infection of non-pDCs such as conventional DCs (cDCs), macrophages and fibroblasts stimulates production of significant amounts of type 1 IFNs, proinflammatory cytokines and chemokines through phosphorylation of interferon regulatory factor (IRF)-3. The activation of IRF-3 occurs as a result of the recognition of AdV DNA by a yet unidentified cytosolic DNA sensor which promotes phosphorylation of IRF-3. The phosphorylation initiates IRF-3 dimerization and translocation to the nucleus where it

causes transcriptional activation of several IRF-3 responsive inflammatory cytokine and chemokine genes (Hiscott, 2007; Nociari et al., 2007). However, AdV vectors can also induce IRF-3 phosphorylation directly through the capsid-cell membrane interactions prior to the endosomal escape of viral DNA, thereby providing an additional IRF-3 activation signal for stronger IFN and inflammatory response (Nociari et al., 2009). Evidently, AdV infection induces type 1 IFN response through multiple pathways and, therefore, causes a very strong activation of IFN responsive genes which might explain the powerful induction of innate immune response following AdV infection.

The NALP3 protein, a type of Nod-like receptor, plays an important role in cytosolic sensing of AdV DNA and the subsequent induction of proinflammatory IL-1β response. However, NALP3 is a general sensor for any kind of dsDNA including viral, bacterial, or even host (mammalian DNA) (Martinon et al., 2009). AdV DNA was shown *in vitro* to activate NALP3 signaling events through apoptosis-associated speck-like protein containing caspase recruitment domain (ASC), the adaptor protein for NALP3, resulting in the recruitment of the inflammatory caspase-1 into the molecular complex called the inflammasome which activates caspase-1. The activated caspase-1 initiates the proteolytic cleavage of pro-IL-1β into an active and secreted form of IL-1β. A similar response was detected upon infection with first generation AdV vectors, helper-dependent (HD) AdV vectors, and by transfecting cells with AdV DNA implying that the nature of DNA did not affect inflammasome activation. However, empty capsids failed to elicit a similar response suggesting that mere internalization of the capsid proteins was not enough for the inflammatory response. Furthermore, NALP-, ASC- and caspase-1-deficient mice systemically injected with AdV vectors developed only a blunted inflammatory response in the liver and showed a remarkable reduction in the expression of NF-kB regulated pro-inflammatory genes confirming the role of NALP3 and inflammasome in AdV-induced innate immune response (Muruve et al., 2008). Furthermore, recognition of AdV DNA through endosomally expressed TLR9 upregulated expression of NALP3, ASC and pro-IL-1β thereby tuning the cellular environment for a stronger IL-1β response.(Barlan et al., 2011)

2.2 AdV interactions with blood factors

In addition to immune responses, successful gene therapy will depend on organ specific delivery and an adequate expression level of the gene of interest. Following intravascular delivery, AdV vectors first come in contact with blood and its components. Considering the diverse cell types and myriad of proteins with roles in processes as complex as immune response, coagulation, cell signaling and others, it is not surprising that interactions between AdV vectors and blood components are pivotal in shaping the biodistribution profile of intravascularly delivered AdV and, also, the complexity of the ensuing host immune response (Parker et al., 2008). Therefore, a thorough understanding of AdV and blood factor interactions is advantageous in devising novel strategies to preclude deleterious interactions and to efficiently target vector delivery to the organ.

Based on *in vitro* experiments, the classical pathway for AdV internalization involves a primary interaction of fiber knob domain with Coxsackievirus and adenovirus receptor (CAR) resulting in viral attachment to cell surface, followed by a secondary interaction of the RGD motif on a penton base with integrins promoting internalization of the attached virus via receptor-mediated endocytosis. However, the *in vivo* biodistribution pattern cannot be attributed to knob-CAR interaction alone since CAR follows a ubiquitous expression profile, whereas the majority of intravascularly delivered AdV is sequestered primarily in

the liver. Further, HAdV5 vectors modified by inserting retargeting peptides and ablated for CAR binding fail to efficiently retarget to intended sites (Nicklin et al., 2005). These observations suggest involvement of CAR-independent pathways of cellular transduction *in vivo* by AdV. Heparan sulfate proteoglycans (HSPG) and LDL receptor-related proteins (LRP) were identified as alternative receptors for AdV transduction of liver cells. Initially, the blood coagulation factor IX and complement factor C4BP were shown to bind to HAdV5 and HAdV35 fiber knobs and bridge them to HSPG and LRP, thereby providing a 'CAR-independent' pathway of AdV infection *in vivo*.

Transduction of Kupffer cells which sequester the majority of the AdV transducing the liver is CAR-independent; hepatocytes rely on CAR-dependent, as well as CAR-independent, pathways for AdV uptake (Shayakhmetov et al., 2005). Consequently, abrogation of the factor IX- and factor C4BP-mediated AdV transduction can be an effective strategy for precluding AdV sequestration in Kupffer cells which contributes significantly to a proinflammatory cytokine and chemokine response to systemic administration of AdV vectors (Lieber et al., 1997; Manickan et al., 2006). Preventing Kupffer cell transduction can simultaneously provide dual benefits for effective liver gene therapy by mitigating the innate immune response and enhancing hepatocyte transduction through greater bioavailability of the vector.

The Vitamin K-dependent blood coagulation factors FVIII, FIX, FX and protein C also appear to play crucial roles in hepatocyte transduction through interactions with the HAdV5 viral capsid. Of note, these coagulation factors contain a conserved domain with an identical, defined structure comprising of a γ-carboxyglutamate (Gla)-EGF-1-EGF-2-serine protease domain which supposedly arose due to gene duplication. All of these factors significantly enhance hepatocyte transduction *in vitro* with factors FX and protein C being more efficient than factors FVIII and FIX through mediating interactions between the capsid, and the alternate AdV receptors, HSPGs and LRP. Interestingly, the factors did not exert an additive effect on transduction as they all seem to bind at a common site on the capsid and, therefore, might have an overlapping role in mediating AdV entry. Depletion of vitamin K-dependent blood coagulation factors in mice through warfarin treatment prior to intravascular AdV administration resulted in a remarkable reduction in hepatocyte transduction. This effect could be reversed by restoring physiological levels of factor FX just prior to AdV inoculation (Parker et al., 2006). The relevance of factor FX - hexon interaction to AdV vector biology is justified by swapping the hypervariable regions (HVRs) of HAdV5 with those from HAdV48, a non-factor FX-binding serotype, which absolutely abrogates the interaction and also decreases the hepatocyte transduction by more than 150-fold. The interaction between factor FX and the capsid hexon is calcium-dependent and mediated by the (Gla)-EGF1 domain of factor FX and the HVRs of hexons. No other capsid proteins play any role in the factor FX-capsid interaction. Furthermore, the hexon from all human AdV serotypes does not bind factor FX equally; serotypes 5, 2, 50 and 16 show strong binding, while serotypes 35 and 3 show weak binding, and serotypes 26 and 48 show no binding (Waddington et al., 2008). Construction of hexon swapped AdV vectors should be performed accordingly, keeping in mind the factor FX binding ability of the serotype and the intended clinical use of the vector. While constructing AdV vectors targeted to specific sites, tissue-specific fiber modifications must be accompanied by appropriate hexon HVR modifications large enough to ablate factor FX binding yet small enough to allow the chimeric virus rescue.

A recent study suggested that AdV sequestration in the liver is a collective result of a defined set of molecular mechanisms occurring in a redundant, sequential and synergistic pathway (Di Paolo et al., 2009). In addition to the trapping of intravascular AdV by Kupffer cells and

hepatocytes through interactions with blood factors, the AdV penton base-β3-integrin interaction becomes predominant in the absence of other virus clearance mechanisms. Combined treatment with chlodronate liposomes and warfarin removes Kupffer cells and inactivates vitamin K-dependent blood factor, prevents AdV entry into Kupffer cells and hepatocytes, and, therefore, might force AdV to accumulate in the liver sinusoidal space. Here the interaction between penton base RGD motif and β3-integrin may promote AdV uptake by sinusoidal endothelial cells (Di Paolo et al., 2009). These observations led to the proposal of a three step, dose-dependent model of AdV sequestration in the liver. At low doses, Kupffer cells are the primary niche for AdV retained in the liver. When the dose is higher and exceeds Kupffer cell capacity, the excess AdV enter hepatocytes in a blood factor-dependent manner. At an even higher dose, both Kupffer cells and hepatocytes become fully loaded, and AdV start entering sinusoidal epithelial cells. Following these principles, complete elimination of liver retention of systemically delivered AdV through simultaneous blocking of all three mechanisms might enhance the bioavailability of AdV vectors for gene therapy applications of extrahepatic organs and preclude induction of innate immune-mediated hepatotoxicity from reaching dangerous levels.

3. Adaptive immune responses to AdV vectors

AdV vector-mediated transduction *in vivo* results in efficient but transient transgene expression in various organs with the exception of those in newborn, immunocompromised or immunodeficient animals. The loss of transgene expression is attributed to a low basal level expression of AdV proteins despite the absence of E1A and E1B (Yang et al., 1994). Apart from induction of a strong innate immune response, AdV vectors also elicit adaptive immune responses directed towards the capsid components. The induction of adaptive immunity is profoundly influenced by the activated innate immune response (Descamps & Benihoud, 2009). The AdV-specific cellular immune response is induced through the uptake of AdV particles by antigen-presenting cells such as macrophages and DCs and the presentation of peptides derived from capsid proteins through MHC class I and class II pathways. Subsequently, the CTLs recognize and destroy the host cells displaying AdV-specific peptides, thereby leading to diminished transgene expression in immunocompetent hosts (Schagen et al., 2004). Macrophages also play a role in transporting AdV to draining lymph nodes, where AdV-specific B lymphocytes are activated resulting in an AdV-specific humoral immune response comprising of anti-AdV antibodies directed against capsid proteins (Junt et al., 2007).

The HD-AdV vectors, due to a lack of all viral genes, effectively remain unseen by the immune system and consequently cause a severely attenuated adaptive immune response. However, a transgene-specific immune response can be induced causing elimination of the host cells expressing the target protein in addition to antibody-mediated clearance of secreted target protein from circulation. The problem of anti-transgene immunity is unique and no less confounding than vector-associated primary complications since immunity may vary with the nature of transgene, animal model or even individual (Brown et al., 2004). On the positive side, HD-AdV vector-based gene therapy studies in various mouse and rat models of diseases such as diabetes, hemophilia, familial hypercholesterolemia, ornithine transcarbamylase deficiency, and Crigler Najjar syndrome produced favorable results through long-term phenotypic correction implying transgene-specific immunity may not be a significant hindrance (Seiler et al., 2007).

3.1 Significance of pre-existing vector immunity

More than 80% of the human population has been exposed to one or more serotypes among over fifty different serotypes of human AdV (Harvey et al., 1999; Xiang et al., 2006). The adjuvant effect of AdV results in development of strong anti-viral humoral and cellular immune responses in the pre-exposed individuals. These neutralizing antibodies are serotype-specific and are directed toward the viral capsid proteins. Similarly, the AdV-specific CD8+ T cells against both viral structural and non-structural proteins as well as the transgene will eliminate AdV-transduced cells (Tang et al., 2006). In the absence of pre-existing immunity, re-administration of homologous vector mimics vector administration in the presence of pre-existing immunity. Therefore, first and second generation AdV vectors are not suitable for correction of disorders requiring multiple vector inoculations and are limited to only those applications requiring short-term transgene expression or circumvention of vector immunity, either natural or induced. The presence of anti-AdV antibodies also enhances AdV uptake by DCs and macrophages via interaction with the Fc-receptor (FcR), but without a consequent increase in transgene expression. Pre-immunization with homologous vectors has been shown to exacerbate vector-induced hepatotoxicity and increase mortality rates (Vlachaki et al., 2002) which might be the indirect consequences of stronger innate immune activation.

4. Evolution of AdV vector systems

Despite early observations, it took several years following the advent of recombinant technology before the therapeutic potential of AdV was truly realized. The first generation AdV vectors (FGAdV) have a deleted early region (E) 1 (E1). The E1 region has two subunits E1A and E1B (Dormond et al., 2009). E1A deletions impair vector replication, down regulate transactivation of other early units, and deregulate cell cycle controls. However, this deletion does permit transgene expression capacity up to 5kb. E1B primarily inhibits apoptotic cell signaling. Most of these vectors also have a deletion in E3, which does not hinder *in vitro* growth but adds 3kb worth of transgene capacity. The different types of AdV vectors are described below and illustrated in Figure 1.

The Frank Graham Laboratory in 1977 developed a human embryonic kidney-derived 293 cell line that expresses the E1 gene products *in trans* thereby enabling the production of the first recombinant AdV (rAdV) in a helper-free environment (Graham et al., 1977). The 293 cell line used in vector manufacturing has nucleotides 1-4344 of the left end of the AdV genome which provides considerable overlap in the sequences with conventional vectors. These overlapping sequences within the cell promote homologous recombination between the vector and the AdV sequences which can generate replication-competent AdV (RCAdV) recombinants during the process of amplification.

Currently, the FDA has ruled that there must be less than one RCA per 3×10^{10} vector particles (Jolly et al., 2008). New cell lines, such as PER.C6 (Fallaux et al., 1998) and SL0036 (Howe et al., 2006), have been developed which abrogate sequence overlap and reduce RCAdV occurrence. Another method to diminish sequence overlap and reduce RCAdV risk has been to transfer a protein encoding gene into the cell genome which encodes for pIX, a minor AdV capsid protein (Hehir et al., 1996). The introduction of stuffer DNA into the E3 region is an additional method which increases the FGAdV DNA size but renders the system unpackageable in E1-recombined viral DNA progeny.
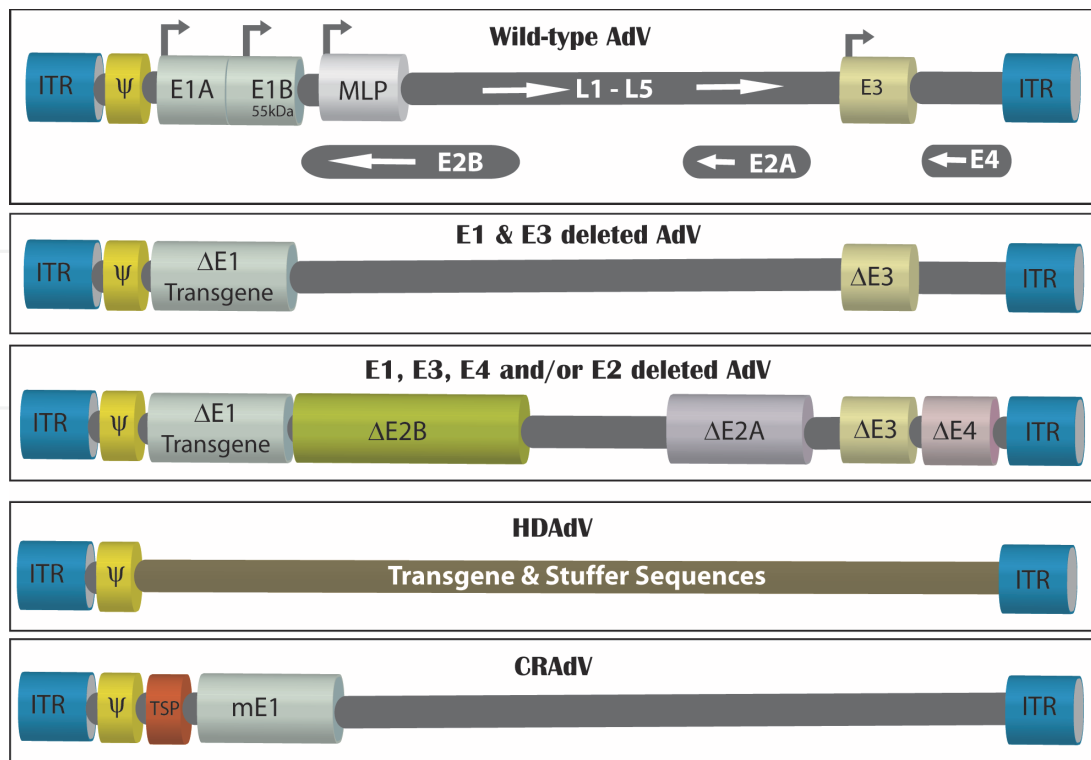
Fig. 1. Representative Diagram of the wild type AdV genome and different deletions in the various AdV vector constructs. E1 - E4: early transcription regions; L1 - L5: late transcription regions; ITRs: inverted terminal repeats; ψ: packaging signal; mE1: mutated E1; TSP: tumor-specific promoter.

In order to reduce the *in vivo* toxicity associated with the FGAdV, the second generation AdV vectors (SGAdV) were developed with additional deletions in their vector constructs. The initial E1 or E1/E3 deletions are supplemented with full or partial deletions in E2 and/or E4. Complementing cell lines are designed to constitutively express the matching E2/E4 region, but isolating these cell lines is a time-consuming process. The propagation of these vector constructs is less likely to generate RCAdV but will also have a lower yield. Although production possesses its own problems, it ultimately limits effects on immunogenicity resulting in the loss of interest for these multiple regions-deleted vectors (Lusky et al., 1998; Morral et al., 2002; O'Neal et al., 1998). Ultimately, long term expression and added safety were not attained with SGAdV (Dormond et al., 2009).

The further deletions of viral encoding genes that were gradually introduced to address safety concerns gave rise to the third generation AdV vectors (TGAdV). TGAdV lack all non-essential viral sequences. The TGAdV or helper-dependent AdV (HD-AdV) rely on helper-dependent systems in order to be generated. Along with the packaging signals, the inverted terminal repeats (ITRs) of AdV are required *in cis* to initiate replication (Grable & Hearing, 1992). Non-coding stuffer DNA is introduced into the 36kb of space that is available for transgene insertion. Helper functions are furnished by FGAdV which are also referred to as helper AdV. Initially, this class of AdV yielded a low number of TGAdV and a high level of contamination from helper AdV (Kochanek, 1999; Mitani et al., 1995). The purification of TGAdV from FGAdV by density gradient is a difficult task (Kumar-Singh & Chamberlain, 1996; Mitani et al., 1995; Parks & Graham, 1997). To facilitate better separation, the size of TGAdV should not exceed 32 kb which permits a better separation from the 36 kb FGAdV.

A recombinase system has been introduced into the TGAdV platform in order to reduce the amount of contamination (Hardy et al., 1997; Lieber et al., 1996; Parks et al., 1996). Adding a recombinase recognition site next to the packaging signal of the helper AdV yields an unpackageable FGAdV while co-infecting into HEK293 cells that constitutively express the respective recombinase. Currently, three recombinase systems have been employed: (1) Cre/LoxP, (2) FLP/frt and (3) C31/attB-attP (Alba et al., 2007; Ng et al., 2001; Umana et al., 2001). The two latter systems possess similar potential for the removal of FGAdV (Ng et al., 2001; Umana et al., 2001). Certain sequences on the TGAdV genome influence the yield. The stuffer DNA that is selected is of non-coding human DNA origin with minimized repeating sequences (Parks et al., 1999; Sandig et al., 2000; Schiedner et al., 2002). A promoter in the E4 region has provided amplification enhancement to TGAdV (Sandig et al., 2000). Additional amplification has been accomplished by the strategic development of FGAdV (Zhou et al., 2002). Sequence homology between packaging signals of the FGAdV and TGAdV should be avoided in order to limit the recombination events (Hardy et al., 1997; Sandig et al., 2000).

Complementary cell lines used in the production of TGAdV were derived from those that produce FGAdV and SGAdV. Furthermore, these cell lines express the respective recombinase enzyme such as Cre, FLP, or C31 (Alba et al., 2007; Ng et al., 2001; Umana et al., 2001). Generating competent cell lines that amplify TGAdV, while limiting FGAdV contamination has been difficult. Investigators have demonstrated that FGAdV contamination is due to the AdV-mediated shutoff of the host cell (Hartigan-O'Connor et al., 2002). The appearance of RCAs in TGAdV preparations has been attributed to a low level of FGAdV serving as helper AdV. Yet, production methods that necessitate that two viral constructs be present during serial passages increases the incidence of RCA. The use of PERC.6, a cell line which minimizes homology (Sakhuja et al., 2003), and the addition of stuffer DNA into the E3 region of FGAdV DNA (Parks et al., 1996) which limits encapsidation are useful solutions to minimizing RCAs.

Some AdV constructs have been changed to focus on the targeting potential instead of deleting multiple regions. Various methods are based on the rationale of expanding the otherwise limited tropism of the AdV while also reducing the potential toxicity seen in non-target tissue during over expression thereby improving the odds for systemic delivery. There are two approaches used in targeting: the modification of the virion to specifically transduce targeted cells or the use of gene regulation to restrict transgene expression to desired tissues. Tissue restriction modifications can be accomplished by genetically modifying the fiber-knob structure or through the covalent binding of a "bridging" molecule to the virion (Fattori et al., 2006; Folgori et al., 2006; Gherardi et al., 2003; Heeney et al., 2000; Lemckert et al., 2005; Liu et al., 2009; Shiver et al., 2002; Yu et al., 2008). Modification possibilities are limitless and range from the addition of de novo peptides or fiber-knob hybrids that combine other human and nonhuman AdV to exploitation of common receptor-ligand interactions to variations in RGD or similar motifs (Jolly et al., 2008). The bridging molecule method is advantageous since multiple antibodies and ligands can be implemented to form bispecific (target and AdV) bridges between the vector and the target cell type. Despite the advantages, each bispecific molecule and virion combination will possess unique effects, distribution, and toxicity profiles which will have to be defined both individually and as a constituent of a biological complex used in clinical data for drug trials. Retargeted or unique serotype vectors may prove efficacious in bypassing anti-vector immunity which could otherwise disturb transgene delivery used in

vaccine applications. Promising preclinical data with canine and nonhuman primate AdV exists (Alejo et al., 2006; Beveridge et al., 2007; McElrath et al., 2008).

Transcriptional targeting is achieved by placing a gene of interest under the control of a tissue- or tumor-specific promoter. This construct possesses potential for desired long term gene expression especially when small levels of expression in nontargeted cells can significantly impact potency or toxicity. One of the first constructs implemented in a human lung cancer cell line used the carcinoembryonic antigen promoter (CEA) to drive expression of the thymidine kinase gene derived from the herpes simplex virus (Liu et al., 2008a). Other promoters have been used which possess similar efficacy while simultaneously reducing the vector toxicity (Geiben-Lynn et al., 2008; Jego et al., 2003).

Conditionally replicating AdV vectors (CRAdV) have been implemented in cancer therapeutics because lysis of solid tumor masses requires a replicating vector in order to penetrate and spread through the tumor cells (Everts & van der Poel, 2005). The first class of deletion mutant CRAdV was designed so that a portion of E1 remains intact. Either E1A or E1B are mutated in order to confer replicative capacity only in human tumor cells which possess an impaired retinoblastoma tumor suppressor (Rb) or p53 gene. The second class of CRAdV possesses a tissue specific promoter upstream of E1A which restricts replication only in the target cells. Class 1 and 2 CRAdV have been adequately produced in E1-containing cell lines. RCAdV-preventing cell lines such as HeLA or A549 have also been implemented in CRAdV production (Longley et al., 2005; Yuk et al., 2004).

5. AdV vectors as a gene therapy tool

One of the important applications of gene therapy is the treatment of patients with monogenic recessive disorders by delivery of the mutated or non-functional gene. Hemophilia, cystic fibrosis and muscular dystrophies are some of the several thousand inherited disorders that can, in principle, be corrected by gene therapy. The various techniques used for delivery of therapeutic genes include viral vectors, physical methods, chemical methods and naked nucleic acids. A successful gene therapy program requires an efficient gene delivery system and targeting of a specific cell type without dissemination of the therapeutic gene to other cells thereby allowing longer persistence and adequate expression of the replaced gene. In the case of viral vectors, the host immune response may become a significant barrier to gene delivery and impede the overall success of the gene therapy effort.

AdV-based vectors were initially thought to be very promising candidates for gene therapy applications for genetic disorders. However, the progress of AdV vectors have been hampered by vector immunity and the toxicity induced by AdV vectors following systemic administration. The pre-existing anti-AdV neutralizing antibodies in the majority of the human population induced following natural exposure to AdV clears the AdV vectors soon after systemic administration. In the absence of pre-existing anti-AdV antibodies, the AdV vectors persist longer following initial inoculation. Repeated administration results in development of a neutralizing antibody response similar to natural infection preventing subsequent inoculations with the same vector from being effective.

The death of the patient in the ornithine transcarbamylase (OTC) clinical trial (Raper et al., 2003) and the results of several subsequent pre-clinical trials shifted the focus of AdV research towards the molecular mechanisms underlying AdV-induced innate immune pathways leading to development of AdV vectors, such as HD-AdV vectors, particularly suited for gene therapy applications. These HD-AdV vectors, due to the deletion of most of

the viral genome, can accommodate inserts as large as 36 Kb, making them the vector of choice for delivering very large genes, multiple genes or ta tissue-specific regulatory promoter which may be very long (Shi et al., 2002; Shi et al., 2006). The host cells transduced by HD-AdV vectors do not express the viral proteins and, therefore, cells carrying HD-AdV are not recognized by the host immune system resulting in longer persistence and transgene expression.

5.1 HD-AdV for liver gene therapy

The liver is the most affected organ in several genetic diseases including Crigler-Najjar syndrome and OTC deficiency. HD-AdV vectors have shown tremendous promise for liver directed gene therapy in several pre-clinical studies in small and large animal models. Their abilities to support long-term transgene expression, low chronic toxicity, natural liver tropism of AdV vectors and the fenestrated structure of liver endothelium allowing efficient hepatocyte transduction are the main factors (Brunetti-Pierri et al., 2008; Dimmock et al., 2011; Toietta et al., 2005). HD-AdV vectors were shown to be very effective for liver gene therapy in Gunn rats, the animal model for Crigler-Najjar syndrome. A single injection of a HD-AdV vector expressing uridine diphospho-glucuronosyl transferase 1A1 (UGT1A) was sufficient to induce life-long normalization of hyperbilirubinemia (Toietta et al., 2005). Similarly, an HD-AdV vector expressing canine glycogen-6-phosphatase was able to correct the hypoglycemia and prolong survival up to seven months in a mouse model of glycogen storage disease type 1 (Koeberl et al., 2007). Results of several other studies have supported the clinical utility of HD-AdV vectors in the gene therapy of liver diseases (Brunetti-Pierri et al., 2008; Gau et al., 2009; Hu et al., 2011; McCormack, Jr. et al., 2006; Oka et al., 2007).

However, the prospects of liver directed gene therapy using HD-AdV vectors are hampered by the high vector doses necessary to result in efficient hepatocyte transduction. Such high doses may not be clinically relevant for human use. A non-linear relation is observed between the vector dose and hepatocyte transduction; at low doses, there is very little to undetectable transgene expression, yet there are disproportionately high levels of transgene expression when vector dose is increased (Morral et al., 2002; Sullivan et al., 1997) with the Kupffer cells sequestering more than 85% of the intravascularly administered AdV vectors. AdV vectors also associate with platelets, red blood cells and other binding components in circulation resulting in eventual elimination of the vector. Sequestration by Kupffer cells and binding to the blood components determines the 'threshold dose' which must be administered for efficient hepatocyte transduction. Unfortunately, such high doses precipitate acute toxicity due to activation of the innate immune system and might result in the death of patient as observed during the human clinical trial for gene therapy of OTC deficiency (Raper et al., 2003). This conundrum warrants a thorough understanding of mechanisms underlying AdV-mediated innate immune system induction and calls for development of novel strategies to achieve high level hepatocyte transduction at clinically relevant doses.

5.2 HD-AdV for brain gene therapy

Due to the complexity of tissue organization in the nervous system, treatment of neurological disorders imposes a huge challenge to both healthcare providers and clinical researchers alike. HD-AdV gene therapy is currently being employed in preclinical trials as well as in animal models in the cases of brain cancer, sensory neuronopathies, neurodegenerative diseases, and multiple sclerosis, to name a few. Despite pre-exposure to

AdV, the sustained transgene expression in the brain for at least one year (Barcia et al., 2007) and the ability to infect the cells of the central nervous system renders HD-AdV vectors particularly appealing for brain gene therapy.

5.2.1 Gliomas

The tumor originating from glial cells is called a glioma which is highly lethal and associated with a median survival of mere 9-12 months (Furnari et al., 2007). Among gliomas, Glioblastoma multiforme (GBM) is the most common and so far incurable; aggressive primary brain cancer in adults has a median survival time of 15-21 months (Grossman et al., 2010). GBM is highly invasive with a limited potential for complete resection and a high recurrence rate owing to its resistance towards conventional chemotherapy and radiotherapy. A combination of HD-AdV-TK (cytotoxic herpes simplex type 1 thymidine kinase/HSV1-TK) and HD-AdV vector encoding Flt3L (human soluble fms-like tyrosine kinase ligand 3) under the control of a tightly regulatable mCMV-TetOn expression system (HC-AdV-TetOn-Flt3L29–31) was tried in rats bearing GBM (Muhammad et al., 2010). As a result of intratumoral administration of HD-AdV vectors, tumor cells were rapidly removed from the brain, and ~70% of the animals showed a reduction in the tumor mass within thirty days of the treatment leading to a long-term survival rate. The treatment also ensured the safety of the protocol with a one-year follow-up indicating the lack of behavioral deficits, chronic inflammation in the brain or alteration in the brain architecture. Furthermore, in order to lower the vector dose and improve production yield, a bi-cistronic HD-AdV vector has been developed "that encodes both constitutively expressed HSV1-TK and inducible Flt3L from a single HD-AdV vector genome" - the first one of its kind (Puntel et al., 2010).

5.2.2 Sensory neuronopathies

Dorsal root ganglion (DRG) neuron dysfunction is commonly associated with a number of sensory neuronopathies, and therefore, DRG comprises the target of choice for their treatment. Undesirable side effects of conventional treatments can be minimized by targeted gene delivery towards DRG neurons (Waehler et al., 2007). Fiber modified HD-AdV vectors have been engineered to specifically target DRG neurons in mice (Terashima et al., 2009) and were also tested in *Hexb*–/– mice, an animal model exhibiting neurological impairment leading to death before the age of twenty weeks (Sango et al., 2002). DRG-targeted HD-AdV vectors were injected through an intra-thecal route and were shown to have a significantly higher transduction of DRG neurons compared to unmodified HD-AdV vectors. Testing in Hexb–/– mice also showed production of β-hexosaminidase in the original Hexb-deficient mice as well as a reversal of gangliosidosis and improvement in peripheral sensory dysfunction (Terashima et al., 2009).

5.2.3 Neurodegenerative diseases

A helper-dependent canine AdV (CAdV-2) vector was designed and tested by Soudais et al (2003) and was proposed to be useful in the treatment of neurodegenerative diseases (Soudais et al., 2004). The advantages associated with this particular vector include preferential transduction of the neurons and retrograde transportation through the axons. Due to the chronic nature of neurodegenerative diseases, it is of particular interest whether the treatment can be offered over a long period of time. HD-AdV vectors have been administered into the cerebrospinal fluid (CSF) of non-human primates through lumbar

puncture, and it was shown that the intervention allowed a long-lasting (three months) infection of the neuroepithelial cells without any systemic or local toxicity (Butti et al., 2008).

5.2.4 Multiple sclerosis

The ability of HD-AdV vectors to express anti-inflammatory molecules can be useful in inflammatory disorders of the central nervous system. Experimental autoimmune encephalomyelitis (EAE) is an animal model of multiple sclerosis that has been adopted for pre-clinical studies. HD-AdV vectors expressing IL-4 were administered into the CSF of immunocompetent mice that allowed transduction of neuroepithelial cells and prolonged (five months) transgene expression without any adverse effects (Butti et al., 2008).

6. AdV vectors for cancer therapy

Not long after the discovery of existence of AdV, AdV were noted for their oncolytic nature in the clinical treatment of cervical carcinoma. Since that time, AdV have shown promise in the cancer vaccine field and currently offer a variety of methodologies which are being implemented to combat various forms of cancer (Table 1). The field of cancer vaccinology is fraught with complications since an effectively persistent immune response must be stimulated. One complication lies in the fact that immune tolerance must be overcome against self-antigens. These self-antigens, which are weak stimulators of the immune system or completely lack the ability to be immunogenic in an immunocompromised environment, pose quite a hurdle in the cancer vaccine development.

AdV	Condition	Intervention	Route of Inoculation	Phase	Sponsor
		Cancer			
Replication defective AdV5 [E1-, E2b-]-CEA(6D) [ETBX-011]	Advanced or metastatic malignancies expressing CEA (colon cancer, lung cancer, breast cancer)	Biological: Ad5 CEA Vaccine	s.q.	I	Etubics Corp.
Replication defective, Adv/CMV.rhENDO (AdVE10A)	Head and Neck Squamous Carcinoma Nasopharyngeal Carcinoma	Drug: E10A Drug: Cisplatin Drug: Paclitaxel	i.t.	II	Sun Yat-sen University
Replication defective E1-,E3-deleted HAdV5/CD40L gene driven by RSV promoter	Bladder cancer	Genetic: AdCD40L	Intravesical	I / II	Uppsala University
E1a and E1b-deleted CRrAdV5-p53 (SCH-58500)	recurrent, or progressive glioblastoma multiforme, anaplastic astrocytoma, or anaplastic mixed glioma	Biological: recombinant adenovirus-p53 SCH-58500	i.t.	I	National Cancer Institute (NCI)

AdV	Condition	Intervention	Route of Inoculation	Phase	Sponsor
Infectivity enhanced CRAdV5/tk (GliAtak) (PancAtak) (ProstAtak)	malignant glioma, glioblastoma multiforme, anaplastic astrocytoma, pancreatic cancer, prostate cancer	Biological: AdV-tk Drug: Valacyclovir + Standard Chemo-/Radio- therapy	i.t.	II	Advantagene Inc.
Replication defective E1a/E1b/E3 HAdV5.IL2 into autologous neuroblastoma cells	Neuroblastoma	Biological: autologous neuroblastoma vaccine	s.q.	I / II	Baylor College of Medicine
AdV.IFNgamma (TG1042)	Relapsing primary cutaneous B-Cell Lymphoma	Genetic: Adenovirus Interferon gamma	intralesional	II	Transgene
Replication-defective E1/E3-deleted AdVHer2/neu under the control of MMTV promoter transduced into DC	breast neoplasm	Biological: CD34+ derived DCs	intradermal	I	Hamilton Health Sciences Corporation
E1/E2a/E3-deleted HDAdV expressing rat Her2/neus	metastatic breast cancer, recurrent breast cancer	Biological: adenoviral vector encoding rat Her-2/neu	intradermal	I	Ontario Clinical Oncology Group (OCOG)
Replication deficient, E1/E3 deleted rAdV/RSV-hIL12 gene	primary metastatic breast cancer spread to liver	Biological: adenovirus-mediated human interleukin-12	i.t.	I	Mount Sinai School of Medicine
Replication-incompetent Ad-sig-hMUC-1/ecdCD40L vaccine	MUC-1 positive cancer cells in metastatic breast cancer	Biological: Ad-sig-hMUC-1/ecdCD40L vaccine	s.q.	I	University of California, Los Angeles
Replication defective, recombinant Ad.hIFNbeta gene	Colorectal carcinoma metastases	Drug: Ad.hIFNbeta (BG0001, IDEC-201)	i.v.	I / II	Biogen Idec
Replication-deficient (E1, E3 and E4 deleted) AdV containing TNF-alpha gene under control of radiation inducible promoter	Esophageal cancer	Genetic: TNFerade	i.t.	II	GenVec
Replication-deficient E1/E3 deleted rAdcuCD40L	Esophageal neoplasms	Genetic: AdcuCD40L	i.t.	I / II	Weill Medical College of Cornell University
(INGN 201) (Advexin®)HAd5CMV/p53	Head and Neck Cancer: premalignant carcinoma of the oral cavity or pharynx	Biological: Ad5CMV-p53 gene	oral rinse	I / II	M.D. Anderson Cancer Center
HAd5F35.LMP1/LMP2-DC	Head and Neck Cancer: metastatic nasopharyngeal cancer	Biological: Ad5F35-LMP1/LMP2-transduced autologous DC Drug: celecoxib	intradermal	II	NCC Head and Neck Clinic

AdV	Condition	Intervention	Route of Inoculation	Phase	Sponsor
Replication defective AdV w/TK, TK99UN suicide gene therapy	Carcinoma, hepatocellular	Genetic: TK99UN	i.t.	I	Instituto Cientifico y Tecnologico de Navarra, Universidad de Navarra
AdVhAFP	Locoregionally pre-treated hepatocellular carcinoma	Drug: AFP + GM-CSF Plasmid Prime and AdV/hAFP Boost	i.m.	I / II	University of Pittsburgh
AdV.p53-DC	Extensive Stage Small Cell Lung Cancer	Biological: Autologous DC transduced w/ AdVp53 + chemo Drug: w/ or w/o all trans retinoic acid (ATRA)	intradermal	II	H. Lee Moffitt Cancer Center and Research Institute
E1b 55kD-, E3- HAdV5/RSV-TK	Cutaneous Metastatic Malignant Melanoma	Biological: adenovirus RSV-TK Drug: ganciclovir	Intra-lesional	I	National Human Genome Research Institute (NHGRI)
Replication-defective AdV.IFNalpha gene	Malignant Pleural Mesothelioma	Drug: SCH 921015 + chemotherapy	intrapleural infusion	0	Abramson Cancer Center of the University of Pennsylvania
Replication-defective AdV.TNFalpha controlled by chemoraditation inducible promoter	unresectable locally advanced pancreatic cancer.	Genetic: TNFerade 5-FU	i.t.	III	GenVec
CRhAdV5/SSTR/TK.RGD (imaging gene/infectivity enhanced suicide gene)	ovarian cancer	Genetic: Ad5.SSTR/TK.RGD Drug: Ganciclovir (GCV)	Intra-peritoneal	I	University of Alabama at Birmingham
Replication defective AdVhIL12	prostatic neoplasms prostate cancer	Genetic: Il-12 gene	prostatic injection	I	Baylor College of Medicine
Ad-REIC/Dkk-3	Prostate cancer	Biological: Ad-REIC/Dkk-3	prostatic injection	I	Momotaro-Gene Inc.
Replication competent AdV5-yCD/mut TKSR39 rep-ADP	Prostate cancer	Biological: Ad5-yCD/mutTKSR39rep-ADP Radiation: IMRT	prostatic injection	II / III	Henry Ford Health Systems
Gene Therapies					
Replication deficient AdGVVEGF121cDNA	Coronary Artery Disease	Genetic: AdGVVEGF121cDNA	Intra-myocardial	FU on Phase I	Weill Medical College of Cornell University

AdV	Condition	Intervention	Route of Inoculation	Phase	Sponsor
Replication deficient E1, E4 deleted HAdV5OTCcDNA	Ornithine Transcarbamylase Deficiency Disease	Genetic: AdV Vector-Mediated Gene Transfer	Femoral arterial placement of a hepatic intraarterial catheter	I	FDA Office of Orphan Products Development
Replication deficient E1 deleted rHAd5-CB-CFTR	Cystic Fibrosis	Genetic: Ad5-CB-CFTR	nasal cavity	I	National Center for Research Resources (NCRR)
E1 deleted, E3 substituted (d1309) AdV5/OTC	Amino Acid Metabolism, Inborn Errors	Behavioral: Protein and calorie controlled diet	i.v.	I	National Center for Research Resources (NCRR)
Replication deficient, E1, E3 and E4 deleted, AdV/PEDF (pigment epithelium-derived factor) protein	Macular Degeneration	Drug: AdGVPEDF.11D	intravitreal injection into one eye	I	GenVec
Replication deficient deleted for all of E1a, most of E1b, and E3 sequences AdV/VEGF-D gene and a biodegradable local delivery device (collar) made of collagen	End Stage Renal Disease	(RAVE trial) Procedure: Graft placement surgery plus Trinam therapy	Near anasto-mosis	III	Ark Therapeutics Ltd
Replication deficient, E1-,E3-deleted AdV5/PDGF-B	Varicose Ulcer	Drug: Ad5/PDGF-B	intra-ulcer injection	I	National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS)
Replication-deficient AdV/VEGF-D	Angina Pectoris Myocardial Infarction	Biological: VEGF-D gene transfer	endocardial injection system	I	Kuopio University Hospital
Replication-defective E1 deleted HAdV5/hAQP1	Parotid Salivary Dysfunction	Gene Transfer Drug: AdhAQP1	catheter into parotid	I	National Institute of Dental and Craniofacial Research (NIDCR)
Infectious Diseases					
Mix of 2 Replication-deficient, recombinants (Ebola-rAd5) encoding GP from Zaire and SudanGulu strains	Ebola Hemorrhagic Fever	Drug: VRC-EBOADV018-00-VP	i.m.	I	National Institute of Allergy and Infectious Diseases (NIAID)
ADhVN1203/04.H5	Pandemic Influenza	Pandemic Influenza Vaccine	Oral ingestion of enteric capsule	I	Vaxin Inc.

AdV	Condition	Intervention	Route of Inoculation	Phase	Sponsor
Replication-Competent, recombinant AdV4/H5N1 (Ad4-H5-Vtn)	Avian Influenza	Biological: Ad4-H5-Vtn	Oral ingestion of enteric capsule	I	PaxVax, Inc.
Live, Replication-deficient rAdV35 (E1-, partially deleted E3encoding fusion protein of the Mycobacterium tuberculosis antigens	Tuberculosis	Biological: AERAS-402	i.m.	I / II	Aeras Global TB Vaccine Foundation
Replication incompetent, multiclade rAdV5 VRC-HIVADV014-00-VP	HIV	Biologicals: VRC-HIVDNA016-00-VP; VRC-HIVADV014-00-VP; VRC-DILUENT013-DIL-VP; VRC-HIVADV014-00-VP placebo	i.m. via Bioinjector	I / II	National Institute of Allergy and Infectious Diseases (NIAID)
AdV.ZFN/CCR5 (SB-728-T)	HIV	Biological: ZFN modified T cells	Infusion of apheresed T cells	I	University of Pennsylvania
Replication defective MRK Ad5 HIV-1 gag	HIV	Biological: MRK Ad5 HIV-1 gag vaccine	i.m.	II	National Institute of Allergy and Infectious Diseases (NIAID)
rAd26.ENVA.01	HIV	Biologicals: Ad26.ENVA.01 (rAd26); Placebo Vaccine	i.m.	I	National Institute of Allergy and Infectious Diseases (NIAID)
rAd35, rAd5	HIV	DNA Vaccine; DNA Vaccine placebo; rAd35; rAd35 placebo; rAd5; rAd5 placebo	i.m.	I	National Institute of Allergy and Infectious Diseases (NIAID)
rAd5	HIV	Biological: NYVAC-B (poxvirus) Biological: Placebo Biological: rAd5	i.m.	I	HIV Vaccine Trials Network
Replication defective simian Ad	HIV	Biological: MVA.HIVconsv low dose Other: Placebo low dose Biological: MVA.HIVconsv high dose Other: Placebo high dose	i.m.	I	University of Oxford

AdV	Condition	Intervention	Route of Inoculation	Phase	Sponsor
Ad26.ENVA.01, Ad35-ENV	HIV	Biological: Ad35-ENV vaccine Biological: Ad26.ENVA.01 vaccine Biological: Placebo Control	i.m.	I	International AIDS Vaccine Initiative

DC=dendritic cell; h=human; LMP= latent membrane protein; PEDF = pigment epithelium-derived factor protein; OTC: ornithine transcarbamylase; r=recombinant; VEGF=Vascular Endothelial Growth Factor D; SSTR=somatostatin receptor type 2

Table 1. Clinical Trials of AdV vectors

6.1 Tumor suppressor therapies

Several genes (p53, Rb,BRCA1, PTEN, etc) regulate the cell cycle and apoptotic pathways. The cellular machinery can inhibit tumor formation by suppressing aberrant cellular proliferation. Several malignancies have a loss-of-function mutation in one or more of the cell cycle control genes. Gene delivery can rescue a cell from a tumor phenotype by restoring the dysfunctional gene or a tumor-suppressor gene. One of the most extensively studied tumor suppressor genes, p53, is mutated in more than 50% of all human malignancies. The administration of wild-type p53 through gene transfer with a replication-defective AdV has demonstrated significant tumor suppression (Roth, 2006). The repair of p53 activity also has impaired angiongenic activity, inhibited the growth of nontransduced cells and directed a local immune response against tumor cells. In addition, replacement of p53 increased the vulnerability of tumor cells to chemotherapy and radiotherapy by reestablishing pro-apoptotic pathways. Furthermore, the potency of an anti-tumor response is enhanced when p53 gene replacement therapy is supplemented with available cytotoxic chemotherapeutics (El-Deiry, 2003). The first AdV gene therapy construct for the treatment of cancer has been approved by the State Food and Drug Administration (SFDA) of China. This AdV construct contains a p53 expression cassette in the deleted E1 region and has demonstrated remarkable synergistic effects when combined with radio-/chemotherapy, surgery, or hyperthermia for the management of cancer (Peng, 2005).

Another way to inhibit tumor growth is to manipulate ligand-receptor interactions. AdV have been engineered to deliver ligands to oncogenic receptors in the form of antibodies against the receptor or by mimicking natural interactions. Peptides with either natural or recombined ligand moieties have been particularly efficacious in receptor-ligand mimicry (Pasquale, 2010). Receptor-ligand interactions typically lead to down-regulation of that receptor either directly or through signal transduction complexes. One example is the EphA2 receptor, a tyrosine kinase protein, which is elevated in breast (Zelinski et al., 2001), prostate (Walker-Daniels et al., 1999), pancreatic, (Van Geer et al., 2010) and many other cancers. Thus, EphA2 is a good therapeutic candidate for treating cancer. Under normal conditions, the EphA2 receptor is localized at intracellular junctions where it binds to its membrane-anchored ligand, EphrinA1, resulting in phosphorylation of the receptor. E-cadherin, a cell adhesion molecule, is also able to phosphorylate EphA2 and aid in proper localization of the receptor. In many cancer cells, EphA2 is over expressed, unphosphorylated and unable to bind to its ligands due to its altered localization (Kinch & Carles-Kinch, 2003; Zantek et al., 1999). Monoclonal antibodies directed against EphA2 have

been used in order to mimic ligand-induced downregulation. These antibodies caused elevated levels of EphA2 phosphorylation which encouraged its degradation (Carles-Kinch et al., 2002). AdV-mediated delivery of the secretory form of EphrinA1 produced autophosphorylation, degradation, and subsequent inhibition of tumor growth and metastasis (Noblitt et al., 2004; Noblitt et al., 2005). Likewise, AdV-mediated antibodies directed against ErbB2 (HER2/neu) demonstrated that ErbB2 down-regulation increased apoptosis and cytotoxicity both *in vitro* and *in vivo* (Arafat et al., 2002; Jiang et al., 2006).

6.2 CRAdV therapy

An effective approach to cancer therapy is the use of RCAdV that selectively replicate inside a tumor and ultimately kill tumor cells while leaving normal cells intact. As previously mentioned, therapeutic CRAdV are either engineered by deletion mutagenesis or engineered for the tumor- or tissue-specific replication by use of transcriptional regulatory elements (TREs).

6.2.1 Deletion mutation of CRAdV

CRAdV have expanded our knowledge regarding AdV proteins and how they interact with cellular proteins. E1 proteins of AdV adjust cell cycle controls in order to promote virus replication. The product of the E1A gene binds to the wild type Rb protein which causes the release and activation of E2F, a transcription factor (Whyte et al., 1988). Once E2F is activated, the transcription of S phase entry genes facilitates the hijacking of cellular machinery involved in AdV replication (Flint & Shenk, 1997). Additionally, E2F transactivates the p14ARF gene which increases p53 levels within the cell by inhibiting its degradation by murine double minute 2 (MDM2) (Bates et al., 1998). Although increased p53 levels can cause cellular apoptosis or arrest of the cell cycle, it also inhibits viral replication. To circumvent this replication inhibition, AdV E1B 55 kDa proteins work with E4 open reading from (ORF) 6 to bind to p53 and inactivate it preventing apoptosis and permitting virus replication and spread (Dobner et al., 1996; Yew & Berk, 1992). A *bcl-2*-related protein, E1B 19 kDa, also prevents apoptosis stimulated by E1A (Boyd et al., 1994; Rao et al., 1992). This knowledge combined with known tumor suppressor genes (i.e., p53 and Rb), tumor-associated antigens (CEA) or specialized promoters has facilitated the development of efficacious CRAdV.

The first CRAdV to enter clinical trials was ONYX-15 (also known as dl1520), a E1B 55kDa/E3B deleted vector. This mutant CRAdV selectively replicates in cancer cells that possess a defective p53 while leaving cells with intact p53 alone. It has undergone numerous clinical trials (Phase I to III) to test its therapeutic potential in head and neck squamous cell carcinoma, glioblastoma, hepatocellular carcinoma, colorectal carcinoma, sarcomas, ovarian, pancreatic, and hepatobiliary cancer (Aghi & Martuza, 2005; Kirn, 2001). Data indicate that ONYX-15 is safe and selective for cancer (Kirn & Thorne, 2009), but the strategy has failed to demonstrate extensive therapeutic effects or significant systemic spread (Liu et al., 2008b). Other oncolytic vectors that possess the same E1B/E3B mutations have exhibited the same capabilities, but only H101 was finally approved by the Chinese SFDA for use in combination with chemotherapy to treat late-stage refractory nasopharyngeal cancer (Kirn et al., 2008; Liu et al., 2008b).

There is a weak correlation between p53 status and cellular susceptibility to E1B/E3B mutant CRAdV (Geoerger et al., 2002; Hay et al., 1999; Rothmann et al., 1998). Some

researchers have observed that when E1B 55kDa binds to p53, AdV-mediated death is followed by a productive infection (Dix et al., 2000; Hall et al., 1998). Others argue that the preferential killing by ONYX-15 types may be due to infectivity variances of the vector, permissiveness, upregulation of AdV early proteins (Steegenga et al., 1999), p14ARF failure (Ries et al., 2000), mutation features of p53 (Hann & Balmain, 2003), or late export of viral RNA (O'Shea et al., 2004) rather than solely the status of p53 (Sharma et al., 2009b). Replication of ONYX-15 is attenuated when compared with wild-type virus (Dix et al., 2001) which may be attributable to other E1B functions such as translation, nuclear exports of late viral mRNA, and compromised inhibition of host cell protein synthesis (Babiss & Ginsberg, 1984). Other mutations have been identified in the E1B 55 kDa protein that enhanced selectivity for tumors without hindering viral replication (Shen et al., 2001).

An alternative method involves the use of CRAdV mutants that possess a deletion in E1A and Rb-binding domain (CR-2) which ultimately targets cancers that have aberrant Rb pathways (Fueyo et al., 2000; Heise et al., 2000). When anti-tumor potential is compared between E1B mutations and CR-2 deletion vectors which do not inhibit vector replication in cancerous cells, the CR-2 vectors demonstrate better efficacy *in vivo* and *in vitro* (Alemany & Curiel, 2001). There are safety concerns, however, that exist with CR-2 deletion vectors since their replication is not restricted to cancer cells alone (Heise et al., 2000). Incorporation of tumor-specific promoters which restrict viral gene expression (i.e., ONYX-411) or the incorporation of Arg-Gly-Asp(RGD) motifs are beneficial to enhancing tumor cell selectivity and safety (Johnson et al., 2002; Page et al., 2007).

CRAdV targeting CEA-expressing malignancies in the colon, lung, and breast tissues have been successful and are in Phase I clinical trials with HAd5[E1-,E2b-]-CEA(6D) or ETBX-011. The HAdV5 has been engineered to express the CEA protein which is found in some cancerous cells. The goal is to direct the immune system to target cancer cells producing CEA. [http://clinicaltrials.gov/ct2/show/NCT01147965]

6.2.2 Transcriptional regulation of CRAdV

Another class of CRAdV replaces viral promoters that control vital transcriptional units with transcription regulating elements (TREs). Transcriptional targeting drives selection according to the vector gene expression and replication parameters set in motion by the TREs. The most obvious gene to be selected for TRE regulation is E1A since it is responsible for viral replication and adaptations which favor virus replication in the host cell environment. CN706 was the first TRE-regulated AdV to demonstrate potent anti-tumor effects (Rodriguez et al., 1997). In this construct, E1A expression was under the control of the prostate-specific antigen (PSA)-derived minimal enhancer/promoter which permitted selective replication in PSA-expressing prostate cells. Several promising TREs under investigation today include those that regulate expression of human telomerase reverse transcriptase (hTERT), transcription factor E2F, alpha-fetoprotein (AFP), and many more which tailor vector replication to be selective for a specific tumor phenotype.

Leaky replication has been observed in normal cells even though a single essential viral gene is under exogenous control. To improve tumor specificity and safety of CRADV, other essential viral genes (e.g., E1B, E2, E4) can be manipulated (Brunori et al., 2001; Doronin et al., 2001; Kawashima et al., 2004; Kuppuswamy et al., 2005; Li et al., 2005). A more complete list is available in a previous review (Sharma et al., 2009b). Other deletion mutations have been made in an effort to exploit specific oncolytic potentials while also retaining the replicative capacity of the virus. However, more work is warranted in all areas of CRAdV

development before they can be used commercially. Additional safety features should include external regulation of oncolytic CRAdV through activation of inducible promoter systems such as TET or MMTV promoters (Avvakumov & Mymryk, 2002; Chong et al., 2002; Fechner et al., 2003). Furthermore, tissue-specific promoters can regulate where the vector replicates (Hernandez-Alcoceba et al., 2000; Hsieh et al., 2002). Radiation, chemical or heat-inducible promoters have also been employed to restrict transgene expression (Lee et al., 2001; Rasmussen et al., 2002).

Various regulatory elements have been fused in order to artificially design promoters which restrict TRE size while also preserving or amplifying rigid control of vector replication (Nettelbeck et al., 2000; Nettelbeck, 2008). In one artificial design, bidirectional promoters that simultaneously exert control over two key viral genes (i.e., E1A/E1B or E1A/E4) have been applied. Another promoter concept includes a dual-specific hybrid with regulatory elements that react to a hypoxic environment and estrogen thereby conferring greater discriminatory capabilities to oncolytic CRAdV (Hernandez-Alcoceba et al., 2002). Engineering of these transcriptionally-regulated CRAdV is an onerous task and poses several limitations due to the variety of viral and non-viral factors which can influence the response of the heterologous promoter. Safety concerns arise when *cis*-acting enhancer elements or cryptic transcription initiation sites lie in the left ITR or packaging signal. These regions that are upstream of E1A can influence transcriptional read-through of the E1A or transgene region even in the presence of a TRE (Yamamoto et al., 2003). Other elegant designs which address the limitations include the insertion of additional transcriptional terminators or insulators which are upstream of heterologous promoters, orientation changes of the E1A expression cassette, or the translocation of the packaging signal from the left ITR to the right ITR.

Post-transcriptional methods which control mRNA stability or translation are also under investigation for CRAdV development. Within the tumor microenvironment, proliferative signals are the driving force for expression of specific tumor-associated proteins. Expression of these proteins is boosted partially by the stabilization of mRNA by a 3'UTR which is regulated by an activated mitogen-activated protein kinase (P-MAPK) pathway. The 3'UTR regulates mRNA stability in several genes; hence, ligation of the 3'UTR with crucial AdV genes confers the vector with the ability to better target tumors (Sharma et al., 2009b).

No CRAdV have currently been developed that completely lack the ability to replicate or cause some toxicity in normal cells. They do however confer a great deal of selectivity and safety. Future conceptions should involve the enhancement of tumor-specific replication.

6.3 Suicide gene therapy

Suicide gene therapy selectively orchestrates a gene-directed enzyme for prodrug therapy (GDEPT) to deliver chemotherapeutic moieties to specific tumors. Nontoxic drugs (i.e., chemotherapeutic agents) are converted by AdV loaded with a GDEPT into cytotoxic agents within the tumor. Classic chemotherapy is often harmful to normal cells and, thus, promotes deleterious effects with the high doses needed for maintaining therapeutic indices. Since nontoxic prodrugs can be administered without harmful side effects to normal cells, this is a preferred methodology for chemotherapeutic delivery.

Two suicide genes which have been well characterized include the herpes simplex virus, type I thymidine kinase (HSV-TK) and the Escherichia coli, cytosine deaminase (CD) genes. HSV-TK phosphorylates ganciclovir (GCV) which cooperates with DNA polymerase to interfere with DNA synthesis ultimately leads to cell death in rapidly dividing cells. CD transforms 5-fluorocytosine (5-FC) into a very toxic metabolite 5-fluorouracil (5-FU), which is a frequently

used chemotherapeutic (Aghi et al., 1998). Although direct toxicity is imparted by GDEPT-mediated cell death, there is also significant growth inhibition/killing of uninfected neighboring tumor cells due to the transfer of the toxic drug. The causes of this event are hypothesized to involve transfer via gap junctions or diffusion and stimulation of an anti-tumor response against lysed tumor cells (Lumniczky & Safrany, 2006).

Replication-defective AdV were initially used in GDEPT but demonstrated poor anti-tumor efficacy. RCAdV have had a better outcome, and the combination of suicide genes with standard therapies has further enhanced anti-tumor efficacy. However, GDEPT loaded AdV must be administered intratumorally in order to protect normal cells from cytotoxic events.

Other methods have evolved which can be used alone or in combination with the methods described above. The transductional targeting of tumors, the selective expression of suicide genes, or the addition of tumor specific promoters to AdV essential genes have improved anti-tumor potential of AdV vectors. These suicide gene/prodrug combinations are under investigation in both preclinical and clinical trials. Better dosage regimens and vaccination protocols must be formulated in order to improve the therapeutic potential of this system.

Another approach to consider for improvement involves thorough documentation of the kinetics of the prodrug and vector transgene expression. Timing prodrug administration in order to maximize benefits is of crucial importance. One novel method that has been proposed involves sequential administration of prodrug 5-FC followed by GCV. This double suicide gene therapy produced significant synergistic cytotoxicity. Avoidance of premature cell death is of critical importance since AdV will not be able to replicate. If virus proliferation is inhibited, yield will decrease markedly affecting the benefit of AdV-mediated suicide gene therapy (Sharma et al., 2009b).

6.4 Cancer immunotherapy

An intact immune system is capable of recognizing and eliminating tumor cells in the body; however, cancer cells possess the ability to evade immune detection. Tumor cells have several methods to avoid immune detection which include but are not limited to the release of immunosuppressive or anti-inflammatory proteins, flawed antigen presentation and processing caused by mutations within the mechanisms of antigen presentation or decreased expression of MHC molecules. The ineffectual anti-tumor immune response (humoral or cell-mediated) along with the progression of immunosuppression during oncogenesis demonstrates how the immune system needs to be magnified and specificity-modified to target tumor cells and the process of oncogenesis. The objective of cancer immunotherapy is to stimulate an endogenous immune response against already established or rapidly developing tumors. AdV have been employed to promote an immune response against cancerous cells by transporting immunostimulatory molecules or by arming dendritic cells (DC) with the appropriate tumor-associated antigen (TAA) (Sharma et al., 2009b).

6.4.1 Delivery of cytokines/co-stimulatory molecules via AdV

The administration of cytokines augments the cytokine environment within the tumor cell while also rallying immune cells to elicit an anti-tumor response. Many cytokines and membrane bound receptors (IL-12, IL-2, IFN-gamma, CD40L, etc) are very potent immunomodulators but elicit systemic toxicity during exogenous delivery. They also have a short half-life *in vivo* and are maintained at subtherapeutic levels at the tumor site. AdV loaded with cytokine expressing genes which are delivered intratumorally have

demonstrated increased patient survival and decreased tumor growth. IL-2 suppresses tumor growth by stimulating cell-mediated killing activities in cytotoxic T lymphocytes (CTLs), lymphokine-activated killer (LAK) cells, or tumor infiltrating lymphocytes (TILs) (Eberlein & Schoof, 1991). A few clinical trials have exploited the potential of IL-2 in order to build an immune response to eventually kill tumor cells. IL-12 is another cytokine under investigation which elicits a potent anti-tumor response when administered locally by stimulating proliferation and cytotoxicity of CTLs and natural killer (NK) cells. A replication defective AdV (AdV.TNF) which does not target any particular tumor-associated antigen but rather expresses TNF-α under a chemoradiation-inducible promoter is in Phase III of clinical trials. The AdV.TNF is administered intratumorally along with radiation and 5-FU to induce an anti-tumor response. Co-stimulatory molecules, such as CD40L, have also demonstrated an ability to suppress tumor growth and are in Phase I/II of clinical trials (Habib-Agahi et al., 2007; Kikuchi & Crystal, 1999; Loskog et al., 2004; Martinet et al., 2000; Xu et al., 2005; Yoshida et al., 2003).

6.4.2 AdV Infection of DCs facilitate cancer immunotherapy

DCs are efficient and specialized APCs which present MHC-tagged epitopes to T cells in order to generate a specific immune response. These cells have been modified by AdV *ex vivo* so that the DCs either present TAA to effector cells of the immune system and/or introduce various immunomodulatory genes (i.e., cytokines or co-stimulatory receptors). Several of the anti-cancer therapies utilizing AdV have coupled the vector with the DC's abilities in order to generate an effective anti-tumor therapy. This method offers several advantages: (1) the AdV demonstrates adjuvant activity and has the ability to activate DCs and promote maturation which facilitates the induction of strong anti-tumor response (Geutskens et al., 2000; Kanagawa et al., 2008) , (2) AdV expression of TAA within DCs permits processing and loading onto both MHCI and MHCII molecules which stimulates the appropriate and relatively persistent activation of CD8+/CD4+ responses (Xia et al., 2006), and (3) *ex vivo* treatment of DCs with AdV evades any potential problems with pre-existing vector immunity (Wan et al., 1999).

7. AdV vaccines for infectious diseases

Significant advancements have been made in recombinant viral-vaccine strategies for infectious disease. AdV vaccines have demonstrated remarkable levels of T cell immunogenicity. AdV possess the ability to prime the immune response to target a transgene. This immune response can be boosted to high levels with a second vector that is recombined with the target antigen or with a serotype from a heterologous AdV (Jolly et al., 2008).

7.1 HIV vaccines

HIV is a constantly evolving virus that is an imposing challenge in the realm of engineering vaccines. The goal of HIV-1 vaccines is to reduce viral load, prolong survival, and minimize transmission. Vaccines that induce the HIV-1-specific cellular immune response have a delicate balance to maintain with regard to population preservation and selective stimulation. The vaccine construct should preserve the CD4+ T cell population and also increase the degree to which the CD8+ and CD4+ T cells recognize and bind HIV-1 antigens such as Gag and Pol. In addition, an elevated titer of neutralizing antibodies which possess high-avidity for the native trimeric Env protein of the virus needs to be attained.

A HAdV5-based HIV-1 vaccine was tested in humans in a Phase II trial to evaluate the risk for acquiring HIV infection. Unfortunately this trial was halted because the monitoring board determined that the vaccine was not able to demonstrate protection (Buchbinder et al., 2008; McElrath et al., 2008; Priddy et al., 2008). Future goals are focused on additional optimization of vaccine immunogens and technologies (Bradac & Dieffenbach, 2009). Since other researchers report the ability to induce a high level of cell-mediated immune response in non-HIV formulations, this is the time to apply this proof of principle to the area of HIV vaccine research.

In other areas of research, an AdV vector with chimeric fiber is used to infect autologous T cells to facilitate HIV therapy. This chimeric fiber contains both the HAdV5 fiber tail and the HAdV35 fiber shaft and knob domains (Perez et al., 2008). Due to its specificity to CCR5, CD4+ T-cells treated with this vector are relatively resistant to HIV infection. The data, while preliminary, are encouraging. Also, the modified CD4+ T cells treated with this vector are well-tolerated and engraft, replicate, and persist within the body after a single infusion. They behave like unmodified T-cells in so much as they migrate to the gut mucosa where they undergo selective expansion (Barouch, 2010). When used in combination with conventional anti-HIV therapies, this strategy might prove beneficial.

Different immunization strategies include a prime-boost approach with AdV-modified vaccinia virus Ankara (MVA) or heterologous HAdV5 and HAdV35 regimens or triple combinations which further elicit both T and B cell responses. The use of HAdV26 and HAdV35 in a prime-boost approach has shown promise in avoiding pre-existing vector immunity (Barouch, 2010). Immunization with HAdV26-Gag and HAdV35-Gag resulted in increased levels of CD127, CD62L, and Bcl-2 on T cells compared to HAdV5-Gag inoculation. These markers are indicative of improved functioning in T lymphocytes. There is also increased proliferation potential as well as increased antigen specificity.

7.2 Malaria vaccines

Malaria is characterized by several molecular interactions between the parasite and host during the parasite's incubation. An effective malaria vaccine will vigorously induce protective antibody and T cell responses to various malaria antigens that are expressed during the parasite's differentiation at the various life stages. Currently a mixture of two HAdV-5 vectors which express the pre-erthrocytic-stage malaria antigen circumsporozoite protein (CSP) or blood-stage malaria antigen apical membrane antigen 1 (AMA1) have demonstrated some success and are in Phase I/II of clinical trials. After immunization, human volunteers had a remarkable response to each antigen. Depletion studies determined that the response was due to a mixed population of CD8+ and CD4+ T cells. The CD8+ T cell lineage was five times greater than the CD4+phenotype when IFN-γ secreting cells were assayed within the response population (Draper & Heeney, 2010).

Another malaria vaccine platform involves the use of rAdV of rarer human serotypes or of non-human origin. These designs are engineered in such a way as to avoid pre-existing vector immunity against HAdV5. Some preclinical trials investigate priming with one AdV (i.e., HAdV5) and boosting with a heterologous AdV (i.e., HAdV35). Another strategy uses chimpanzee AdV serotype 63 (AdVCh63). AdVCh63 expresses the pre-erthrocytic-stage antigen thrombospondin-related adhesion protein combined with a multi-epitope string (ME-TRAP). A preclinical study utilizing a HAdV5 vector for priming followed with a boost by a MVA vector to promote the antibody and T cell responses. This particular HAdV5-MVA protocol used vectors that had been recombined with blood-stage malaria

antigen merozoite surface protein 1 (MSP1) which induces a T cell response which was partially effective against the liver-stage infection (Draper et al., 2009) and a protective antibody response effective against the blood-stage infection (Draper et al., 2008). Future studies will have to address the immunogenicity problems of the AdV system that may exist when it is used in a target population of African children between the ages of one and six. This age group lives in a highly endemic region for malaria. It has already been shown that vector immunogenicity, provided by sequential immunization with two attenuated poxvirus vectors, was less effective in affording protection when immunized children were compared with naturally immune adults in Africa and malaria-naïve volunteers from developed countries (Bejon et al., 2006).

7.3 Tuberculosis (TB) vaccines

AdV vaccine programs are being investigated for human TB, but these programs are slightly hindered due to the absence of a good experimental model for humans during challenge. Currently, *M. bovis* BCG is being developed as a representative challenge for human *M. tuberculosis* (Draper & Heeney, 2010). The AdV in clinical trials for *M. tuberculosis* aims to circumvent vector immunity by utilizing HAdV35 in place of HAdV5. AERAS-402 utilizes a live, replication-deficient rHAdV35 that expresses a fusion protein of three Mycobacterium tuberculosis antigens, 85A, 85B, and 10.4 (Radosevic et al., 2007). This platform has demonstrated safety and efficacy in adults that were primed by BCG or recombinant BCG. The system intends to increase T cell-mediated immunity and, thus, protection from tuberculosis. Preclinical studies are exploring the potential of aerosolized delivery for AERAS-402 in an attempt to intensify mucosal immunity to provide greater protection against *M. tuberculosis* infection. However, in order to address these issues, more preclinical data needs to be collected from non-human primates.

7.4 Influenza vaccines

The establishment of an influenza vaccine that protects against multiple strains is a major undertaking and a long sought after goal. Given the current circumstances of the seasonal emergence of new permutations of influenza, the past pandemic involving the 2009 H1N1 influenza type A strain, and the possibility of highly pathogenic avian influenza to attain pandemic potential, accomplishing this goal is imperative. Inducing variant-specific antibody responses against the surface antigens, hemagglutinin and neuraminidase, is not the best way to combat influenza viruses which are constantly changing their surface proteins due to antigenic drift. Initiating a strong T cell response in addition to neutralizing antibody response to conserved T and B cell epitopes is important for protection against influenza variants.

AdV-based influenza vaccines provide particular advantages over egg-based influenza vaccines due to their low production costs and the quick and easy manufacturing of a substantial amount in validated cell lines. Replication-competent as well as replication-defective AdV vectors have been developed to express distinct influenza antigens. Each construct has been evaluated for its immunogenicity and efficacy in a variety of animal models, and a few formulations have been evaluated for the safety and efficacy in clinical trials in humans.

Immunization of mice with an AdV expressing the HA gene from swine influenza virus - A/Swine/Iowa/1999 (H3N2) resulted in high levels of influenza-specific neutralizing antibodies. Also, the immunization led to partial protection against a lethal challenge with a

heterologous virus [A/HK/1/1968 (H3N2)] (Tang et al., 2002). Intranasal or epicutaneous immunization of humans with a replication-defective AdV encoding the HA gene of A/PR/8/1934 (H1N1) influenza virus resulted in a fourfold increase in hemagglutination inhibition (HI) titers in 83% of the participants (Van Kampen et al., 2005). The presence of pre-existing vector immunity did not seem to have a significant impact on the resultant hemagglutinin (HA)-specific immune response.

A replication defective HAdV5 influenza A (HAd-H5HA) vaccine containing the HA gene from HK/156/97 (H5N1) was evaluated for immunogenicity and protection in a mouse model (Hoelscher et al., 2006). The immunization resulted in the development of epitope-specific CD8 T cells and virus neutralizing antibodies. HAd-H5HA expresses HA from H5N1 influenza strain A/Hong Kong/156/1997 (HK/156/97) and is administered intramuscularly or intranasally. This vaccine construct provided complete protection to mice when challenged with homologous (HK/156/97) or heterologous (VN/1203/04) H5N1 influenza virus challenge indicating that HAd-H5HA afforded cross-protection to antigenically distinct strains of highly pathogenic H5N1. Immunized mice with HAd-H5HA were fully protected following challenge with a homologous H5N1 virus even after one year following immunization (Hoelscher et al., 2007). Inclusion of the HA genes from clade 1 and clade 2 H5N1 influenza viruses and the NP gene from one of the clades in the AdV-based vaccine resulted in expanded protection (Hoelscher et al., 2008). A replication-defective AdV vector expressing the HA gene from a H5N1 influenza virus has been tested in a Phase 1 clinical trial. The replication competent HAdV4 vector-based vaccine containing the HA gene of a H5N1 virus induced both humoral and mucosal antibodies as well as a cellular immune response when administered in a enteric capsule (Palkonyay, 2009).

An AdV vector provokes both humoral and cellular immune responses. If an AdV is engineered to carry multiple genes from different influenza strains, this will enhance the cross-protective efficacy against multiple strains. An AdV that is loaded with cross-reactive potential will provide a stockpiling option to address the vaccine need for a potential H5N1-based pandemic influenza (Vemula & Mittal, 2010).

7.5 Filovirus vaccine

In the effort to battle Ebola and Marburg viruses, a Phase I study has been completed using a mixture of two replication-deficient, rAdV5 (Ebola-rAdV5) encoding glycoproteins (GP) from Zaire and Sudan-Gulu strains, as well as the nucleoprotein (NP) of filovirus. It demonstrated excellent pre-clinical results with 100% protection in monkeys, but, apparently, it did not do well in human clinical trials (Martin et al., 2006). Virus neutralizing antibodies that were specific for the virus strains used in the trial were undetectable in the persons vaccinated. It was also determined that NP was not necessary for inclusion in the vaccine construct and may actually dampen the protective immune response (Sullivan et al., 2006).

8. Improving adenovirus based gene transfer- strategies for immune evasion

Staggering innate and adaptive immune responses have undoubtedly limited the therapeutic potential of AdV based gene transfer. However, the extensive promise that Ad vectors offer for improving human and animal health has fueled tremendous interest in acquiring a deeper understanding of molecular mechanisms underlying the immune

activation. Several promising innovative strategies to eliminate or minimize the acute inflammatory responses induced by AdV vectors, as well as to circumvent the vector immunity in order to prolong the vector persistence in host are being developed.

8.1 Immunosuppression and immunomodulation

Elimination of a significant fraction of intravascularly administered AdV vectors within initial twenty-four hours is attributed to elements of non-specific innate immune response, particularly the liver Kupffer cells, acting as a buffer against invading pathogen. Hepatocyte transduction occurs only after the saturation of kupffer cells at higher doses (Di Paolo et al., 2009). Removal of Kupffer cells is expected to result in higher hepatocyte transduction efficiency. In fact, transient depletion of macrophages prior to vector inoculation using pre-treatment with gadolinium chloride ($GdCl_3$) or dicholoromethylene bisphosphate (Cl_2MBP) not only reduces the initial vector clearance, but also seem to prolong the long-term transgene expression, possibly through attenuating the vector-specific adaptive immune responses (Kuzmin et al., 1997; Lieber et al., 1997). Although an effective macrophage depletion strategy may not be able to prevent vector elimination entirely since other mechanisms are also involved, this strategy might be used effectively in combination with other immune suppression methods.

The anti-vector humoral and cytotoxic immune responses are primarily responsible for preventing long-term transgene expression and act by eliminating the vector-transduced cells. Several studies have shown that treatment with non-specific immunosuppressive agents such as cyclosporine A, cyclophosphamide, deoxypergualin, dexamethasone and FK506 at the time of vector administration in presence of pre-existing immunity can suppress progression of humoral and/or cellular immune response and prolong the persistence of transgene in the host (Kaplan & Smith, 1997; Kuriyama et al., 2000; Seregin et al., 2009; Smith et al., 1996; Thomas et al., 2008). Similar effects can be obtained through transient immunosuppression by using specific antibodies targeted against molecules such as T lymphocyte antigens, CD4, CD40, or CD86 to block the receptor-ligand interaction (Chirmule et al., 2000; Haegel-Kronenberger et al., 2004; Ye et al., 2000). Many AdV vector applications require administration of high vector doses and, therefore, necessitate prevention or mitigation of an AdV-induced acute inflammatory response. Simultaneous administration of suppressor of cytokine signaling 1 (SOCS-1) results in generalized lower serum levels of cytokines, including IL-6, MCP-1, RANTES and TNF-α (Sakurai et al., 2008). Other studies have targeted suppression of specific cytokines, for example, TNF-α (Wilderman et al., 2006) and IFN-α/β (Zhu et al., 2007) resulted in significant reduction of the inflammatory response. Interestingly, a regimen for the inhibition of multiple cytokines at same time may have an additive or synergistic effect on inhibition of the inflammatory response. However, caution must be exercised for interpreting the results of such studies as species-specific variations may exist, and the observation made in animal models may not be replicated in humans. Each immunosuppressive or immunomodulatory approach needs to be thoroughly evaluated for potential side effects before their transition to clinical trials. Additionally, although transient, a generalized immunosuppression might render the patients more vulnerable to other diseases.

8.2 Vector modifications

Strategies involving vector modifications impose lesser risk to the host than those that involve attenuating or modifying host immune responses. Vector modifications can be

achieved through several different strategies, each with its own advantages and disadvantages, and will vary pertinent to the specific application. Vector modifications can consist of many alternatives such as (1) those intended to ablate interactions between AdV and its primary and/or secondary receptor, (2) fiber knob modifications to retarget vector to specific cell types, (3) replacement of fibers using serotype chimerism, (3) covalent modifications, (4) vector pseudotyping, and (5) vector microencapsulation.

8.2.1 Fiber knob modification

Binding of the HAdV5 fiber knob domain to CAR on the cell surface is the foremost step in virus entry. Multiple strategies are available for altering the natural biodistribution pattern of AdV vectors and retargeting them to specific cell types. Complexing the vector with a bi-specific antibody for recognizing the fiber knob or a specific cell surface molecule will serve the dual purpose of ablating knob-CAR interaction and targeting the modified vector to an alternative receptor (Curiel, 1999; Mizuguchi & Hayakawa, 2004). Alternatively, the vector can be treated with a CAR ectodomain-single chain antibody targeted against a tissue-specific antigen. The CAR ectodomain will bind to the fiber knob preventing interaction between CAR on cell surface. The single chain antibody will retarget the vector to the specific cell type (Li et al., 2009). *In vivo*, the fiber knob domain also interacts with coagulation factor IX and factor C4BP, targeting AdV vectors to HSPGs and LRP on hepatocytes and Kupffer cells. Consequently, fiber knob modifications might also ablate these interactions, thereby reducing liver transduction and subsequent hepatotoxicity (Shayakhmetov et al., 2005).

8.2.2 Serotype chimerism

Chimeric AdV vectors are most commonly constructed by replacing the knob domain or whole fiber with one from an alternative AdV serotype. Remarkable similarities among fibers of different Ad serotypes allows for the 'fiber chimerism'. Since the subgroup B viruses use cell surface molecules other then CAR as their primary attachment receptor, it is credible to replace HAdV5 fiber knob with one of a subgroup B virus, or vice versa, and expect a shift in vector biodistribution. Expectedly, fibers from subgroup B AdV have been successfully used for constructing HAdV5-based fiber chimeric vectors with an altered biodistribution pattern compared to HAdV5 alone. For example, HAdV5 carrying a fiber knob from HAdV16p, a subgroup B virus, showed greater transduction of cardiovascular and synovial tissues (Havenga et al., 2001). Similarly, HAdV5 with fiber from HAdV35, another subgroup B virus, showed enhanced transduction of hematopoeitic cells and human pancreatic cancer cells *in vitro* (Shayakhmetov et al., 2000; Toyoda et al., 2008).

Alternatively, the replacement of only the knob domain instead of the whole fiber has also been evaluated. A HAdV5 vector with fiber knob domain from HAdV3 proved to be remarkably efficient for gene transfer in animal models of breast cancer, ovarian cancer, gastric cancer and renal cancer and also showed good oncolytic ability (Ranki & Hemminki, 2010). A recent study explained the possibility of exploiting non-human AdV vectors for constructing fiber chimerism. Replacing the HAdV5 shaft and knob domains of HAdV5 with those from a bovine AdV ablated the vector interaction with blood factors IX and X and the subsequent HSPG-mediated hepatocyte transduction. As a result, serum levels of many of the pro-inflammatory cytokines and chemokines at early time points after the intravascular inoculation were significantly reduced (Rogee et al., 2010).

The major capsid protein hexon of HAdV5 interacts with factor X and mediates hepatocyte transduction. Being the most abundant capsid protein, it is expected to contribute significantly to non-CAR-dependent tropism. However, not all AdV serotypes bind factor X, and therefore, have variable hepatocyte transduction profiles *in vivo*. Chimeric HAdV5 27 vectors whose HVRs were replaced with HVRs of HAdV26 or HAdV27, which do not bind factor X, ablated factor X binding and showed a reduced hepatocyte transduction at a comparatively high dose (10^{11} vp/mouse). However, a greater degree of macrophage transduction was observed, giving a possible reason for the concomitant higher degree of proinflammatory cytokine and chemokine response following intravascular administration of the hexon chimeric HAdV5 vector. Interestingly, inserting HAdV35 fiber in place of native fiber of this chimeric HAdV5 vector dramatically increased lung transduction by more than 16000 times compared to the vector with HAdV5 fiber (Alba et al., 2010). Such a vector may prove very useful for lung gene therapy applications. Furthermore, this study also highlighted an interesting possibility of combining hexon and fiber chimerism to construct novel vectors with decreased immunogenicity and suitability for a particular application. Recently, another study reported a chimeric HAdV5 vector with the hexon from HAdV3. This chimeric vector showed ablation for factor X binding, which translated into reduced liver transduction and enhanced anti-tumor activity, owing to greater bioavailability of the vector for tumor transduction (Short et al., 2010).

8.3 Covalent modification of Ad capsid

Covalent modification of AdV capsids is an attractive alternative to genetic modification for preventing induction of immune responses and the acute toxicity following systemic administration. Addition of chemical groups to AdV capsids masks the natural immunodominant epitopes and the molecular patterns required for interaction with Ad-neutralizing antibodies along with the pattern recognition receptors, thereby preventing antibody-mediated vector clearance and induction of innate immune responses. Several standardized methods for covalent modifications are available for the generation of high titer stocks of modified vectors. Furthermore, simultaneous modification of a large number of capsid amino acids is feasible with covalent modification which may not be possible through genetic modification. Multiple studies have reported the so-called 'stealth AdV vectors' modified with synthetic polymers, for example, polyethylene glycol (PEG) (Kreppel & Kochanek, 2008). In accordance with the above principles, covalently modified AdV vectors have been shown to prevent or attenuate the induction of innate and adaptive immune responses, as well as, to evade pre-existing AdV-neutralizing antibodies (Croyle et al., 2002; Croyle et al., 2005). In addition, the use of bifunctional PEG allows for tissue-specific targeting of AdV vectors by coupling one functional group to capsid proteins and another functional group to a ligand of a specific tissue receptor (Park et al., 2008).

8.4 Alternative Ad vectors

Since AdV-neutralizing antibodies are serotype-specific and the prevalence of some of the HAdV serotypes in the human population is rare, HAdV vectors derived from the rare HAdV serotypes can potentially circumvent the pre-existing vector immunity, and, therefore, be more effective than vectors derived from common HAdV serotypes such as HAdV5. Vector systems based on HAdV serotypes in all subgroups have been reported (Appledorn et al., 2008a).

8.5 Non-human Ad vectors

Vectors based on nonhuman AdV serotypes are very promising candidates to serve as gene delivery vehicles and are frequently derived from AdV infecting bovine, porcine, ovine and chimpanzee. The nonhuman AdV do not cause disease in humans and are not neutralized by the HAdV-specific neutralizing antibodies (Moffatt et al., 2000; Sharma et al., 2010; Singh et al., 2008). In addition, nonhuman AdV vectors have been shown to transduce human cells in culture and express transgene (Bangari et al., 2005; Bangari & Mittal, 2004; Farina et al., 2001; Klonjkowski et al., 1997; Mittal et al., 1995; Rasmussen et al., 1999). Advances in the past decade signify the potential of nonhuman AdV vectors as effective gene delivery vehicles without any significant toxicity and interference from pre-existing immunity. A bovine AdV serotype 3 (BAdV3) vector has been shown to possess low liver tropism, prolonged vector genome persistence and, thereby, higher transgene expression than a HAdV5 vector in the heart, lungs, and kidneys of mice following intravenous inoculation (Sharma et al., 2009a). The humoral as well as cellular immune responses generated against various non-human AdV are expected to be non cross-neutralizing (Sharma et al., 2010). Sequential use of alternative non-human AdV-derived vectors might prolong the transgene expression and, therefore, the desired therapeutic effect.

9. Conclusion

The future of AdV vectors is very promising. Much research has been conducted on the various constructs of the vectors with the hope of finding therapies that will enable specific targeting of diseased cells or tumors thereby causing minimum harm to surrounding cells or organs. With continued research on the mechanisms of AdV gene expression and tissue tropism, the medical and scientific communities have great reason to anticipate breakthroughs in cancer and vaccine therapies.

10. Acknowledgement

11. References

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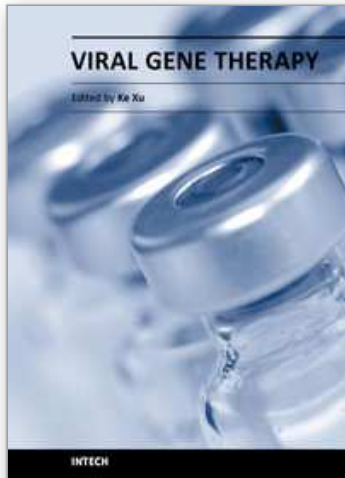
Viral Gene Therapy

Edited by Dr. Ke Xu

The development of technologies that allow targeting of specific cells has progressed substantially in recent years for several types of vectors, particularly viral vectors, which have been used in 70% of gene therapy clinical trials. Particular viruses have been selected as gene delivery vehicles because of their capacities to carry foreign genes and their ability to efficiently deliver these genes associated with efficient gene expression. This book is designed to present the most recent advances in viral gene therapy

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