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Helper Dependent Adenovirus Vectors: Progress and Future Prospects

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Abstract: Sixteen years after Graham and coworkers described the most used system for generating helper-dependent adenovirus (HDAd) vectors, production systems have evolved considerably, and most resulting preparations have titres of 1×10^{13} IU/ml (infection units/ml) and very low helper contamination levels (<0.1%). These advances in production, as well as the attractive characteristics of these vectors (large insert capacity and low cell immune response compared with first-generation Ad vectors) make them very interesting for many research purposes as they have become more accessible to the scientific community. In this review we summarise the latest strategies for producing HDAd vectors, describe the main areas of interest for which HDAd vectors are being used, and comment on the future prospects for HDAd vectors in gene therapy.

Keywords: Adenovirus, gene therapy, helper vector, helper-dependent vector, immune response.

INTRODUCTION

HDAd vectors, alternatively referred to as gutless or high-capacity adenoviruses, are one of the most promising vectors for gene therapy because of their lack of viral genes and their large cloning capacity (up to 36 kb), which makes them more useful than other vectors such as the first-generation adenoviruses (up to 8 kb) [1] or adeno-associated viruses (5 kb cloning capacity) [2], especially when the insertion of large genes, combinations of genes or regulatory elements is required. Moreover, in contrast to first-generation adenoviruses (FGAds), the lack of any viral coding region minimises the cellular immune response, promoting safer and more prolonged transgene expression. Finally, because of its non-integrating nature [3], HDAd vectors have a negligible risk of insertional mutagenesis while, like other adenoviral vectors, they still mediate efficient transduction to a wide variety of cell types and organs (e.g. liver, brain, lungs and muscle) (Table **1**), and have great potential in biomedical applications (e.g. vaccine development). Therefore, HDAd vectors have a great therapeutic potential, which makes them very attractive for use in animal models and as well as in clinical gene therapy trials.

PRODUCTION

Since HDAd vectors lack any viral coding region, proteins needed for its genome replication, capsid formation and packaging are provided *in trans* by coinfection of the HDAd vector with a helper adenovirus (helper-Ad). However, as both helper-Ad and HDAd vectors have the same capsid, as well as the same packaging efficiency, their production levels are similar, resulting in a large amount of helper vector contamination in the viral preparation. In order to lower the helper-Ad contamination, different production systems have been developed, all focusing on reducing the packaging of the helper genome. Most of the HDAd vectors preparations are currently produced using variants of the Cre/*loxP* recombination system [4]. However, other strategies have recently been described – such as mutating the packaging signal of the helper-Ad to impair its packaging capacity [5-7], or the insertion of an *attB*-FC31 sequence at the 5′ end of the packaging signal – which specifically delay the packaging process and the viral replicative cycle of the helper-Ad [8, 9].

The classical Cre/*loxP* system is based on the specific excision of the packaging signal of the helper-Ad, flanked by *loxP* sequences, in cells expressing the Cre recombinase. In addition, this strategy may be combined with a physical separation on a final CsCl gradient ultracentrifugation step based on the different size of the virus genome, reducing the levels of helper-Ad contamination to a range of 1.0–0.1% [4]. Despite the success of this strategy, it also has some drawbacks, such as the toxicity of Cre recombinase [10]. These problems have been addressed by the development of an improved system consisting of a suspension-adapted producer cell line expressing high levels of Cre recombinase, the use of a reverse packaging signal in the Ad-helper vector, and a refined purification protocol [5]. With this system, large-scale production of $>1 \times 10^{13}$ highly infectious vector particles was easily achieved in spinner flasks with very low helper-Ad contamination levels (0.01–0.02%) [5]. However, this system still requires considerable time and effort to produce HDAd vectors. In this regard, a method consisting of the use of chamber cell factories with adherent cells was recently developed, in which comparable quantities of HDAd vector preparations were obtained with levels of contamination equivalent to those of the spinner flask approach,

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Table 1. Transduction of Helper-Dependent Adenovirus Vectors in Animal Models fo Human Diseases

Organ	Disease	Species	Outcome	Reference
CNS	Mucopolysaccharidosis type IIIA	Mouse	Expression of SGSH in discrete areas of the brain for at least 8.5 months. Injection of 2×10^9 pp after injection of an HD-CAV-2. SGSH levels were insufficient to correct the neuropathology	[49]
	Multiple sclerosis	Non-human primates	Long-term (3 months) infection of neuroepithelial cells after injection of 5×10^8 transducing units of HDAd by lumbar puncture into the cerebrospinal fluid. No signs of systemic or local toxicity were shown in monkeys bearing a pre-existing anti-adenoviral immunity	[68]
		Mouse	One-year expression of GFP marker protein in neuroependymal and neuronal cells without chronic toxicity after injection of 1×10^{12} vp/kg of HD-Ad by lumbar puncture	[69]
	Huntington disease	Mouse	Attenuation of aggregate formation 4 weeks after stereotactic injection of 1×10^7 IU of HDAd expressing an shRNA targeted to huntingtin. Limited effect due to vector distribution limited to a few millimetres from the needle track	[66]
	Sandhoff neuropathy	Mouse	Reversion of gangliosidosis and amelioration of peripheral sensory dysfunction 8 weeks after a single injection of 1×10^8 vp of dorsal root ganglia-targeted HDAd encoding for Hexβ protein	[121]
	Glioblastoma	Rat	Long-term survival (1 year) after intratumoural injection of 5×10^{19} vp of HDAd encoding for TK and Flt3L without systemic toxicity in rats bearing large intracranial glioblastoma	[122]
Muscle	Duchenne muscular dystrophy	Mouse	Expression of dystrophin at the sarcolemma of > 20% of total fibres in the injected diaphragm bundle for at least 30 days after injection of 5×10^{10} vp of HDAd. Improved resistance to the abnormal force deficits induced by high-stress muscle contractions, despite the presence of mildly increased inflammation	[81]
		Mouse	One year gene expression of full-length dystrophin after a skeletal muscle injection of 10^9 vp of a 5/3 capsid-modified HDAd	[83]
Liver	Haemophilia A	Mouse	Stable expression of factor VIII for more than 1 year in neonatal mice after injection of 5×10^{12} vp of HDAd and subsequent readministration allowed by the operational tolerance to factor VIII	[101]
		Dog	Significant improvement of the whole blood clotting time, plasma FVIII concentration, FVIII activity, and activated partial thromboplastin for 2 years after injection of 3×10^{12} vp/kg of HDAd	[123]
		Human	No evidence of FVIII expression. Significant thrombocytopenia developed, forcing the trial to come to a halt	[124]
	Haemophilia B	Mouse	Therapeutic hFIX levels for 45 weeks after intravenous administration of HDAd (1×10^{12} VP/kg)	[125]
		Dog	Decline of whole blood clotting time.Therapeutic levels of factor IX without spontaneous bleeds for at least 213 days after intravenous administration of HDAd (3×10^{12} vp/kg)	[126]
		Rhesus macaques	Therapeutic hFIX levels for 3 years after an intravenous administration of 1×10^{12} and 1×10^{11} vp/kg HDAd using a balloon occlusion catheter	[127]
	Ornithine transcarbamylase deficiency	Mouse	Metabolic correction of adult OTC-deficient mice, normalisation of orotic aciduria and hepatic enzyme activity and absence of chronic hepatotoxicity for >6 months after an intravenous administration of 1×10^{13} vp/kg of HDAd	[128]
	Arginase deficiency	Mouse	Temporary correction of arginase activity and ammonia and amino acids levels and increased survival (from 14 to 27 days) of newborn mice after neonatal injection of 5×10^9 vp of HDAd	[129]
	Propionic acidaemia	Mouse	Moderately increased survival of mice (from 32 hours after birth until 50–70 hours after birth) after injection of 2.5×10^9 to 5×10^{10} vp of HDAd encoding the α and β subunits of propionyl-CoA carboxylase per neonate	[130]

(Table 1) contd….

Organ	Disease	Species	Outcome	Reference
	Glycogen storage disease Ia	Mouse	Prolonged survival (average of 7 months) and long-term correction of G6Pase levels, body weight, glycaemia, cholesterolaemia and glycogen accumulation in the liver after the intravenous administration of 2 or 5×10^{12} vp/kg of HDAd encoding G6Pase to 2-week-old G6Pase-KO mice	[131]
		Dog	Prolonged survival (36 months) and reversion of hypoglycaemia despite the persistence of long-term complications after neonatal adminstration of 2×10^{12} vp HDAd5 and 22 months later 1×10^{12} vp HDAd2 encoding for G6Pase	[90]
	Crigler-Najjar syndrome	Rat	Correction of hyperbilirubinaemia in the Gunn rat for 60 weeks using clinically relevant low HDAd doses of 5×10^{11} vp/kg or 5×10^{10} vp/kg when the vector is administered by hydrodynamic injection	[89]
	Pompe disease	Mouse	Long-term correction and long-term hepatic secretion of hGAA after intravenous delivery of HDAd-hGAA, resulting in a complete reversal of cardiac glycogen storage and near-complete skeletal glycogen correction for at least 180 days after injection of 2.5×10^{11} vp of HDAd	[132]
	ApoE deficiency	Mouse	Stable correction of hypercholesterolaemia with negligible toxicity for at least 2.5 years after a sigle intravenous injection of 5×10^{12} vp/kg of an HDAd carrying the ApoE gene	[133]
	Familial hypercholeresterolaemia	Mouse	LDL cholesterol lowering and induction of regression of pre-existing atherosclerosis for at least 28 weeks after intravenous injection of 5×10^{12} vp/kg HDAd containing the LDLR gene	[134]
	ApoA1 deficiency hypoalphalipoprotein-aemia	Mouse	Reduction in the development of atherosclerosis with the absence of significant toxicity for at least 2 years after a single intravenous injection of 4.5×10^{12} vp/kg of an HDAd vector containing the entire human APOA-I gene	[135]
	Gulonolactone oxidase deficiency	Mouse	Elevation of ascorbic acid levels in serum, urine and tissue for at least 23 days after intravenous administration of 2×10^{11} vp of HDAd encoding for gulonolactone oxidase gene	[136]
	Chronic B hepatitis	Woodchuck	Sustained viraemia and WHV DNA reduction, loss of the e antigen and the surface antigen and improved liver histology in woodchucks with low viraemia after intrahepatic injection of 2×10^{10} IU of an HDAd encoding IL12	[137]
	Type 1 diabetes	Mouse	Islet neogenesis and reversion of hyperglycaemia in diabetic mice for at least 120 days after intravenous administration of 2 HDAd encoding Neuogenin3 (5×10^{11} vp) and Betacellulin (1×10^{11} vp)	[138]
	Type 2 diabetes	Mouse	Glucose homeostasis improvement without increasing insulin levels for at least 15 weeks after administration of 1×10^{11} vp of a HDAd vector encoding exendin 4	[139]
Vascular system	Hyperlipidaemia	Rabbit	Improvement of endothelium-dependent vasodilation and atheroprotective effects in HDAd-infused arteries for more than 2 weeks by carotidal injection of an HDAd that leads to an over-expression of endothelial nitric oxide synthase	[140]
Lungs	Cystic fibrosis	Mouse	Transgene expression of a reporter gene human alpha-fetoprotein up to 15 weeks with absence of pulmonary inflammation after intranasal administration of the vector	[103]
		Rabbits	Extensive expression of a marker transgene from the trachea to terminal bronchioles for at least 5 days after administration of 5×10^{11} vp encoding the *lac*Z reporter gene mixed with L-alpha-lysophosphatidylcholine using an intratracheal aerosoliser	[113]
		Non-human primates	High expression of the transgene from the trachea to terminal bronchioles	[114]

thus reducing technical complexity, effort and medium requirements [11].

Although major advances in cell system development have been achieved, it is important to mention that even these low levels of helper contamination might be hazardous in clinical trials, depending on specific parameters such as vector dose or route of administration. Also, it is important to note that all systems rely on the adoption of a CsCl gradient ultracentrifugation to lower the inevitable contamination with helper adenovirus, limiting the scalability of the manufacturing process and restricting its possible application in gene therapy protocols, where high-quality clinical-grade vectors need to be produced in large amounts under scalable good manufacturing process conditions [12].

In order to scale up the HDAd vector productions, extensive optimisation work is being performed nowadays. As volumetric productivity is limited by surface area, standard methods requiring adherent cell cultures are not suitable for large-scale productions, and therefore most of these methods are based on suspension cell cultures [13]. Briefly, scalable methods consist of a first rescue step via transfection followed by several amplification steps via coinfection of both helper-Ad and HDAd vectors on suspension-cell-culture bioreactors and downstream processing (Fig. **1**). The rescue step has been improved by adenofection, a transfection/infection method consisting of the use of the HDAd linearised plasmid linked to the helper-Ad with the aid of polyethylenimine (PEI) that outperforms prior protocols by producing higher HDAd vector yields [13]. The amplification steps have also been widely studied, and the identification of the critical infection parameters to improve HDAd vectors yield and limit helper-Ad contamination, such as the optimal multiplicity of infection (MOI) of both HDAd and helper-Ad, the harvesting time, or the cell culture characteristics (cell density, media formulation, and vector production in fed-batch or in perfusion conditions) have been published. More details are available in specialised reviews [14].

The last phase of vector manufacturing, known as downstream processing, includes the steps for recovering and purifying HDAd vectors, from the cell culture harvest to the final product formulation. Unlike the non-scalable systems, where the final preparation is obtained after a CsCl ultracentrifugation, the scalable downstream process consists of methods such as membrane filtration or HPLC chromatography [15]. The use of chromatographic steps allows a scalable clarification, capture and purification of the HDAd vectors, but unlike ultracentrifugation methods these systems cannot separate helper-Ad from HDAd vector particles. In this regard, an ultracentrifugation step has been included in a recent four-step downstream processing system consisting of: (1) release of viral vectors by concentration, cell lysis, DNA clearance and microfiltration; (2) capture of viral vectors by anion exchange chromatography; (3) removal of helper-Ad by ultracentrifugation; and (4) polishing and buffer exchange by size-exclusion chromatography, allowing an 80% recovery and a 10× diminution of helper-Ad contamination from 2 to 0.2% [16].

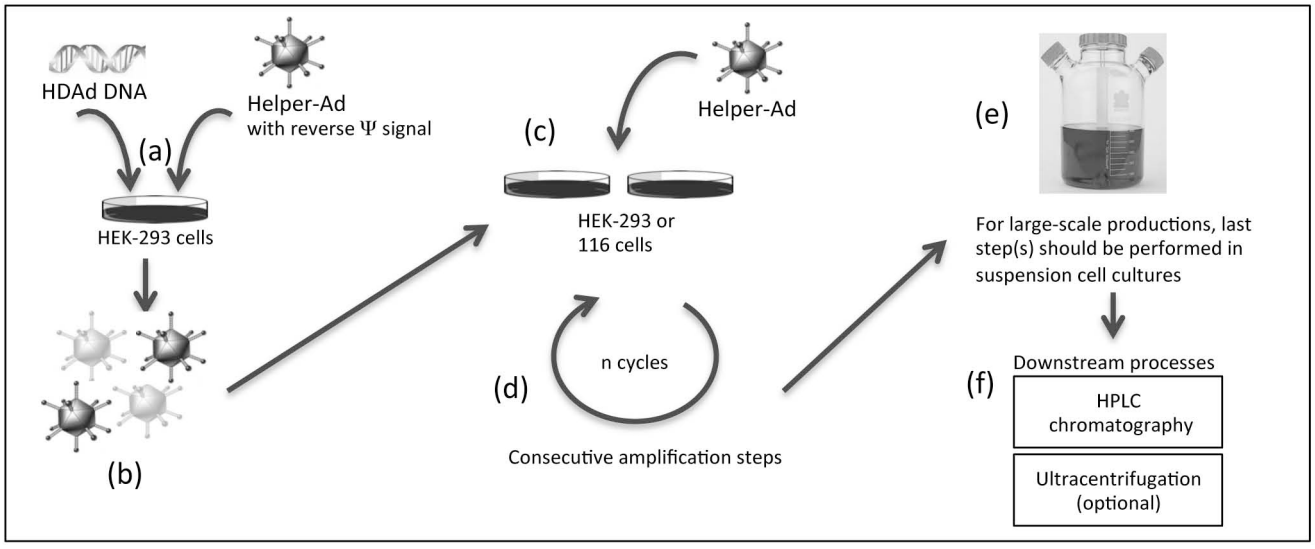

Fig. (1). Production and purification of HDAd vectors. **(a)** Permissive HEK-293 cells are co-transduced with HDAd linearised genomes and Helper-Ad vectors carrying a reverse Ψ signal. To restrict and limit the packaging of helper-Ad genomes, helper Ad vectors must have their Ψ sequences flanked by loxP or *attB* signals. **(b)** Initial HDAd viral stock. Both, HDAd and helper-Ad virions are present. **(c)** Amplification step in HEK-293 or 116 Cre-expressing cells. If required, supplementary helper-Ad vectors can be added in this step. **(d)** Consecutive amplification steps to increase HDAd vector yields. In the classical Cre/*loxP* system, the *loxP*-flanked Ψ signal of the helper-Ad is excised when grown in Cre-expressing cells. In the attB system, helper-Ad vectors have a delayed packaging compared to HDAd vectors. In both systems, additional helper-Ad vectors can be used if required. **(e)** For large scale productions, the final steps must be performed in suspension cultures of HEK-293 or 116 cells. **(f)** Downstream processes. For small-scale productions, purification by ultracentrifugation in CsCl or iodixanol gradients is recommended. In large-scale productions HDAd vectors are purified by HPLC-chromatography. An additional ultracentrifugation step to remove empty viral capsids is recommended.

IMMUNE RESPONSE AGAINST ADENOVIRUS PARTICLES

Despite the fact that first-generation adenoviral vectors are among the most used in gene therapy, they have a considerable toxicity profile [17]. The immune response against FG adenoviruses involve both non-specific innate and adaptive mechanisms and consist of three overlapping phases [18]. The first response, known as acute toxicity, is induced by the adenovirus capsid proteins and therefore is similar for both HDAd and FGAd vectors. It appears within minutes of the administration of the vector and it does not require viral gene expression since psoralen-inactivated UV Ad genomes do not show an attenuated acute toxicity [19, 20]. Finally, this phase is characterised by an acute production of pro-inflammatory cytokines and chemokines as well as a widespread activation of macrophages, neutrophils and Kupffer cells in the liver [21].

The intermediate response, that occurs from several hours to one day after vector administration is characterised by side effects such as thrombocytopenia, periportal polymorphonuclear leukocyte infiltration and elevated liver enzymes (alanine and aspartate aminotransferases), which at certain doses can lead to dramatic effects such as tissue injury, multi-organ failure or even death [22]. Moreover, detection of viral DNA by molecular sensors including the Toll-Like Receptor (TLR) family increases the expression of multiple proinflammatory cytokines (including IL-5, IL-6, IL-8, IL-12, TNFalpha, RANTES, IP-10, MIP-1b and MIP-2 among others), and also activates monocytes and resident macrophages (see reference [20] for an extensive review).

The third phase (delayed chronic toxicity) involves the adaptive immune response, and it occurs from several days to weeks after vector administration. This response is induced by the uptake of adenovirus by antigen-presenting cells (APCs), which process the adenoviral proteins or the adenoviral-encoded transgenes into oligopeptides and present them to the major histocompatibility complex (MHC) class-I molecules. Its successive binding to $CD8^+$ T cells leads to the generation of Ad-specific or transgene-product-specific cytotoxic T lymphocytes (CTLs) [23].

HDAd vectors are devoid of viral genes, and therefore they elicit an attenuated adaptive immune response compared to FGAd vectors. In contrast, since host innate immune responses are induced by both HDAd and FGAd vectors in a similar manner [24], a major limitation of HDAd vectors is their potential to activate a potent innate immune response. Thus, HDAd vectors also interact with bloodborne factors including C3 and C4b proteins, as well as clotting factors IX and X [25-27], in a non-linear toxic dose response [20, 28], indicating a key role for the innate immune sensing cells in the overall toxicity [20]. Additionally, primary macrophages can sense HDAd vectors via the Toll-like Receptor 9 (TLR9), which is essential for early detection of adenoviral infection [20, 29, 30]. TLR9 is activated by viral dsDNA genomes [29] in a process mediated by MyD88 [30], and it increases IL-6, TNFα and IFNβ gene expression, which are key mediators of the acute response [24]. Nevertheless, the knowledge of all the signalling pathways and the interactions involved in Ad infection is still incomplete.

On the other hand, while encoded transgenes may or may not be immunogenic, residual expression of viral genes from first-generation adenoviral vectors is responsible for vector clearance within a few weeks of administration [31, 32]. For that reason, different strategies to avoid innate and adaptive immune responses were rapidly developed. Strategies targeting the immune system – such as the use of immunosuppressive agents (cyclosporine A, cyclophosphamide, dexamethasone, FK506, Interleukin-12 and deoxypergualin) [19, 21, 33-35] blockade of co-stimulatory interactions between APCs, T cells and B cells [36, 37], antibodies to deplete CTLs [38], or macrophage depletion [39-42] – resulted in the impairment of the immune system, which made them unsuitable for use in future clinical trials. Similarly, other strategies such as oral tolerisation [43] and intrathymic administration of adenovirus [44] also seemed promising, but their application have not progressed to clinical trials.

Since most adenoviral vectors are derived from the human adenovirus type 5 (Ad5), and the great majority of the population have notable levels of neutralising antibodies against them, vector uptake by target cells is prevented [45, 46]. To overcome vector neutralisation, use of non-cross reacting serotypes [47, 48], and vectors of non-human origin – such as ovine, canine, simian, chimpanzee and porcine adenoviruses – have been developed and show interesting results in mice [49]. Thus, in contrast to human Ad5, CAV-2 vectors poorly transduced human monocyte-derived dendritic cells and therefore induce minimal upregulation of major histocompatibility complex class I/II and costimulatory molecules (CD40, CD80, and CD86) [50]. An alternative strategy based on a HDAd vector encoding the hyperactive transposase Sleeping Beauty achieved somatic integration of the therapeutic gene and stabilised transgene expression for up to three years in a canine model, thus circumventing the pre-existing immunity associated with vector readministration [51].

Similarly, modification of the physical and chemical properties of biological molecules by covalent attachment of polyethylene glycol, also known as PEGylation, has been widely tested to improve the stability, solubility, pharmacokinetic and immunological/toxicological profiles without compromising their bioactivity [52]. Thus, PEGylated helper-dependent adenoviruses show a significantly reduced toxicity in mice [53], as well as in baboons, an animal model, which has an immune system and pharmacokinetics phylogenetically similar to those in humans [54]. In this study, baboons were intravenously injected with HDAd or PEG-HDAd vectors expressing beta-galactosidase at 5×10^{11} or 3×10^{12} vp/kg. A threefold reduction in IL-6 and a 50% reduction in IL-12 and serum transaminases were observed in animals injected with PEG-HDAd vectors compared with the animals injected with non-PEGylated HDAd vectors. However, the use of PEGylated HDAd vectors in baboons did not seem as promising as it was in rodents, since hepatic transduction and viral half-life were reduced in plasma compared with those in rodents. These results suggest the presence of notable species-specific differences in the biodistribution and response to PEG-HDAd vectors, probably related to differences in binding properties to coagulation factors, receptor density, and tissue architecture of the organs [54].

HD VECTORS AS GENETIC VACCINES

The use of helper dependent adenoviruses has minimised cellular toxicity, allowing a sustained high-level expression of the encoded transgenes in several animal models. A recent study has demonstrated that compared to FGAd, the administration of HDAd vector vaccines results in a lower anti-Ad T-cell response and higher levels of transgenic protein production in dendritic cells, and therefore a stronger cytotoxic T lymphocyte response against the transgene [55, 56]. Thus, HDAd vectors have been successful in boosting anti-HIV immune responses in macaques [56]. However, since most humans have antibodies against Ad5, an adenovirus vaccine using this serotipe may not be efficient. To overcome the preexisting immune response, Weaver and colleagues showed that serotype switching using HDAd serotypes 1, 2, and 6, clearly induce significant mucosal vaccine effects against HIV and therefore, that HDAd vectors are a robust platform for vaccination. Similarly, the ability of mucosally applied HDAd vaccines to induce systemic and local immunity against transgenes has also been examined by Fu and coworkers after intranasal administration to mice of HDAd vectors encoding EGFP. As for systemic administration, a strong anti-immunogen-specific serum and mucosal antibody responses as well as lymphocyte proliferation responses were observed [57].

More recently, the efficacy of HDAd vectors to induce multispecific CTL responses has been demonstrated against the surface antigen of the hepatitis B virus (HBsAg). In contrast, FGAd vectors showed limited multispecificity because de novo expression of viral genes from FGAd vectors mainly induced CTLs against viral epitopes, while primed CTLs against one immunodominant epitope of HBsAg [58]. Also, an HDAd vector genetic vaccine encoding the merozoite surface protein of *Plasmodium falciparum*, the causal agent of malaria tropica, has shown a good therapeutic potential, and it seems to act at two different stages in the parasite's infection cycle: in the liver and in the blood. As expected, in both cases HDAd vectors were more promising than FGAd vectors [59].

GENE THERAPY FOR THE CENTRAL NERVOUS SYSTEM

Adenoviral vectors hold great potential for brain-directed gene therapy because of their high efficiency to infect post-mitotic cells at [60] as well as to mediate long-term transgene expression. However, despite the apparent immune privilege of the CNS, in the case of pre-existing immunity against the vector, administration of FGAd into the brain will lead to a decrease in expression of the transgene 2 months after transduction, correlating with the disappearance of adenoviral DNA and chronic inflammation [61, 62]. Interestingly, this immune response is significantly lower after HDAd vector administration, allowing higher levels of transgene expression than FGAd vectors and suggesting an evident therapeutic potential of HDAd vectors for gene therapy for brain disorders. In this regard, intratumoural administration of 5×10^9 vp of HDAd vectors encoding the conditionally cytotoxic herpes simplex type-1 thymidine kinase and the immunostimulatory cytokine FMS-like tyrosine kinase ligand 3 (Flt3L/TK) led to long-term survival (up to 1 year) of rats bearing intracranial RG2 orthotopic glioblastoma without systemic toxicity [63]. Further experiments on distribution and immune response against this vector and the therapeutic transgenes in naïve rats indicated that 1×10^9 vp of this HDAd vector is the maximum tolerated dose that can be safely administered in the naïve brain parenchyma without adverse effects [64].

To avoid pre-existing immunity and therefore vector neutralisation, non-human adenoviral vectors have been used to achieve high transduction efficiency. Thus, stereotactic injection of canine HDAd vector expressing human N-sulphoglucosamine sulphohydrolase (SGSH) administered in mucopolysaccharidosis type IIIA (MPS-IIIA) mice enabled transgene expression for at least 8.5 months post-treatment in discrete areas of the brain [49].

Furthermore, the limited distribution of the HDAd vector after sterotactic injection [65, 66] represents a major concern for the correction of diseases with global involvement, such as Alzheimer or Huntington disease. Interestingly, adenovirus vectors have also been administered into the CNS by lumbar puncture, which delivers the vector into the cerebrospinal fluid and allows the transduction of neuroepithelial cells. This method holds great promise to treat several neurological diseases, and it can be used to secrete therapeutic proteins into the cerebrospinal fluid and reach non-transduced cells, as shown by Butti and collaborators by lumbar puncture administration of HDAd vectors expressing anti-inflammatory cytokines in animal models of multiple sclerosis [67]. Of note, injection of HDAd vectors by lumbar puncture allows at least three months transduction in non-human primates [68] or up to 1 year in mice, with no chronic toxicity [69].

GENE THERAPY TO THE MUSCLE

Gene transfer of the skeletal muscle with HDAd vectors is very promising for the treatment of inherited skeletal muscle disorders, as well as for systemic gene therapy approaches where muscle is used as a protein production platform. Duchenne Muscular Dystrophy (DMD), a disease caused by genetic mutations in the dystrophin gene, has been considered a prime candidate for gene therapy due to the lack of effective treatments. The length of dystrophin cDNA (14 kb) precludes its cloning into most viral vectors. However, the high capacity of HDAd vectors (up to 37 kb) has opened up the possibility of treating DMD animal models [70, 71]. Thus, *mdx* mice injected with HDAd vectors showed expression of dystrophin in neonate skeletal muscles for up to 1 year, which resulted in the mecanical stabilisation of the sarcolemma by the restoration of the dystrophin-glycoprotein complex, as well as a reduction of muscle degeneration and amelioration of the physiological and pathological indices of muscle disease [70]. Functional correction of the muscular contractility was also reported despite the loss of vector DNA copy number over time as well as the induction of a significant humoral response against the murine dystrophin protein [70].

Notably, while immature or regenerating muscle can be effectively transduced by Ad vectors, adult skeletal muscle is only poorly transduced [72]. Some authors suggest that this is caused by the small mass of immature muscle and because

the basal lamina and other connective tissues are not yet formed [73], while others propose that the low Ad transduction in adult muscle correlates with a down-regulation of CAR during muscle development [74-76]. In any case, high levels of dystrophin expression seem to be required to reverse, at least partially, DMD pathology. Interestingly, Larochelle and collaborators injected AAV2 encoding CAR in adult *mdx* mice skeletal fibers and demonstrated that a moderate increase of CAR expression resulted in a significative increase of expression from Ad in skeletal muscle fibers [77], which could improve Ad-mediated DMD therapy and have a strong potential for non-regenerative skeletal muscle diseases.

Taking advantage of the immaturity of the foetal immune system, together with the robust CAR expression in the foetal skeletal muscle, Bilbao and colleagues analysed the potential of HDAd vectors for *in utero* gene therapy for DMD. In this study, skeletal muscle fibres transduced before birth allowed the expression of the reporter protein for at least 5 months. However, despite the immaturity of the foetal immune system, antibodies against both the vector capsid and the transgene were developed [78].

Another strategy to treat DMD is the over-expression of utrophin, a functional homologue of dystrophin that is widely expressed during early development and is restricted to neuromuscular junctions in mature muscle. Interestingly, when HDAd vectors are administered to neonate *mdx* mice, the utrophin gene is expressed for up to 1 year, leading to a notable physiological improvement in young and adult animals. However, despite the lack of antibodies and low cellular immunity, expression of utrophin decreased over time, probably because of the innate immune response as well as to the relatively short half life of the protein [79]. Importantly, later studies demonstrated that administration of immunomodulatory molecules blocking the interaction between naïve T cells and APC cells allowed prolonged expression of the HDAd-encoded dystropin [80].

In DMD, the transduction of a critical number of fibres in multiple muscles is required, specially the diaphragm and other minor respiratory muscles, to avoid respiratory failure, which is the main cause of death in these patients [81]. Thus, laparotomy administration of HDAd encoding dystrophin in the diaphragm of *mdx* mice resulted in functional amelioration for at least 30 days [81]. Similarly, intraperitoneal injection of the vector in double knock-out mice for dystrophin and utrophin showed efficient transduction of the diaphragm, dystrophin expression for at least 9 weeks, and rescue from ventilatory impairment [82]. Of note, recent advances in vector development have shown that, in adult mice, intramuscular administration of chimeric HDAd5/3 vectors transduced the skeletal muscle significantly better than HDAd serotype 5, and long-term gene expression was observed for at least 1 year, suggesting the feasibility of these vectors for muscle-directed gene therapy [83].

GENE THERAPY TO THE LIVER

The liver is a very attractive target for gene therapy because it is affected in numerous genetic diseases and also plays an important role in many metabolic pathways. Thus, several diseases such as atherosclerosis, diabetes, Crigler–Najjar syndrome type I, glycogen storage disease type Ia, or hemophilia B, among others, have also been targeted with HDAd vectors, demonstrating their therapeutic potential in the majority of cases. A paradigmatic example comes from the studies performed in the Crigler–Najjar syndrome type I, which is a disease caused by mutations in the uridine diphospho-glucuronosyltransferase 1A1 gene, encoding a protein involved in the elimination of bilirubin. This deficiency results in high levels of non-conjugated bilirubin in serum and its accumulation in several organs, causing brain damage – and even neurological impairment and death – in non-treated patients [84]. Liver transplantation is the only cure available, and even though it provides a complete metabolic correction [85] it has some important drawbacks, such as rejection of the transplant and long-term morbidity associated with chronic immunosuppression [86]. Interestingly, long-term correction of hyperbilirubinaemia after administration of high doses ($\geq 3 \times 10^{12}$ vp/kg) of HDAd vectors was described in Gunn rats, a model of Crigler–Najjar syndrome type I [87]. However, these doses are likely to elicit a severe immune response in humans, as demonstrated with the death of a human patient due to the administration of 6×10^{11} vp/kg [88]. Interestingly, in a recent study, Dimmock and collaborators achieved correction of hyperbilirubinaemia in the Gunn rat model using a more potent UGT1A1 cassette and doses of 5×10^{10} vp/kg after hydrodynamic injection [89].

Similarly, HDAd vectors encoding human α-1 anti-tripsin (hAAT) were intravenously injected in baboons and continuous hAAT expression was observed for more than 1-2 years. Interestingly no abnormalities in blood cell counts and liver enzymes were detected in any of the animals. In contrast, FGAd-treated baboons generated a cellular immune response directed against the transduced cells causing loss of hATT expression [48]. Also, intravenous administration of an HDAd vector encoding the glucose-6-phosphatase gene on postnatal day 3 in a dog model of glycogen storage disease type Ia resulted in the correction of the hypoglycaemia and prolonged survival. After 6-22 months, vector-treated dogs developed hypoglycaemia, anorexia and lethargy, suggesting that the HDAd-cG6Pase serotype 5 vector had lost efficacy. Interestingly, a HDAd-cG6Pase serotype 2 vector was administered to two dogs, and hypoglycaemia was reversed and prolonged survival in one GSD-Ia dog to 12 months of age and 36 months of age in another, though unfortunately, did not avoid the development of hepatic adenomas, which typically occur during adolescence in GSD-Ia and, on the other hand, are known not to be prevented by good metabolic control [90].

In addition, it has been hypothesised that liver fenestrations (size about 100 nm) may act as a structural barrier restricting the entrance of the adenovirus type 5 virion (80-120 nm) into the liver parenchyma [91, 92]. Several strategies have been applied to enlarge the fenestration size, including the use of the neuropeptide vasoactive intestinal peptide (VIP) [93], Na-decanoate [91], and N-acetylcysteine [92], or by increasing the intrahepatic pressure during hydrodynamic administration of the vector [94]. Interestingly, the use of VIP prior to HDAd vector administration not only increases liver transduction but also reduces splenic uptake of the HDAd and attenuates the HDAd vector-mediated innate im-

mune response and hepatotoxicity [93]. Of note, while the pharmacological approach will require further studies to determine its potential for human applications, hydrodynamic administration has already been discarded for this use, because the rapid injection of large volumes would not be indicated in humans. Nevertheless, a minimally invasive method based on two balloon occlusion catheters placed in the vena cava has been developed to mimic the high preassure achieved by hydrodynamic injection [95], which allowed long-term transduction in non-human primates (964 days) after administration of HDAd vectors encoding alpha-fetoprotein [96].

Other methods have also been used to confine the administered HDAd vectors into the liver and thus to reduce their diffusion to other organs, such as direct injection into the liver parenchyma [97], or surgical isolation of the liver followed by an intraportal injection of the virus [98]. In all cases, systemic vector dissemination was greatly reduced and high levels of transgene expression were achieved.

In addition to liver fenestrations, the presence of Kupffer cells makes predicting the efficiency of liver transduction difficult, since these cells have the capacity to sequester intravenously administered adenovirus, and play a very important role in the non-linear dose–response characteristic of liver transduction, where low virus doses lead to very low or undetectable levels of transgene expression, and high virus doses result in a very robust expression [99]. In this regard, the blockade of Kupffer scavenger receptor A (SR-A) and scavenger receptor of endothelial cells-I (SREC-1) prior to HDAd infection with antigen-binding specific fragments (Fabs) can be an interesting approach to increase hepatocyte transduction efficiency, allowing the use of lower doses of the vector and thus, lowering toxicity [100].

Finally, neonatal gene therapy of the liver has also been studied to treat congenital diseases during early postnatal development. Initiating gene therapy in the neonatal period has advantages such as early gene expression, before the development of irreversible pathology, and the low or undetectable immune responses against the vector and the transgene as it has been described after neonatal administration of HDAd vectors encoding the clotting factor VIII in haemophilia A mouse models [101].

Last, despite HDAd vectors are usually considered non-integrative vectors, they are able to integrate in the host genome though with a very low frequency. Thus, as reported by Stephen and co-workers, heterologous recombination occurs with a median frequency of 6.7×10^5 per transduced hepatocyte, while homologous recombination occurs more rarely with a median frequency of 3.8×10^7, which represent an important safety feature since compared to the spontaneous mutation frequency, the likelihood that Ad vector integration would result in germ-line transmission and insertional mutagenesis is relatively low [3].

GENE THERAPY TO THE LUNG

Most of the gene therapy strategies in lung have been addressed to Cystic fibrosis (CF), which is the most common autosomal recessive disorder in Caucasoids. The disease, characterised by chronic pulmonary infections, pancreatic enzyme insufficiency, and elevated electrolyte levels in sweat, is caused by mutations in the CFTR gene. As for other organs, administration of FGAd vectors into the airway of animal models induced innate, humoral and cellular imunes responses, which limited transgene expression for 2-3 weeks. In contrast, administration of HDAds vectors carrying the CFTR gene driven by human cytokeratin 18 promoter caused no pulmonary inflammation and provided transgene expression for at least 15 weeks, protecting the lungs from opportunistic infections in mice [102, 103]. In addition, because cystic fibrosis is a chronic disease and the airway epithelium has a constant turnover, the treatment of CF by gene therapy requires repeated administrations of the vector [104]. In this regard, Koehler and collegues showed that in oposition to the initial administration of FGAd followed by a FGAd or HDAd vectors, readministration of moderate doses of HDAd vectors after a prior HDAd administration leads to a reduced immune response, suggesting that readministration of HDAd vectors for lung gene therapy may be feasible [105]. Similarly, immune response against HDAd vector readministration could be improved by rotating Ad serotypes [106], administering cyclophosphamide to temporary modulate the host's immune system [107], or by using PEGylated vectors [108].

Notably, gene therapy strategies in the lung using adenoviral vectors have been hindered by the cellular structure of the epithelial airway, where the location of CAR receptors (the primary receptors of Ad5) on the basolateral surface – and therefore unavailable to the Ad vectors – is an important drawback for adenovirus transduction [109, 110]. Regarding this, different strategies to disrupt the tight-junctions such as the use of ethylenediamine tetra-acetic acid (EDTA), ethylene glycol tetra-acetic acid (EGTA), lysophosphatidycholine (LPC), and polycations prior to the Ad administration have had positive results [111-113]. However, these strategies, usually based on two separate administrations are inefficient as they do not lead to homogenous distribution of the vector and the tight-junction opening agent. This has been recently improved by using an intratracheal nebuliser in a single administration, a delivery method that is clinically relevant for humans and allows a high expression of the transgene from the trachea to terminal bronchioles in rabbits [113], and non-human primates [114].

Last, the gene therapy studies on CF have been performed in animals with healthy airways instead of in the presence of thick deposition of mucus in the lungs, which inhibits Ad transduction [115], and leads to multiple bacterial colonisations. Interestingly, the recently developed pig model for cystic fibrosis shares many features with human CF at anatomic, biochemical, and pato-physiological levels [116, 117], and will allow to test gene therapy strategies using HDAd vectors in more clinical rellevant conditions.

PROSPECTS

HDAd vectors are very attractive vectors for gene therapy because they have a large cloning capacity and can infect a wide variety of cell types, regardless of their proliferation state, and result in long-term transgene expression without chronic toxicity. The production of these vectors has been widely optimised up to a level where clinical-grade large-scale production can be easily achieved with very low helper-Ad contamination levels. However, there is still con-

cern for the clinical application of HDAd vectors because the humoral immune response against the viral capsid limits their efficiency, while the innate immune response limits their biosafety. Strategies to avoid the immune response – such as PEGylation of the vectors or the use of non-human-derived HDAd vectors – seem to be very promising options for their application in clinical trials. However, the large-scale production and downstream processing of non-human HDAd vectors has not been sufficiently optimised yet, generally producing low infectious titres with levels of helper contamination that makes them unfeasible for clinical trials. The development of improved methods to produce and purify these vectors will be essential for their use in the clinic.

The potential of HDAd vectors has proved promising in a wide variety of organs (brain, liver, lung and muscle) for several different diseases (mucopolysaccharidosis, multiple sclerosis, glioblastoma, Crigler–Najjar syndrome, DMD, etc). However, despite encouraging results, most preclinical studies have been performed in small animal models, and this makes the use of these strategies in humans difficult to predict. In this regard, the use of larger and more reliable animal models will be crucial for the future use of these vectors in human trials.

To date only a few clinical assays using HDAd vectors have been reported. In the first assay, one patient suffering from severe hemophilia A was treated with a HDAd vector expressing full-length FVIII, only achieving 1% of normal FVIII levels for several months. Furthermore, the patient developed a transient inflammatory response with hematologic and liver abnormalities [118]. HDAd-vectors have also been used in a Phase I–II clinical assay to treat anemic chronic kidney disease (CDK) patients. Autologous dermal fibroblasts from CDK patients were transduced ex vivo with an HDAd encoding erythropoietin (EPO) and were them reimplanted subcutaneously into the patients. Interestingly, after a single treatment with the HDAd-transduced cells, no adverse events were reported and elevated hemoglobin levels were maintained for up to one year [119]. Additionally, a Glioma Phase I Clinical Trial using HDAd vectors encoding the immunostimulatory cytokine fms-like tyrosine kinase ligand 3 has been recently approved by the FDA (BB-IND 14574; NIH/OBA Protocol # 0907-990; OSU Protocol 10089) [120]. No results are available yet.

CONCLUSION

Finally, to improve the biosafety and efficacy of HDAd vectors, different strategies based on modulating the interaction between viral fibre proteins and the cellular receptors by capsid modification are currently being tested. The higher transduction efficiencies of these chimeric vectors would allow therapeutic levels of the vector using lower doses, making them safer in the clinic. Also, capsid modification could be performed to generate vectors capable of only transducing a specific target cell, allowing the control of vector tropism, and therefore avoiding a widespread distribution of the vector, which would be an important step forward in terms of biosafety.

In summary, recent advances demonstrate clearly the therapeutic potential of HDAd vectors. However, more efforts need to be made to optimise and generalise the use of HDAd vectors, especially in large animal models, which should facilitate their application in clinical assays.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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