Expert Opinion

Therapeutic potential of adenoviral vectors for delivery of expressed RNAi activators

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Importance of the field: Harnessing RNA interference (RNAi) to silence pathology-causing genes has shown promise as a mode of therapy. The sustained gene inhibition that may be achieved with expressed sequences is potentially useful for treatment of chronic viral infections, but efficient and safe delivery of these sequences remains a challenge. It is generally recognized that there is no ideal vector for all therapeutic RNAi applications, but recombinant adenovirus vectors are well suited to hepatic delivery of expressed RNAi activators.

Areas covered in this review: Adenoviruses are hepatotropic after systemic administration, and this is useful for delivering expressed RNAi activators that silence pathology-causing genes in the liver. However, drawbacks of adenoviruses are toxicity and diminished efficacy, which result from induction of innate and adaptive immune responses. In this review, the advantages and hurdles facing therapeutic application of adenoviral vectors for liver delivery of RNAi effectors are covered.

What the reader will gain: Insights into adenovirus vectorology and the methods that have been used to make these vectors safer for advancing clinical application of RNAi-based therapy.

Take home message: Adenoviruses are very powerful hepatotropic vectors. To make adenoviruses more effective for clinical use, polymer conjugation and deletion of viral vector sequences have been used successfully. However, further modifications to attenuate immunostimulation as well as improvements in large-scale production are necessary before the therapeutic potential of adenovirus-mediated delivery of RNAi activators is realized.

Keywords: adenovirus, helper-dependent adenovirus, hepatitis B virus, hepatitis C virus, RNA interference

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1. Introduction

Demonstration that RNA interference (RNAi) may be activated with exogenous effectors to silence pathology-causing genes has generated considerable interest in using this approach for therapy [1,2]. Harnessing RNAi to counter gene expression has been a particularly active field of research, and many diseases, especially those caused by virus infections and cancer, have been shown to be susceptible to RNAi-mediated inhibition. However, one of the major obstacles to realizing the therapeutic potential of RNAi has been the difficulty with which safe and efficient delivery of gene silencing sequences to intended target cells may be accomplished. A variety of methods has been used to achieve this [1]. These include use of non-viral vectors for delivery of synthetic RNAi activators and viral vectors for transfer of antiviral RNAi expression cassettes. The sustained silencing that may be achieved with expressed sequences makes viral vectors attractive for treatment of chronic

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Article highlights.

- Harnessing RNAi to silence pathology-causing genes has enormous therapeutic potential.
- Expressed RNAi activators are compatible with recombinant adenovirus vectors and have the useful property of achieving sustained gene silencing.
- Adenovirus vectors have many features that make them useful for delivery of potentially therapeutic RNAi activators.
- An important drawback of adenoviruses is their stimulation of innate and adaptive immune responses, which is potentially toxic.
- Development of helper-dependent adenoviruses and also polymer conjugation of adenoviruses has contributed to overcoming problems of immunostimulation.
- The efficient delivery of transgenes to the liver should make adenoviruses useful for treating liver diseases such as those caused by HBV and HCV infection.
- The full potential of adenovirus-mediated delivery of RNAi activators will depend largely on establishing their safety and developing convenient methods of large-scale production.

This box summarizes key points contained in the article.

diseases. Viral vectors that are under development include recombinant adeno-associated viruses (AAVs), lentiviruses and adenoviruses. The ability of lentiviral vectors to integrate proviral sequences that stably produce antiviral RNAi activators has been particularly useful for *ex vivo* approaches to treating viral diseases that result from chronic infections, for example, AIDS caused by HIV persistence. This is not suitable for all RNAi-based antiviral approaches, and utility of lentiviral vectors is specific to certain clinical conditions. A further potential complication of using lentiviral vectors is that promoters that are responsible for control of expression of RNAi activators may interfere with transcription required for vector propagation [3]. The lack of pathology that is caused by infection with wild-type AAVs and attenuated induction of immunostimulatory pathways have made these vectors particularly appealing [4]. However, induction of an immune response by AAVs does occur and their lower transgene capacity would compromise ability to incorporate large RNAi expression sequences [5]. Recombinant adenoviruses have features that make them suited to delivering potentially therapeutic RNAi activators to the liver. These include efficient hepatic expression of inserted cassettes, well-established methods of propagating the vector, episomal location of the adenovirus genome in infected cells, compatibility with chemical modification to alter biological properties, ability to infect non-dividing cells, and transient or sustained expression of transgenes. A shortcoming of adenoviruses is their powerful stimulation of the innate and adaptive immune responses, which may result in toxicity and attenuation of efficiency of transgene delivery. The death of a patient in a clinical trial who developed complications arising from immunostimulatory effects of adenoviruses provided an important and hard lesson for researchers in the field [6]. In this review, recent advances that are relevant to the potential therapeutic utility of incorporating expressed antiviral RNAi activators into adenoviruses are discussed. Modifications to reduce vector immunostimulation, and improve stability, specificity, control of vector tropism and expression of transgenes are discussed. An opinion on the existing important challenges in the field is provided.

2. Activating RNAi for therapeutic application

RNAi, which was first described in 1998 [7], is a highly conserved pathway in metazoan cells and is essential for regulation of genes involved in a variety of cellular processes [8]. Transcription of duplex-containing RNA molecules initiates the pathway. These transcripts are processed in a stepwise manner to form mature gene silencing sequences. The mammalian micro-RNA (miR) pathway is a well-characterized mechanism of RNAi. RNA Polymerase II (Pol II)-mediated transcription of long mono- or polycistronic primary miRs (pri-miRs) sets off the pathway (**Figure 1**). These sequences contain hairpin-like RNA structures and are cleaved in the nucleus by Drosha, an RNase III enzyme, and its double-stranded RNA binding partner, DiGeorge Critical Region 8 (DGCR8) [9]. The resulting precursor miRs (pre-miRs) are then transported to the cytoplasm by exportin-5 and are then processed further by Dicer to yield mature miR duplexes comprising 21 – 23 bp RNA. One strand of the mature miR is selected as a guide for incorporation into the RNA inducing silencing complex (RISC). The guide directs RISC to effect degradation or translational suppression of target mRNA.

Silencing of target genes with exogenous RNAi activators may be achieved with chemically synthesized short interfering RNAs (siRNA), which mimic mature endogenous miRs [10], or by expression of RNAi intermediates from exogenous DNA templates (**Figure 1**) [2]. Synthetic siRNAs have the advantage of being compatible with chemical modification to improve stability, specificity, cellular delivery and safety [11,12]. Also, the smaller size of synthetic siRNAs and the cytoplasmic, not nuclear, site of action make their dose control and delivery with non-viral vectors easier to achieve. Exogenous expressed RNAi activators have the advantages of prolonged efficacy from sustained intracellular supply of siRNAs, ease of propagation in plasmid DNA, and better stability and compatibility with viral vectors such as recombinant adenoviruses. Typically, DNA expression cassettes encoding mimics of pri-miRs (pri-miR shuttles) or short hairpin RNA (shRNA) analogues of pre-miR are used to activate the RNAi pathway (**Figure 1**). RNA polymerase III promoters, for example U6 small nuclear RNA and human ribonuclease P RNA component H1 promoters, are commonly used to regulate transcription of RNAi activators. However, overexpression from Pol III promoters may be complicated by toxicity that is attributed to saturation of the endogenous

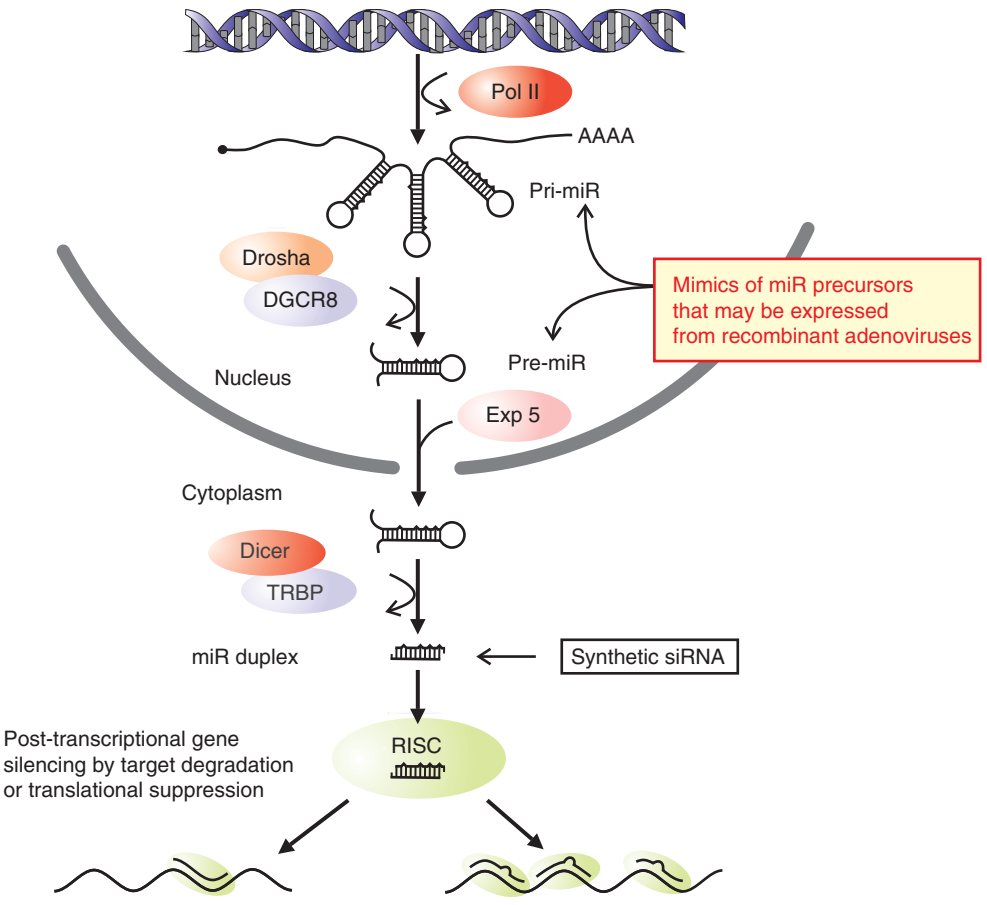

Figure 1. Summarized illustration of miR processing that shows the essential nuclear and cytoplasmic steps of the RNAi pathway. Exogenous expression cassettes that transcribe miR intermediates, which may be pri-miR or pre-miR sequences, are typically incorporated into adenoviruses. Non-viral vectors are normally used to deliver synthetic siRNA analogues of mature miR duplexes.

Adapted from [93,94].

miR: Micro-RNA; pre-miR: Precursor miR; pri-miR: Primary miR; RNAi: RNA interference; siRNA: Short interfering RNA.

RNAi machinery [13-15] or activation of an interferon response [16]. Importantly, unlike the case with delivery of synthetic siRNAs, expressed RNAi activators do not traverse the endosomal compartment and therefore bypass much of the toll-like receptor (TLR)-mediated immunostimulation [17]. Compatibility of pri-miR expression cassettes with versatile Pol II promoters enables improved transcriptional regulation [18] and generation of multimeric RNAi activators [19]. This is particularly useful to achieve tissue-specific expression, regulate the dose of RNAi activators and prevent viral escape. These properties have been utilized effectively to silence hepatitis B virus (HBV) replication *in vivo* [18,19]. In developing RNAi-based antiviral therapy, the focus of research has been on the identification of sequences that are effective at low concentrations (potent), devoid of unintended off-target effects, have limited immunostimulatory effects and can be easily delivered to target tissues in a dose-dependent manner. When used in conjunction with adenoviruses, these considerations are especially important to avoid exacerbating potential undesirable effects of the vectors.

Diseases caused by viral infections that are amenable to RNAi-based therapy are highly varied and this has a bearing on the approach to the development of RNAi-based therapy. Methods of delivery of sequences that silence virus replication and the selection of appropriate RNAi activators need to be tailored to the characteristics of individual infections. Important factors are the tropism of viruses, acute or chronic nature of the infection, whether a virus encodes RNA silencing suppressors (RSSs) and genetic variability as a result of inaccuracies of viral polymerases. Decisions about selection of adenoviruses for delivery of RNAi-activating sequences are guided by these considerations. Adenoviruses have a broad tissue tropism, but are hepatotropic *in vivo* after systemic administration. This means that delivery of anti-HBV or hepatitis C virus (HCV) sequences can be achieved efficiently after intravenous injection of recombinant adenoviruses. The

duration of adenovirus-delivered transgene expression varies and is largely dependent on the host's immune response to the vector. The development of methods to remove adenovirus sequences and chemical modification of vectors to avoid immunodetection of vectors have therefore been very important. These adaptations improve persistence of transgene expression, which is required for treatment of chronic infections.

3. Principles underlying methods of propagating adenovirus vectors

Wild-type adenovirus virions are non-enveloped and have icosahedral symmetry with a diameter of 70 – 90 nm (reviewed in [20]). They belong to the Adenoviridae family and the genome comprises 26 – 44 kb of linear double-stranded DNA. The capsid contains 240 homotrimeric proteins, 12 pentameric penton proteins that are located at each of the apices of the icosahedral capsid, and homotrimeric fiber monomers that extend from the penton base. The fiber proteins are anchored at their N-terminal regions and the protruding C-terminal globular regions interact with cell surface receptors. Human adenovirus serotype 5 is the most comprehensively studied adenovirus, and together with adenovirus 2 has been used to develop the vectors that are widely used for gene transfer. The initiating event during natural adenovirus infection is the interaction of the fiber knob with its cognate cellular receptor. Several adenovirus receptors have been identified (reviewed in [21]), of which the Coxsackievirus and adenovirus receptor (CAR) is the primary binding site. Subsequent to receptor binding, interaction of the penton base with cellular integrins ($\alpha_V\beta_3$ and $\alpha_V\beta_5$) mediates internalization [22]. With the exception of hepatocytes (see below), the susceptibility of cells to adenovirus serotype 5 infection correlates with their CAR expression and induction of expression of the receptor on cells that normally are refractory to infection show increased adenovirus serotype 5 transduction [23]. In addition to CAR, other lower affinity cellular receptors that are involved with adenovirus attachment include heparin sulfate proteoglycans (HSPGs), intergrins, CD46, CD80/86, sialic acid, major histocompatability complex class I (MHC class I) and vascular cell adhesion molecule 1 (VCAM-1) [21].

Adenoviruses bind to blood cellular components such as erythrocytes [24], macrophages [25] and neutrophils [26], as well as plasma coagulation factors [27]. These interactions may sequester adenoviruses and prevent access to extravascular targets. Interestingly, the hepatotropism of adenovirus serotype 5 has recently been shown to result from interaction of blood clotting Factor X (FX) with the hexon, not fiber, protein of the virus [28]. The exact mechanism by which this interaction causes hepatotropism has not been established. However, it is thought that FX forms a mesh over the virion capsid that sterically inhibits interaction of the fiber protein with other receptors.

The adenovirus serotype 5 genome is organized into regions that are transcribed early or late during the infection (**Figure 2A**) [29]. E1A and E1B are the first to be expressed. Their function is to interact with p53 and Rb to prevent cell cycle arrest, inhibit apoptosis and permit establishment of viral replication. The attenuation of viral replication that results from deletion of E1 has been exploited for the generation of replication-defective adenovirus vectors (discussed below). Replication-competent and conditionally replication-competent adenoviruses, which have direct toxic effects on cells, have been used for the treatment of cancer [30]. Although these vectors show promise for the treatment of various malignancies, they are generally not suitable for delivery of RNAi activators. An important consideration for using adenoviruses to deliver RNAi activators is that adenovirus VA RNAI and adenovirus VA RNAII, which are expressed during the late phase of replication, are capable of inhibiting RNAi [31,32]. Adenovirus VA RNAI and adenovirus VA RNAII transcripts are highly folded and contain hairpin motifs that resemble pri-miRs. They are produced in high amounts, act as substrates and competitive inhibitors of Dicer and are also capable of suppressing RISC function [31]. A further effect that impedes RNAi function is the inhibition of exportin-5-mediated export of shRNAs [32]. Of course, expression of VA RNAs in adenoviruses that deliver RNAi activators would be undesirable. However, as recombinant adenovirus vectors are deficient in E1, they are replication-defective and do not express late genes. Production of late adenovirus VA RNAI and adenovirus VA RNAII transcripts is therefore significantly attenuated and disruption of silencing by adenovirus-delivered RNAi expression cassettes should not occur. This is supported further by the demonstration that first-generation adenovirus vectors do not disrupt endogenous miR biogenesis [33].

First-generation adenoviruses have been in use for gene therapy application for several years. These vectors are deficient in the viral *E1* gene, and the function of this sequence is typically provided *in trans* by HEK293 packaging cells that stably produce *E1* (**Figure 2B**). To propagate RNAi-activating adenoviruses, expression cassettes may be accommodated at sites of adenovirus genome deletions. Limitations of first-generation vectors are that target cells are co-transduced with virus protein encoding sequences together with the transgenes. As a result, immunogenic expression of viral proteins occurs, which shortens the duration of transgene expression and predisposes it to toxicity. Second-generation adenoviruses also have the E2 and E4 sequence removed (**Figure 2C**), which partially diminishes immunostimulatory effects and provides extra transgene capacity. Helper-dependent (HD) or 'gutless' vectors have been widely used for the delivery of transgenes. These third-generation adenoviruses have all of the viral protein coding sequences stripped from the genome (**Figure 2D**). Only the packaging signal and inverted terminal repeat (ITR) elements at the ends of the genome are retained. Total gene deletion in adenovirus vectors dramatically reduces immune response stimulation, thereby enabling prolonged

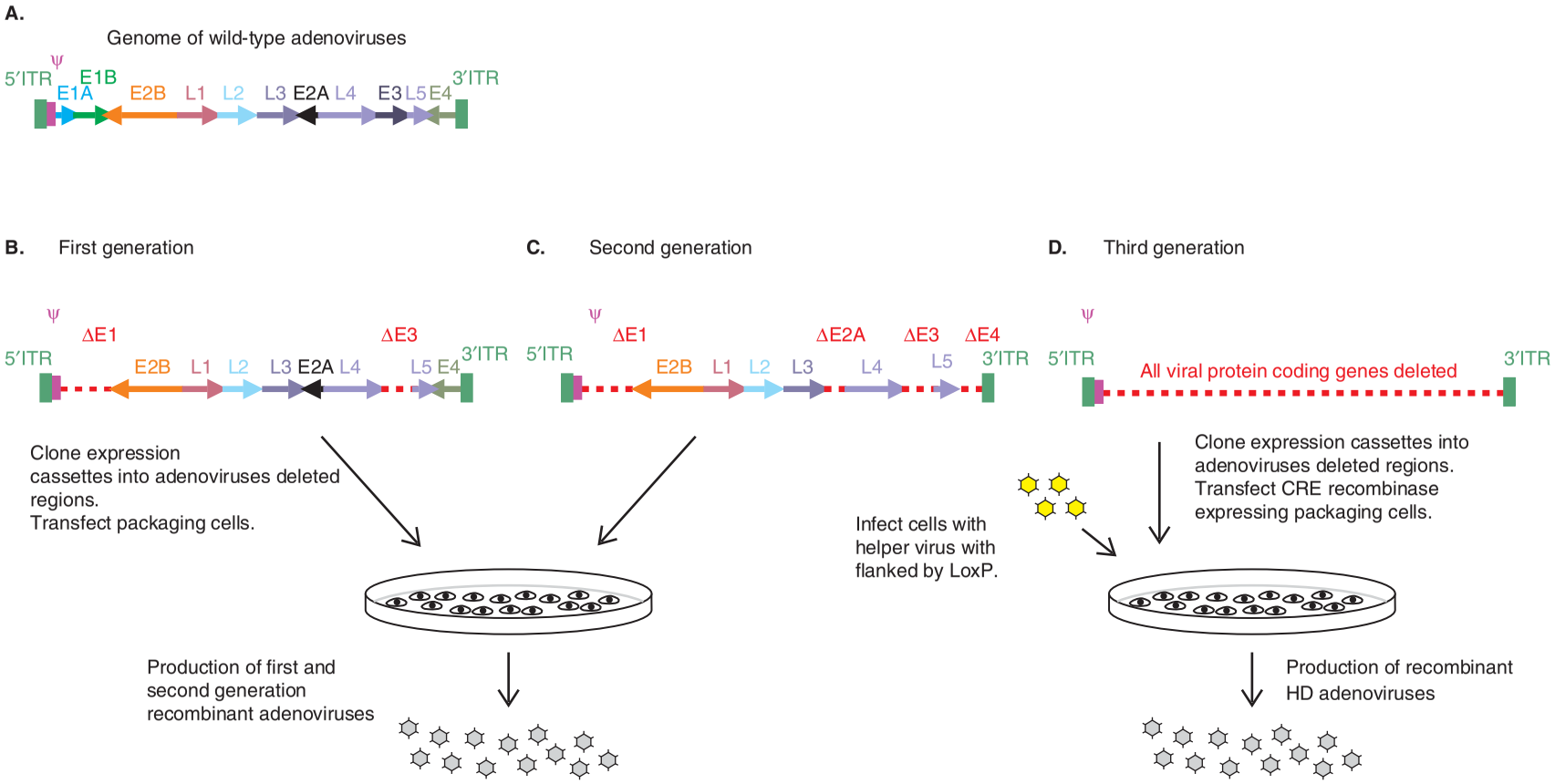

Figure 2. Schematic outline of production of first-, second- and third-generation recombinant adenovirus vectors. A. Linear genome of wild-type adenovirus. **B.** First-generation vectors have E1 and/or E3 deleted. **C.** Second-generation vectors have E1, E2, E4 and/or E3 deleted. Both these categories of recombinant viruses can be produced by introducing recombinant adenovirus DNA containing RNAi expression cassettes into cell lines expressing the missing essential genes. **D.** Third-generation or HD adenoviruses have all the viral sequences deleted, except for the left and right inverted terminal repeats (5′ITR and 3′ITR) and the packaging elements (ψ). To produce HD adenoviruses, a helper virus with its packaging signal flanked by recombinase targets, for example lox P sites, is typically used to complement for deleted adenoviral genes in recombinase-expressing packaging cells. RNAi expression cassettes may be incorporated at sites of Ad gene deletion (dashed lines).

HD: Helper-dependent; RNAi: RNA interference.

transgene expression with reduced vector toxicity [34,35]. To propagate HD adenoviruses, complementing helper viruses (HVs) produce all of the protein components that are required for the formation of the viral particles. Ingenious methods, particularly the incorporation of LoxP [36] and FRT [37] recombinase recognition sites to flank the packaging signal in HVs, have been devised to remove the contaminating complementing virus. Derivatives of the HEK293 cells that stably express CRE or FLPe recombinases remove the packaging signal from the helper virus, and HD adenovirus DNA is preferentially packaged in the recombinant vector. To overcome problems of HV contamination that may result from homologous recombination between the ψ sequences of helper virus and HD adenovirus, Palmer and Ng reversed the orientation of ψ in the helper virus [38]. This approach successfully reduced HV contamination to 0.01 – 0.1%. Availability of packaging cells that are capable of growing in suspension has also facilitated large-scale production of adenoviruses. Nevertheless, generating adenoviruses in sufficient amounts and purity for clinical application remains difficult and costly. However, protocols are continuously being refined to improve current HD adenovirus preparation methods [39].

3.1 Immunostimulation by adenoviruses

After intravenous administration, adenoviruses are readily taken up by antigen-presenting cells (APCs). Their activation of the innate immune response is mediated by mitogen-activated protein kinases (MAPK). Release of cytokines, such as TNF-α, IL-6 and IL-12, causes local inflammation and toxicity [40-43]. Resulting damage to healthy tissues with decreased transgene expression is thus a significant concern for therapeutic use of recombinant adenoviruses. Importantly, this rapidly occurring effect is not dependent on the expression of viral genes, and unmodified HD adenoviruses also cause stimulation of the innate immune response [40]. Nevertheless, this immunostimulatory effect of HD adenoviruses is more transient than that caused by first-generation unmodified adenoviruses [44,45]. APCs also initiate an adaptive immune response by MHC class I presentation of processed viral antigens to $CD8^+$ T cells. This triggers $CD8^+$ T cells' differentiation into cytotoxic T lymphocytes (CTLs) and adenovirus-specific cytotoxic immunoclearance of transduced cells. In addition, presentation of viral antigens by MHC class II activates $CD4^+$ T cells. This leads to cytokine-mediated destruction of the adenovirus-infected cells as well as B-cell differentiation to induce a humoral immune response. Anti-adenovirus immunity, induced by community-acquired infection, is induced by similar mechanisms and may counter efficiency of adenovirus transgene delivery.

Although essential aspects of the mechanism of adenovirus immunostimulation are generic, vector-induced immune responses are complex, variable and influenced by several factors [46]. These include adenovirus dose, route of administration and tissues that are targeted. Overcoming problems of immunostimulation may be achieved more easily in situations where local and low dose adenovirus administration is adequate for therapeutic benefit. Preventing immunostimulation in situations that require systemic administration of high doses of adenoviruses is more complicated. In such cases, pre-administration of immunosuppressive drugs, such as steroids, has been found to be useful [47]. A major focus of adenovirus vectorology has therefore been on the development of methods to diminish induction of immunostimulation and modification of vector tropism.

3.2 Adenovirus tissue tropism

Targeting RNAi activators to specific tissues is essential to limit unintended effects of adenoviruses. Benefits of specific target tissue tropism include: i) minimizing the required dose of adenoviruses; ii) avoiding cells that mediate immunostimulation; and iii) escape from pre-existing adenovirus immunity [46]. A major focus of adenovirus vector development has therefore been to improve specificity of transduction of intended target cells. Adenovirus tropism is complex and varied. As new receptors are identified and new serotype-specific cellular transduction processes are elucidated, so the repertoire of adenoviruses will increase. This should enable more specific vector targeting and also allow for serotype switching to avoid vectors' interaction with neutralizing antibodies [48].

Much of the knowledge on adenovirus transduction, particularly the binding of CAR by adenovirus serotype 5, comes from studies carried out on cells in culture. Extrapolation to use *in vivo* has not been easy as adenoviruses display a strong liver tropism when injected systemically, despite low expression of CAR in hepatocytes [49]. It was only recently that the central role of FX in liver targeting by adenoviruses was identified (discussed earlier) [28]. Direct binding of FX to the adenovirus serotype 5 hexon is required for liver transduction. This is supported by the reduction in adenovirus serotype 5 liver transduction following inhibition of FX interaction with hexon [28,50], which is not observed after CAR disruption [51].

So far three methods have been used to alter vector tropism [46]: i) genetic modification of the capsid; ii) chemical conjugation of the vector particles with polymers such as polyethylene glycol (PEG); and iii) introduction of a two-component modification that comprises a fiber knob binding domain at one end and a retargeting moiety at the other. Changing sequences of receptor-interacting capsid proteins, particularly the fiber knob domain, have been used successfully to modify vector tropism [52]. However, this approach may be complicated by defective fiber trimerization. PEGylation of adenoviruses is a convenient approach that provides a hydrophilic coat to adenoviruses, which decreases the proteolytic degradation and shields vector epitopes [53]. PEGylated adenoviruses are less immunogenic and their blood clearance rate is fourfold lower than unPEGylated adenoviruses. The increased circulation time in blood also facilitates transduction of non-hepatic tissue [54]. Liver tropism can

also be altered by using PEG of different molecular masses. Adenovirus serotype 5 conjugated to PEG_{5000} (PEG with molecular mass of 5000 Da) maintains normal liver transduction, but PEG_{20000} and PEG_{35000} reduce hepatocyte transduction [55]. Attaching targeting ligands to the end of the PEG chain has also enabled tissue-specific targeting. For example, integrin-binding RGD (Arg-Gly-Asp) peptides attached to a PEG chain (RGD-PEG-adenovirus) demonstrated a 200-fold increased transduction in both CAR^+ and CAR^- negative cells [56].

4. HD adenovirus vectors are attractive for achieving sustained RNAi-based therapeutic gene silencing

As indicated earlier, HD adenoviruses have the dual advantages of a high capacity (~ 36 kb) for accommodating transgene elements and a lack of sequences encoding viral proteins. Although typical RNAi expression sequences are small, inclusion of extra cassettes in HD adenoviruses may be useful to make vectors more functional. The feasibility of such an approach was demonstrated in a study that generated an adenovirus vector that encoded both transforming growth factor-β3 and a shRNA targeted to type I collagen [57]. This particular application to treating articular cartilage degeneration facilitated both chondrocyte growth stimulation and inhibition of type I collagen production. The resulting formation of cartilage that contains type II collagen and proteoglycans has greater mechanical strength. Extension of this method to incorporate immunomodulatory transgenic elements may be useful to modulate toxicity of HD adenovirus vectors. Proof of principle of such an approach was provided by a study showing that silencing the Cys-X3-Cys chemokine ligand could be used to prevent acute liver injury caused by adenoviruses [58]. In another impressive study showing the utility of HD adenoviruses for delivery of RNAi expression cassettes, shRNAs targeting the insulin-responsive *SREBP-1* gene resulted in sustained 90% *SREBP-1* knockdown [59]. Therapeutic benefit in a type 2 diabetes mouse model was manifested as reduced body weight gain, which was observable at week 3 after HD adenovirus administration. Similarly, a recent study explored using HD adenoviruses to express shRNA against mutated huntington protein (htt)-encoding mRNA as a possible Huntington's disease therapeutic strategy [60]. This study reported 90% reduction in formation of htt aggregates at 4 weeks after vector administration. These silencing effects of HD adenoviruses are typically more sustained than what can be achieved with first-generation adenoviruses [61-63]. Interestingly, unlike the disruption of the endogeneous RNAi pathway and liver or neuronal degeneration caused by shRNAs overexpressed from an AAV [13,15], high levels of shRNA from HD adenoviruses did not appear to affect the exportin-5 pathway or to induce liver toxicity in mice [16]. Together, these observations showing prolonged transgene expression and good safety support the notion that HD adenovirus vectors are useful for RNAi-based therapeutic application.

5. Viral infections as targets for adenovirus vector-based RNAi therapy

The natural targeting of hepatocytes by adenoviruses following systemic administration *in vivo* makes them well suited to delivery of RNAi expression cassettes that target liver-specific virus infections. Globally, chronic infections with either HCV or HBV are the chief causes of hepatocellular carcinoma (HCC) and cirrhosis [1,64]. Despite the similarities in the sequelae of chronic infection by these viruses, they differ in genetic structure and life cycles, which necessitates different approaches to developing RNAi therapy.

Several studies have been carried out aimed at utilizing RNAi to treat HBV infection (reviewed in [65,66]). Approximately 6% of the world's population is chronically infected with HBV. Drugs available at present have limited efficacy, and carriers of the virus are at risk of HCC. Despite implementation of vaccination programs in parts of the world where HBV infection is endemic, persistent HBV infection is likely to be a significant global problem for many years, and improved treatment of the infection remains a priority. The virus has a compact genome that comprises partly double-stranded relaxed circular DNA (rcDNA), which is converted to covalently closed circular DNA (cccDNA) in infected hepatocytes. This cccDNA then serves as a template for transcription of viral RNAs from which *core*, *surface*, *polymerase* and *X* overlapping open reading frames (ORFs) are translated.

Several different target sites within all four HBV transcripts have been found to be suitable for RNAi-mediated inhibition of HBV replication [67-73]. As there is no convenient small animal model of HBV infection, use has been made of HBV transgenic mice to simulate the clinical condition. These animals have HBV DNA stably integrated into their genomes, and constitutively produce the virus in a manner that is similar to the HBV carrier state in humans. Initial studies demonstrated that recombinant adenoviruses carrying U6 promoter-driven anti-HBV shRNA cassettes efficiently inhibited HBV replication in transgenic mice [61,74]. Comparison with the efficacy that was achieved after injection of similar mice with siRNA-containing lipoplexes [75] reveals that inhibition of HBV replication was considerably better with recombinant adenoviruses. To address concerns about the immunostimulatory effects, polyethylene glycol modification of anti-HBV shRNA-expressing adenoviruses was tested [62]. Successful attenuation of mouse immune responses to the vector, a better safety profile and improved anti-HBV effects after repeat adenovirus administration were demonstrated. Although adenoviruses encode VA RNAI and RNAII, which act as RSSs [31], these sequences do not appear to compromise silencing efficacy of adenovirus-delivered RNAi activators [33]. This is likely to be a result of VA RNA production during late

adenovirus infection, which does not occur after replication-defective adenovirus vector infection of cells. Assessment of anti-HBV HD adenoviruses has been carried out in one study reported so far [76]. Modest silencing was observed, and is likely to have resulted from poor antiviral efficacy of the RNAi expression cassette used in this study. Nevertheless, proof of principle of the utility of HD adenoviruses for the treatment of HBV infection was demonstrated in an immunotherapy-based approach. A mifepristone-inducible expression cassette was used to produce IFN-α and IL-12 in murine and woodchuck models of HBV infection. In these studies, prolonged and specific intrahepatic expression of transgenes with sustained antiviral effects was observed [34,77]. Most importantly, when compared with first-generation adenoviruses carrying the same IFN-α cassette, HD adenoviruses resulted in superior liver damage protection in acute infection [77].

The liver tropism of HCV should make infection with this virus amenable to RNAi-based therapy using adenoviruses. As with HBV, HCV infection is a significant cause of global health problems. Approximately 170 million people are chronically infected with HCV [78]. The virus is typically transmitted percutaneously and infection persists in 60 – 80% of cases, which places individuals at high risk of cirrhosis and HCC. Efficacy of licensed HCV therapies, which include PEG IFN-α and ribovirin, is variable and ranges from 45 to 80% [79].

HCV is a member of the Flaviviridae family and has a single sense strand uncapped RNA genome of 9.6 kb [80]. An internal ribosomal entry site (IRES) located within the 5′ non-translated region (5′NTR) is responsible for initiating translation of the one HCV ORF. The large precursor polyprotein is cleaved by host cellular and viral proteases to form individual viral proteins. The entirely cytoplasmic life cycle of this RNA virus makes it a good candidate for RNAi-based treatment. A difficulty with the development of RNAi-based anti-HCV treatment has been the paucity of suitable animal models of the infection. Subgenomic replicons have been used commonly to study efficacy of antiviral therapeutic agents in cell culture, and assessment of antiviral efficacy with HCV-infected cells in culture has not been possible until recently. Available models of HCV infection *in vivo* are limited to chimpanzees [81] and chimeric immunodeficient mice that are grafted with human hepatocytes [82]. The complex nature of these models makes convenient comprehensive long-term studies difficult to undertake. As a result of its important function as an IRES, evolution of this 5′NTR sequence is constrained [80], and despite being highly structured, the IRES is a favored target of RNAi activators [83-87]. As with HIV-1, HCV has a plastic genome and overcoming HCV escape by using combinatorial RNAi approaches has been an active area of research [88,89]. Silencing of host dependency factors [90,91], including endogenous miR-122 [92], may also be useful to counter the emergence of resistant HCV strains. So far, there have been few if any studies that have utilized adenoviruses to deliver HCV silencing sequences. Given the dearth of available models to test anti-HCV efficacy *in vivo*, the slow progress in developing anti-HCV adenoviruses is perhaps not surprising. Nevertheless, lessons learned from studying HBV silencing will no doubt be useful and directly applicable to advancing anti-HCV RNAi-based therapy.

6. Conclusion

Adenoviruses have been used widely in the development of gene therapy and according to current information they are the most commonly used vector in gene therapy trials. Applications have been varied and include use for treatment of cancer and infectious diseases. As expected, adenoviruses are well suited to incorporation of RNAi-activating expression cassettes. Useful properties of adenoviruses that have contributed to the popularity for gene therapy include the following.

- The molecular biology of adenoviruses is understood well, and this has facilitated engineering of vectors to confer specific intended biological properties.
- Adenoviruses are capable of infecting both dividing and non-dividing cells *in vivo*.
- Lack of interference by viral transcriptional regulatory sequences enables rapid unimpeded transgene expression.
- Methods of propagating the vectors are continually being improved.
- The vectors are typically stable and transduced DNA exists episomally, with minimal risk of insertional mutagenesis.
- HD adenoviruses in particular have a high capacity for incorporation of large transgene sequences or multiple cassettes, and are capable of prolonged transgene expression with reduced toxicity at high vector doses.

Successful propagation of HD adenoviruses was a significant step in the improvement of safety and efficacy of this class of viral vector. Lack of expression of viral proteins means that vector antigens are not presented by MHC class I/II antigens and more sustained transgene expression can be achieved. Concomitant modification of HD adenoviruses with polymers, such as PEG, may be used conveniently to attenuate innate immunostimulation. The very large capacity of HD adenoviruses allows for incorporation of up to 36 kb into these vectors. Although this may not be particularly important for propagation of vectors that contain small RNAi expression cassettes, the extra capacity allows for incorporation of more expression cassettes that could be used to modify host immune responses to vectors.

Hepatotropism of adenovirus serotype 5-derived vectors is very useful for treatment of viral infections that occur primarily in the liver. However, transduction of liver tissue is a disadvantage for the treatment of extrahepatic diseases, and creative methods continue to be developed to redirect the

tropism of adenoviruses. Despite these positive attributes, the potential of adenoviruses for gene silencing applications has not been explored fully, and the reason is their powerful stimulation of host innate and adaptive immune responses. The toxic effects that result may be serious, and the cautious approach to using adenoviruses for systemic administration is justified. Nevertheless, recent developments, particularly in the field of modification of viral capsid proteins to evade cells of the immune system and efficient means of propagating HD adenoviruses, have provided the field with the technology for producing safer vectors that may be applied successfully in the future.

7. Expert opinion

Demonstration that adenovirus vectors are capable of highly efficient gene transfer to cells *in vivo* led logically to their use for delivery of expressed RNAi activators. Using adenoviruses for delivery of RNAi effectors has benefited from the wealth of research on the topic of general applicability of adenoviruses for gene therapy. There are many positive features of adenoviruses, summarized above, which make them appealing for delivery of expressed RNAi activators. For certain applications the highly efficient delivery and expression of transgenes is not surpassed by other vectors. However, harnessing of this very important feature for clinical application has been complicated. Enthusiasm for the use of adenoviruses has been reduced by concerns about toxicity that may result from their immunostimulatory effects, as well as costliness of scalable synthesis of these vectors. Consequently, use of non-viral vectors for delivery of synthetic siRNAs and AAVs or lentiviral vectors to deliver RNAi expression cassettes has been favored by researchers. Nevertheless, recent advances in modifying adenoviruses have succeeded in moderating toxic effects, and it is appropriate that their utility for therapeutic transfer of expressed RNAi activators should be revisited.

In summary, clinical application of adenoviruses to RNAi therapy will depend on improvements that achieve the following.

- Conclusive demonstration that the innate and adaptive immune responses to adenoviruses can be sufficiently attenuated to avoid toxicity and achieve sustained gene silencing.
- Improvement in methods for convenient large-scale preparation of pure adenoviruses that are suitable for clinical use is essential. Current procedures are costly, time-consuming and difficult to implement on a scale that is large enough for human use.
- Development of methods to evade interaction with cells of the reticuloendothelial system and avoidance of pre-existing immunity to wild-type adenoviruses. Infection with adenoviruses is common in general populations, and immunity from community-acquired infections attenuates efficacy of the recombinant viral vectors.
- Comprehensive understanding of the clinical conditions that are best suited to adenovirus-mediated delivery of RNAi activators. It is clear that one vector will not be suitable for all applications within the field of RNAi therapy, and identification of those diseases that are best suited to adenovirus-mediated RNAi activator delivery is important.

The field of RNAi-based treatment of diseases has advanced at a rapid pace over the past 10 years. There is now substantial enthusiasm for the vast potential of this method of therapeutic silencing of gene expression. Limitations of the available delivery methods and lack of information on the long-term effects of RNAi activators' expression *in vivo* are the main obstacles to RNAi-based gene silencing realizing its full potential. It is likely that the considerable momentum that has been gained in the field of RNAi-based gene silencing will enable rapid progress to overcome current difficulties with harnessing RNAi for treatment of viral infections. Adenoviruses, and in particular availability of HD derivatives, have very useful features that should contribute significantly to progress in the exciting field of RNAi-based therapy. Overcoming the obstacles listed above, which is a reasonable expectation, should make adenovirus delivery of anti-HBV and anti-HCV RNAi activators a feasible future therapeutic option.

Declaration of interest

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