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Invited Mini Review

Helper virus-free gutless adenovirus (HF-GLAd): a new platform for gene therapy

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Gene therapy is emerging as a treatment option for inherited genetic diseases. The success of this treatment approach greatly depends upon gene delivery vectors. Researchers have attempted to harness the potential of viral vectors for gene therapy applications over many decades. Among the viral vectors available, gutless adenovirus (GLAd) has been recognized as one of the most promising vectors for *in vivo* gene delivery. GLAd is constructed by deleting all the viral genes from an adenovirus. Owing to this structural feature, the production of GLAd requires a helper that supplies viral proteins *in trans*. Conventionally, the helper is an adenovirus. Although the helper adenovirus efficiently provides helper functions, it remains as an unavoidable contaminant and also generates replication-competent adenovirus (RCA) during the production of GLAd. These two undesirable contaminants have raised safety concerns and hindered the clinical applications of GLAd. Recently, we developed helper virus-free gutless adenovirus (HF-GLAd), a new version of GLAd, which is produced by a helper plasmid instead of a helper adenovirus. Utilization of this helper plasmid eliminated the helper adenovirus and RCA contamination in the production of GLAd. HF-GLAd, devoid of helper adenovirus and RCA contaminants, will facilitate its clinical applications. In this review, we discuss the characteristics of adenoviruses, the evolution and production of adenoviral vectors, and the unique features of HF-GLAd as a new platform for gene therapy. Furthermore, we highlight the potential applications of HF-GLAd as a gene delivery vector for the treatment of various inherited genetic diseases. [BMB Reports 2020; 53(11): 565-575]

INTRODUCTION

The first approved clinical application of gene therapy took place in the US almost three decades ago (1-3). Since then, advances in gene therapy have led to new therapeutic opportunities for once untreatable inherited genetic diseases. To date, three *in vivo* gene therapy products—Glybera (4), Luxturna (5), and Zolgensma (6)—for the treatment of inherited genetic diseases have been approved in Europe and the US, and more products are expected to be available soon.

The fundamental principle of gene therapy is to deliver a functional copy (a therapeutic transgene) of the mutant gene to physiologically relevant target tissues or organs of the patient to compensate for the mutant gene. A therapeutic transgene can be delivered into the patient's body via two methods: (1) an *ex vivo* approach, in which the patient's cells are first modified by therapeutic transgenes outside the body and then transplanted back into the patient's body; (2) an *in vivo* approach, in which therapeutic transgenes are directly delivered into the patient's body and cells are modified *in situ*. In either method, a vehicle called 'vector' is needed to deliver the therapeutic transgenes.

Safety is the highest priority in every therapeutic intervention. The vectors for gene therapy are no exception, and thus, the safety of the gene delivery vectors should be carefully and continuously monitored. Additionally, the gene delivery vectors should be able to sustain high-level and persistent transgene expression in host organisms to achieve therapeutic efficacy of the transgenes delivered.

Among all the currently available vectors for *in vivo* gene delivery, gutless adenovirus (GLAd) has been recognized as one of the most promising vectors (7). GLAd does not integrate into the host genome, and instead remains as an episome in the nucleus, which eliminates concerns related to insertional mutagenesis and germline transmission. GLAd also exhibits a broad tropism and high efficiency in gene delivery. Importantly, GLAd induces negligible immune responses in host organisms, enabling high-level and persistent transgene expression in many types of tissues. Furthermore, GLAd can accommodate transgenes of up to 36 kb, which allows the delivery of a large transgene or multiple transgenes. These advantages of GLAd as a gene delivery vector have drawn the tremendous attention of researchers toward gene therapy applications.

However, despite the obvious benefits, the currently available GLAd has a notable drawback: contamination with adenovirus and RCA in its final product. Safety concerns raised by these

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contaminants have hindered its clinical applications. Recently, we successfully developed helper virus-free gutless adenovirus (HF-GLAd), a new version of GLAd, which is produced by a helper plasmid. HF-GLAd, free of helper adenovirus and RCA contaminants, will facilitate its clinical applications.

In this review, we discuss the characteristics of adenovirus, the evolution of adenoviral gene delivery vectors, and the host immune responses against adenoviral vectors. Moreover, we highlight the unique features of HF-GLAd as a new platform for *in vivo* gene therapy in various inherited genetic diseases and discuss the possible applications of HF-GLAd-based gene therapy for other diseases.

CHARACTERISTICS OF ADENOVIRUS

The first adenovirus was isolated from the tissue culture of human adenoids in 1953 and characterized by Rowe *et al*. (8) and Hilleman *et al*. (9). To date, 57 human adenoviruses (HAd-1 to HAd-57) have been identified and classified into seven serotypes (HAd-A to HAd-G) (10). All of these adenoviruses exhibit the same overall architecture. They measure 90-100 nm in diameter and are non-enveloped viruses containing a linear, non-segmented double-strand DNA genome wrapped in an icosahedral capsid.

The capsid of adenovirus is composed of 292 capsomeres with 20 triangular facets and 12 vertices. These capsomeres consist mainly of 240 hexons (trimer of protein II) on the facet and 12 pentons on the vertices of the capsid (Fig. 1A). The penton unit consists of a penton base (pentamer of protein III) anchored in the capsid and a projecting fiber (trimer of protein VI) with a knob at its distal end (Fig. 1B). Besides, several other minor structural proteins, including IIIa, VIII, IX, vIII, terminal protein (TP) and V, are located on the internal and external surface of the capsid (Fig. 1B).

The capsid contains a relatively large adenoviral genome (30-40 kb). For example, human adenovirus type 5 (HAd5), a member of HAd serotype C, contains an approximately 36 kb genome. The HAd5 genome carries an inverted terminal repeat (ITR) (~100 bp) at both ends, each of which is covalently attached to a TP at the 5′-terminus of each DNA strand (Fig. 1C). The ITR sequence on the left end of the adenoviral genome is followed by the ψ packaging signal that controls the encapsidation of the viral genome (Fig. 1C). In addition to the ITRs and the ψ packaging signal, there are 38 viral genes organized in 17 transcriptional units classified into early, intermediate, and late categories (Fig. 1C). The early (E) transcriptional units (E1-E4) encode proteins that regulate viral gene transcription, viral DNA replication, and the suppression of host immune responses against adenoviral infection. The intermediate transcriptional units code for two proteins, IX and IVa2. The late (L) transcriptional units (L1-L5) encode the structural proteins of adenovirus.

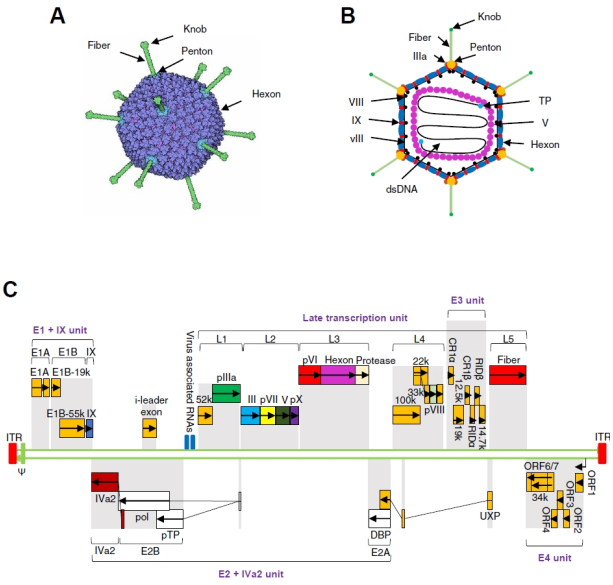

Fig. 1. The capsid and genomic structure of adenovirus. (A) The capsid of adenovirus. The icosahedral capsid of adenovirus is composed of 3 major types of proteins: 12 knobbed fibers, 12 pentons, and 240 hexons. (B) Cross-section through the capsid of adenovirus. The adenovirus is 90-100 nm in diameter. Several types of minor proteins, such as IIIa, VIII, IX, vIII, bind in the grooves between the major proteins, hexons, and pentons. The viral genome DNA binds covalently to the terminal protein (TP) at 5′ ends and is wrapped in nucleocapsid consisting of histone-like proteins, such as protein V. (C) The genomic structure of HAd5. The HAd5 genome is a linear, non-segmented, double-stranded DNA molecule with a length of 36 kb. The 38 viral protein-coding genes are organized in 17 transcriptional units, categorized into early units (E1-E4), intermediate units, and late units (L1-L5) in both directions. The early transcriptional units encode proteins that are involved in the initiation of viral DNA replication (E1), the regulation of viral transcription (E2 and E4), and the suppression of host responses to adenoviral infection (E3). The intermediate transcriptional units encode two proteins, IX and IVa2. The late transcriptional units (L1-L5) encode the components of viral capsid.

EVOLUTION OF ADENOVIRAL GENE DELIVERY VECTORS

Most adenoviral vectors are derived from HAd5, and classified into two categories: replication-competent and replication-defective. The replication-competent adenovirus (RCA) has been developed typically as a tool for anti-cancer therapy. Since RCA can replicate by itself and is strongly immunogenic, it plays a role in the lysis of infected and adjacent tumor cells when injected into tumor tissues. In contrast, the replication-defective adenovirus has been primarily developed as a gene delivery vector, following the modifications by deleting viral genes partially or entirely to reduce or eliminate the expression of viral proteins. These modifications have been shown to attenuate the host immune responses. Based on the modifica-

tions, several generations of adenoviral vectors have been constructed as follows.

The first- and second-generation adenoviral vectors

The first-generation adenovirus (FGAd) was constructed by deleting the E1 region (from nucleotide 400 to 3500) and the E3 region from the adenoviral genome (Fig. 2). The E1 region encodes proteins essential for the expression of other early and late genes, and thus, is crucial in initiating the life cycle of adenovirus (11). The E3 region encodes proteins that protect adenovirus from the host antiviral immune responses (12). Since these E3 proteins are dispensable for adenovirus production, the E3 region is generally deleted in the FGAd to increase its cargo capacity for the transgene.

Due to the deletion of the E1 region, FGAd cannot replicate by itself. Therefore, the production of FGAd requires packaging cell lines that express E1 proteins *in trans* to compensate for the lack of E1 region. The cell line most commonly used for FGAd production is the human embryonic kidney (HEK) 293 (13) that contains an insertion of the E1 region (from nucleotide 1 to 4334) at 19q13.2 in its genome (14). Unfortunately, utilization of this cell line has a potential drawback, RCA generation, resulting from homologous recombination between the FGAd genome and this cell line (15). Although the occurrence of RCA is low during the initial passages of FGAd (16), the RCA is rapidly amplified at higher passages, leading to serious safety concerns in clinical applications (17). According to the FDA guidelines, there should be less than 1 RCA in 3×10^{10} viral particles (vp) (18). Despite the safety concerns associated with RCA, HEK293 is still the most frequently used cell line for FGAd production. An alternative cell line, PER.C6 (19), has been developed to limit RCA generation; however, this cell line has not been widely used due to strict licensing.

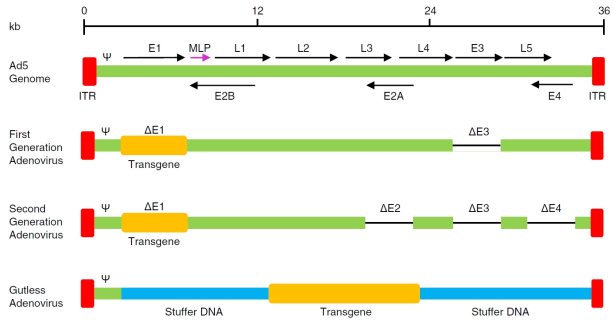

Fig. 2. Three generations of adenoviral vectors. The first-generation adenovirus (FGAd) is constructed by removing the E1 and E3 regions from the adenoviral genome. The second-generation adenovirus (SGAd) is generated via further deletions in the E2 and E4 regions. The third-generation adenovirus, referred to as gutless adenovirus (GLAd), is constructed by deleting all the viral protein-coding genes, leaving only the ITRs and the ψ packaging signal in its genome backbone.

Even though FGAd is devoid of the E1 region, the E1A-like factors present in many cell types can still induce the expression of other adenoviral proteins in transduced host cells (20), eliciting strong host immune responses and resulting in transient transgene expression and chronic toxicity (21). Therefore, FGAd has been recognized as a suitable platform for the delivery of transgenes in anti-cancer therapy (22) rather than a platform for delivering therapeutic transgenes to treat inherited genetic diseases, which requires high-level and persistent transgene expression.

In an attempt to attenuate the host immune responses against adenoviral proteins, the second-generation adenovirus (SGAd) was generated via additional deletions of the E2 and E4 regions (Fig. 2). The E2 region encodes three proteins related to the replication of viral DNA (23), including DNA-binding protein (DBP), terminal protein (TP), and DNA polymerase. The E4 region codes for control proteins that regulate the transcription of adenoviral DNA (24). These deletions significantly reduce the synthesis of adenoviral proteins. Nonetheless, SGAd still induces host immune responses due to the proteins expressed from the residual adenoviral genes, which results in reduced transgene expression in transduced cells (25).

The third-generation adenoviral vector: gutless adenovirus (GLAd)

Despite the deletion of early transcriptional units (E1-E4), the early-generation adenoviral vectors still exhibit strong immunogenicity and toxicity in host organisms. These undesirable safety issues led to the development of the third-generation adenoviral vector, referred to as gutless adenovirus (GLAd). GLAd is constructed by deleting all the viral genes from an adenovirus, only leaving the ITRs and the ψ packaging signal in its genome backbone (Fig. 2). This structural characteristic eliminates the expression of viral proteins in transduced cells and only induces negligible immune responses, enabling high-level and persistent transgene expression in host organisms (26). Importantly, this large deletion also increases the cargo capacity for the transgene up to 36 kb, which allows the delivery of a large transgene or multiple transgenes.

In general, most therapeutic transgenes do not reach 36 kb. Therefore, the deleted viral genes should be replaced with a stuffer DNA to stably maintain the genome of GLAd within the size range (27-37.8 kb) for efficient encapsidation (27-29). The nature of stuffer DNA in GLAds appears to affect the expression of transgenes *in vitro* and *in vivo*. Parks *et al*. (30) showed that the GLAd containing a eukaryotic stuffer DNA leads to enhanced and persistent transgene expression compared with a similar vector containing a prokaryotic stuffer DNA. In contrast, Schiedner *et al*. (31) demonstrated that the origin of the stuffer DNA does not affect the transgene expression. Regardless of this controversy, the candidate for stuffer DNA must be carefully selected to avoid coding sequences, repetitive sequences, recombination sites, and immunogenic se-

quences. Notably, the inclusion of a scaffold/matrix attachment region (S/MAR) (32) into the stuffer DNA can stabilize the genome of GLAd as an episome in the host cell nucleus.

Since GLAd is devoid of all the viral genes, its production requires a helper that supplies the viral proteins *in trans*, and thus, the GLAd is also called 'helper-dependent adenovirus (HDAd)'. Currently, the most commonly used helper is an adenovirus (33-35) that is usually an E1-deleted FGAd. Recombinant GLAd can be generated by co-infection (or co-transfection) of helper adenovirus (or helper adenoviral genome) and GLAd (or GLAd genome) into the packaging cell lines, such as HEK293 and HEK293T (Fig. 3A). This helper adenovirus, which can replicate in the packaging cell lines, robustly supplies viral proteins. Unfortunately, however, helper adenovirus can remain as an undesirable contaminant in the final GLAd product (Fig. 3A). Furthermore, the helper adenovirus can be converted into RCA via homologous recombination between identical sequences present in the genomes of helper adenovirus and packaging cell lines (33) (Fig. 3B), which is similar to RCA generation in the FGAd production (16).

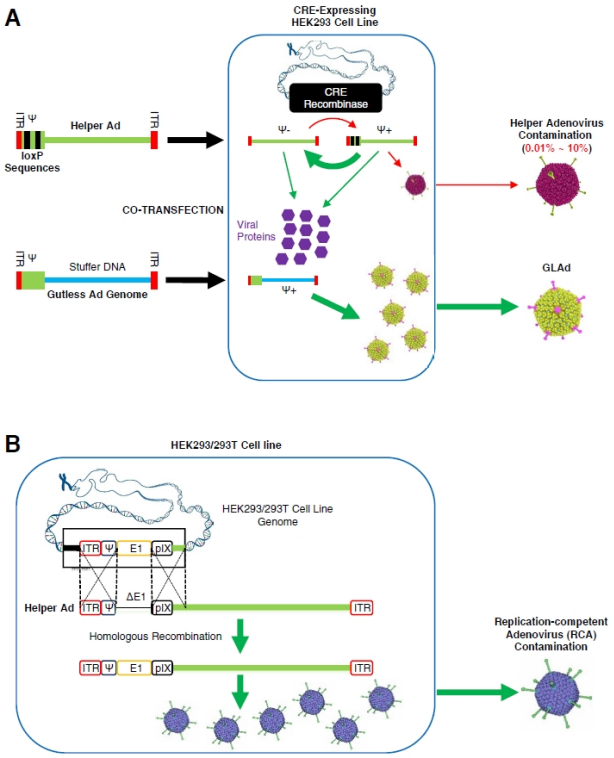

Fig. 3. Helper virus-dependent production of GLAd and the generation of RCA in the final product. (A) Helper virus-dependent production of GLAd using the Cre/loxP system. Genomes of GLAd and helper adenovirus are co-transfected into packaging cells, Cre recombinase-expressing HEK293 cell line, in which both viruses are amplified and the helper adenovirus produces viral proteins. During this process, the ψ packaging signal flanked by loxP sites is excised by Cre recombinase, preventing the packaging of helper adenovirus genome, while the GLAd genome is packaged. The helper adenovirus contamination in the production of GLAd ranges from 0.01% to 10%. (B) The generation of RCA. Two homologous sites are identified in the genomes of E1-deleted helper adenoviruses and E1-expressing packaging cells. Homologous recombination can occur in these two homologous regions, resulting in the generation of RCA as the helper adenovirus acquires the E1 region from the packaging cells.

The helper adenovirus and RCA contaminants are hazardous, especially to the immunocompromised patients because the adenoviral proteins expressed by these two contaminant viruses can induce toxic immune responses. Besides, the strong host immune responses against helper adenovirus and RCA contaminants can limit the expression of the therapeutic transgene delivered by GLAd, even though GLAd *per se* induces negligible immune responses.

Indeed, the advantages of GLAd as a gene delivery vector are enormous. However, the safety concerns raised by the helper adenovirus and RCA contaminants have hindered its clinical applications. Accordingly, no clinical data are available for GLAd. Therefore, any clinical application requires the elimination of these two contaminants from the final preparation of GLAd.

To date, the most elegant strategy to prevent helper adenovirus amplification entails the deletion of the ψ packaging signal (33). In this strategy, the ψ packaging signal flanked by two loxP sites is excised when the helper adenovirus infects Cre-recombinase-expressing cell lines, such as 293Cre (Fig. 3A). The amplification of helper adenovirus is significantly reduced (0.01-10% of total virus produced) by blocking the encapsidation of helper adenoviral genome. Based upon this Cre/LoxP production system, Palmer *et al*. (34) developed a method for large-scale production of GLAd, with a yield exceeding 1×10^{12} blue forming units (BFU), and helper adenovirus contamination of 0.01% - 0.02% following purification by a two-step CsCl gradient ultracentrifugation (36). However, despite the dramatic reduction, the contamination of helper adenovirus was unpreventable even with this sophisticated production system.

A similar strategy utilizing the FLP/frt system was also developed (37). However, it was still very difficult to completely remove the contaminated helper adenovirus.

In an attempt to address the safety concerns associated with helper adenovirus contamination, researchers have developed other strategies utilizing non-adenoviral helpers, such as baculovirus-adenovirus hybrid (38) and herpes simplex virus-1 (HSV-1) (39). Unfortunately, however, these two helpers also generated undesirable RCA contaminants and were shown to be inefficient in the production of GLAd.

Taken together, it is clear that the presence of helper viruses in the GLAd production system is an unavoidable risk. Therefore, establishing a system devoid of any helper viruses is of utmost importance for the production of GLAd that is desirable for clinical applications.

Helper virus-free gutless adenovirus (HF-GLAd)

To address the aforementioned safety issues of GLAd, we developed helper virus-free gutless adenovirus (HF-GLAd), a new version of GLAd, which is produced in a helper virus-free manner (40). In this novel system, the helper function required for the HF-GLAd production is provided by a helper plasmid instead of a helper adenovirus. This helper plasmid does not contain the ITRs and the ψ packaging signal, both of which are essential for viral genome replication and packaging. Therefore, this helper plasmid exclusively supplies viral proteins *in trans*, but cannot be converted into active adenovirus particles (Fig. 4A). Moreover, compared with the helper adenovirus, this helper plasmid contains only a single region for homologous recombination, which prevents conversion of this plasmid to RCA in the packaging cell lines, such as HEK293T (Fig. 4B).

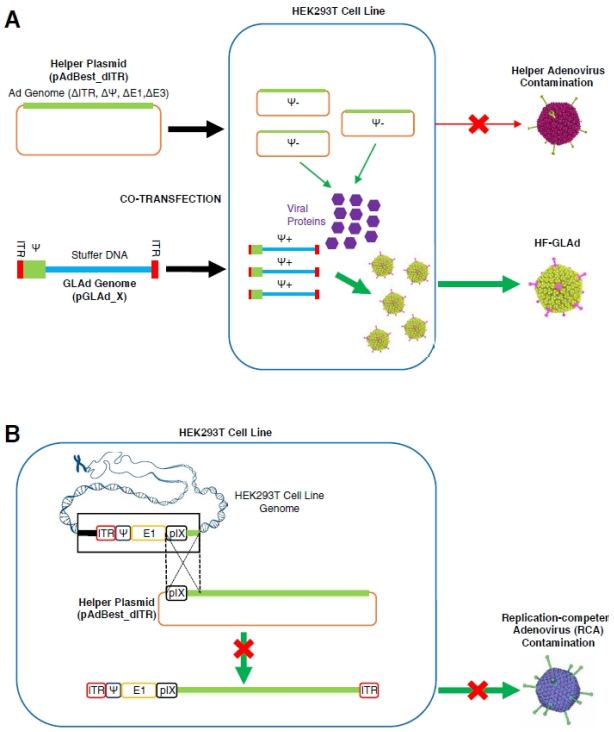

Fig. 4. Production of HF-GLAd with no RCA generation in the final product. (A) Production of HF-GLAd. GLAd genome (pGLAd_X) and helper plasmid (pAdBest_dITR) are co-transfected into a packaging cell line, HEK293T, in which the helper plasmid produces viral proteins for packaging of the GLAd genome. The helper plasmid cannot be amplified or packaged into the active viral particles, resulting in the production of HF-GLAd free of helper adenovirus. (B) No RCA generation during HF-GLAd production. Only one homologous recombination site is identified in the helper plasmid and E1-expressing packaging cells. Helper plasmid cannot acquire the E1 region from the genome of packaging cells via homologous recombination, which eliminates RCA generation in HF-GLAd production.

Most importantly, this helper virus-free production system successfully produced large quantities of HF-GLAd free of helper adenovirus and RCA contaminants (40).

Briefly, the production of HF-GLAd requires two plasmids (Fig. 4A): (1) pAdBest_dITR (~31 kb), a helper plasmid that provides adenoviral proteins *in trans*; (2) pGLAd_X ('X' stands for the gene of interest), a GLAd genome plasmid, part of which is packaged into the active viral particles. HF-GLAd can be generated by co-transfection of the helper plasmid and the linearized GLAd genome plasmid into the HEK293T packaging cells (Fig. 4A). Besides, an auxiliary plasmid, the pAd5pTP, is included to overexpress the precursor terminal protein (pTP) of HAd5 in HEK293T cells for increasing the yield of HF-GLAd (41).

In an attempt to produce large-scale HF-GLAd, a serial amplification process was established (40). This procedure is similar to the standard amplification process used for the helper adenovirus-dependent large-scale GLAd production (34). However, the packaging cells are transfected with helper plasmid in each round of amplification (40) instead of being infected with helper adenovirus (34). This serial amplification method (P0-P3) routinely achieved large-scale production of HF-GLAd with a yield of 5×10^{10} - 1×10^{11} infectious units (ifu) (50-100 ifu/cell) in P3 (40). This yield is merely 10- to 20-fold lower than that of the helper virus-dependent method (34), indicating that the helper plasmid supplies a sufficient amount of viral proteins for HF-GLAd production, although it cannot replicate in the packaging cells.

Since the selection of stuffer DNA is pivotal for sustaining high-level and persistent expression of transgenes, we selected fragments from the second intron of the mouse *E-cadherin* gene (42) as a stuffer DNA, which do not encode any proteins or carry homologous recombination sites. Additionally, we added an S/MAR element (43) to this stuffer DNA to stabilize it as an episome in the cell nucleus and also to increase the transgene expression.

We have successfully established a two-column chromatographic purification method to obtain highly pure recombinant HF-GLAd for preclinical and clinical applications. We are also investigating the possible adaptation of the culture dish-based serial amplification method (40) to the multi-layer Cell Factory-based (44) approach for large-scale production of HF-GLAd.

ADENOVIRAL VECTOR-ASSOCIATED IMMUNE RESPONSES

Viral vectors are the optimal gene therapy platforms because viruses have evolved to deliver their genetic material into permissive cells of other organisms. In parallel, the immune system of the host organism has also evolved to resist invasion of viral pathogens. An immune response against viral pathogens may benefit the application of vaccines (45-50) or anti-cancer therapy (51-53). However, an immune response against viral vectors used in gene therapy can eliminate the vectors and the

transduced host cells. Such phenomena interfere with high-level and persistent expression of therapeutic transgenes in host organisms. Therefore, circumventing the host immune responses against viral vectors is critical for the success of *in vivo* gene therapy.

The immune responses against adenoviral vector-based gene therapy can be summarized into two main classes: a rapid and non-specific innate immune response, and a relatively slow but highly specific adaptive immune response.

Innate immune responses against adenovirus: the prologue

The adenovirus-mediated host innate immune responses induced by virion components (*i.e.*, viral capsid proteins and DNA genome) are dose-dependent (54), and lead to upregulation of inflammatory gene expression (*i.e.*, type I interferons) and cytokine secretion. These type I interferons and cytokines recruit immune cells to the administration sites (55-57), resulting in a rapid clearance of 80-90% vectors from the blood circulation and transduced tissues (58).

Adaptive immune responses against adenoviral vectors: climax

The adaptive immune responses induced by adenoviral vectors or adenovirus-based gene therapy are activated within a week (59). These immune responses can be elicited by the viral proteins expressed from the adenoviral vectors, or the products expressed by the therapeutic transgenes.

The viral proteins expressed by the early-generation adenoviral vectors induce cellular immune responses. In the early phase, the cytokines and chemokines are upregulated, leading to the infiltration of CD4+ and CD8+ T lymphocytes to the administration site of adenoviral vectors (60) and the generation of adenovirus-specific cytotoxic T lymphocytes (CTLs) (61). These cellular immune responses are initiated by antigen-presenting cells (APCs), resulting in the elimination of transduced host cells and the generation of memory immune cells against the adenoviral vectors (62).

Administration of adenoviral vectors also induces humoral adaptive immune responses via presentation of MHC-II/adenoviral capsid antigen complexes at the surface of B lymphocytes to CD4+ T lymphocytes, which results in the activation of CD4+ T lymphocytes. Following the activation, CD4+ Th2 lymphocytes promote the proliferation of B lymphocytes and their differentiation to plasma cells that secrete antibodies against adenoviral capsid proteins. The pre-existing neutralizing antibodies (NAbs) in host organisms (63) may interfere with adenovirus infection and thereby decrease the efficacy of adenoviral vector-based gene therapy (64-66).

The products encoded by therapeutic transgenes may also be immunogenic in patients with *null* mutations. These products may be presented by APCs to CD4+ and CD8+ T lymphocytes and recognized as neo-antigens by the host immune system (67). From a therapeutic point of view, it is expected that repeated administration of gene delivery vector is required to maintain a sustained expression of therapeutic transgenes in tissues or organs with a high regeneration rate (*i.e.*, respiratory and gastrointestinal epithelium). The repeated administration of gene delivery vectors or sustained expression of therapeutic transgenes acts as a 'prime-boost vaccination', which decreases the duration of therapeutic transgene expression and the efficacy of gene therapy.

Immune responses against HF-GLAd

GLAd is devoid of all the viral genes. Thus, GLAd does not express any adenoviral proteins in transduced host cells, which minimizes the induction of adenovirus-specific adaptive immune responses, enabling high-level and persistent transgene expression in host organisms. Importantly, as HF-GLAd is produced in the absence of helper adenovirus, its final product is free of helper adenovirus and RCA contaminants. Therefore, HF-GLAd is clinically more desirable than the GLAd produced by the helper adenovirus-dependent system.

Nevertheless, HF-GLAd can still induce innate immune responses, since it shares an identical capsid structure with wild-type and early-generation adenoviruses (68). Also, pre-existing NAbs and/or adenovirus-specific CTLs present in the patients previously exposed to adenoviruses can decrease the efficacy of HF-GLAd-based gene therapy (63).

Strategies to circumvent immune responses against HF-GLAd

Several elegant strategies have been developed to circumvent the host immune responses against adenoviral vectors. These strategies include transient immune modulation in the host organism before administrating these vectors, and selective modification of these vectors *per se*.

Transient immune modulation entails either pre-deletion of immune cells or induction of immunosuppression (or immune tolerance). For example, transient depletion of specific immune cells (*i.e.*, CD4+ and CD8+ T lymphocytes, B lymphocytes, and NK cells) by injecting antibodies can significantly increase the transduction and re-administration efficiency of GLAd in mouse liver (69). Transient treatment with immunosuppressants or agents inducing immune tolerance, such as glucocorticoids, FK506, dexamethasone, cyclosporin A, cyclophosphamide, mCTLA4-Ig, or mycophenolate mofetil, has been shown to increase the levels and duration of GLAd-mediated transgene expression in various animal models (70-73).

As an approach for selective modification, adenoviral capsid proteins can be conjugated with chemicals, such as polyethylene glycol (PEG) (74, 75). The adenoviral vectors containing such modified capsid proteins have already been shown to improve the vector safety and transduction efficiency. Therefore, these approaches, individually or in combination, can also be adopted in HF-GLAd-based gene therapy.

Several other strategies were also investigated to minimize adenovirus-mediated host immune responses. For example, since host innate immune responses against adenoviral vectors are dose-dependent, it is crucial to establish a threshold dose

to minimize the acute toxic immune responses. Also, it is preferable to select immune-privileged tissues or organs, especially the eye and central nervous system (CNS), as *in vivo* administration targets, given their significantly low immune responses against foreign antigens (76, 77). Therefore, the eye and CNS are ideal targets for HF-GLAd-based gene therapy, owing to their immune-privileged characteristics and the need for relatively low vector doses to achieve sufficient therapeutic efficacy (78).

HF-GLAD AS A GENE DELIVERY VECTOR FOR *IN VIVO* GENE THERAPY

Recombinant adenoviral vectors have been extensively investigated in preclinical and clinical applications. However, the tragic death of Jesse Gelsinger, who was treated for ornithine transcarbamoylase (OTC) deficiency, has severely damaged the reputation of adenovirus-based gene therapy (79). Although these vectors have shown tremendous advantages and technological advances (*i.e.*, the advent of GLAd), safety concerns have led to a significant decline in their clinical applications for inherited genetic diseases. Since then, the majority of *in vivo* gene delivery has shifted to the vectors with less immunogenicity and toxicity. As a result, adeno-associated viruses (AAVs) have become mainstream in development of gene therapy (80, 81), and three AAV-based gene therapy drugs have been approved (4-6). Similar to GLAd, the AAVs are also 'gutless'; however, AAVs carry a small cargo (~4.5 kb) appropriate for only small transgenes (Table 1), which poses an undeniable limitation in large transgene delivery. Therefore, the focus of AAV-based gene therapy applications is on the delivery of small or truncated transgenes, such as the *SMN1* gene (~1.5 kb, a target for spinal muscular atrophy) (82), the *FIX* gene (~2.8 kb, a target for hemophilia B) (83), the *RPE65* gene (~2.6 kb, a target for Leber's congenital amaurosis 2) (84), and the micro-*DMD* gene (~4.2 kb, a target for Duchenne and Becker muscular dystrophy) (85).

Gene therapy using GLAd has attracted tremendous attention in recent years. In particular, in addition to a substantial capacity for transgenes (Table 1), the safety of HF-GLAd is comparable to that of AAV, which is expected to restore the reputation of adenoviral vectors as well as facilitate its applications for *in vivo* gene therapy. Although the HF-GLAd remains to be clinically evaluated, it has already shown high efficiency in *in vitro* and *in vivo* gene delivery (40).

HF-GLAd is capable of delivering transgenes regardless of size because no human gene exceeds its carrying capacity. HF-GLAd can accommodate a small or a large transgene, and even multiple transgenes in a single construct. In theory, HF-GLAd might be an ideal vector to safely deliver large transgenes to treat inherited genetic diseases, such as Duchenne and Becker muscular dystrophy (with mutations in the *DMD* gene, ~11 kb) (40, 86-88), Huntington's disease (with mutations involving the *HTT* gene, ~9.4 kb) (40, 89), Leber's congenital amaurosis 10 (associated with *CEP290* gene mutations, ~7.5 kb) (90), Stargardt disease type I (associated with *ABCA4* gene mutations, ~6.8 kb) (91-93), retinitis pigmentosa 25 (with mutations involving the *EYS* gene, ~9.5 kb) (94), and retinitis pigmentosa 39 (associated with *USH2A* gene mutations, ~15.6 kb) (95). We have successfully constructed the recombinant HF-GLAds harboring codon-optimized transgene for each of these diseases. Currently, the safety and efficacy of HF-GLAd-based gene therapies for the treatment of inherited genetic diseases are being evaluated under consideration of clinical applications.

Table 1. Comparison of helper virus-dependent GLAd, HF-GLAd, and AAV

	Helper virus-dependent GLAd	HF-GLAd	AAV[a]
Use of helper adenovirus in production	YES	NO	n/a[d]
Use of helper plasmid in production	n/a[d]	YES	n/a[d]
Helper adenovirus contamination	YES	NO	n/a[d]
RCA[b] contamination	YES	NO	n/a[d]
Cargo capacity for transgenes	~36 kb	~36 kb	~4.5 kb
Efficiency[c] of transduction	100%	100%	40%
Broad tropism	YES	YES	YES
Random integration into host genome (potential of insertional mutagenesis)	NO	NO	YES[e]
Expression of viral proteins	NO	NO	NO
In vivo acute toxicity by viral capsid	YES	YES	YES
In vivo chronic toxicity	NO	NO	NO
Long-term *in vivo* transgene expression	YES	YES	YES

[a]Production by three plasmid-based transfection, [b]Replication-competent adenovirus, [c]Relative activity, [d]Not applicable, [e]Although frequency is low.

CONCLUSION AND FUTURE PERSPECTIVES

GLAd is one of the most promising vectors for *in vivo* gene therapy, given its advantages. However, the safety concerns raised by undesirable helper adenovirus and RCA contaminants have hindered its clinical applications. Recently, we have successfully created HF-GLAd as a new platform for gene therapy, which is devoid of helper adenovirus and RCA contaminants. This revolutionary advance will facilitate clinical applications of the HF-GLAd.

Currently, many recombinant HF-GLAds are under investiga-

tion in animal studies for the treatment of various inherited genetic diseases. Further, HF-GLAd also carries the potential for delivery of therapeutic transgenes to treat other diseases, such as Parkinson's disease (96, 97), Alzheimer's disease (98), and cancer (*in situ* vaccination) (99). We sincerely hope that the application of HF-GLAd will unlock the full potential of gene therapy and open new vistas in the treatment of a much broader spectrum of diseases.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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