

# Construction and application of adenoviral vectors

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**Adenoviral vectors have been widely used as vaccine candidates or potential vaccine candidates against infectious diseases due to the convenience of genome manipulation, their ability to accommodate large exogenous gene fragments, easy access of obtaining high-titer of virus, and high efficiency of transduction. At the same time, adenoviral vectors have also been used extensively in clinical research for cancer gene therapy and treatment of diseases caused by a single gene defect. However, application of adenovirus also faces a series of challenges such as poor targeting, strong immune response against the vector itself, and they cannot be used repeatedly. It is believed that these problems will be solved gradually with further research and technological development in related fields. Here, we review the construction methods of adenoviral vectors, including “gut-less” adenovirus and discuss application of adenoviral vectors as prophylactic vaccines for infectious pathogens and their application prospects as therapeutic vaccines for cancer and other kinds of chronic infectious disease such as human papilloma-virus, hepatitis B virus, and hepatitis C virus.**

## INTRODUCTION

Adenovirus (Ads), which were first isolated in 1953,<sup>1</sup> are widespread and endemic in nature. Studies have found that Ads have over 100 serotypes and can be divided into two genera: mammalian adenoviruses and avian adenoviruses.<sup>2,3</sup> In this review, we focus on the human adenovirus (HAd).

HAd is a kind of double-stranded DNA virus without an envelope and susceptible to all age groups of the population, with a relatively high incidence in children, the elderly, and immunocompromised individuals, who are also at a higher risk of complications from infection.<sup>4–6</sup> HAd is transmitted mainly by droplet, fecal-oral, and through contact with objects contaminated with the virus. The spread of HAd is particularly rapid in crowded or enclosed areas.<sup>7–9</sup>

HAd is capable of causing symptomatic infections of the upper and lower respiratory tract, gastrointestinal tract, or eyes. Most HAd-infected individuals only exhibit mild symptoms of the common cold, conjunctivitis, and tonsillitis.<sup>10–14</sup> Currently, there are no specific drugs available for the treatment of HAd-infected patients, but the vast majority of infected individuals recover naturally; even severe Ad pneumonia does not cause death, except for rare outbreaks of primary pneumonia that occur in infants and immunocompromised populations. In particular, hematopoietic stem cell and solid organ transplant recipients, and people with congenital immunodeficiency

or human immunodeficiency virus (HIV) infection are at a higher risk of death from Ad infection than the general population.<sup>15–19</sup>

It was not until the 1980s that the therapeutic potential of Ad as a gene delivery vector was discovered.<sup>20</sup> Ad is the first viral gene delivery vector used in humans.<sup>21</sup> Among more than 90 serotypes of HAd, serotype 5 (HAd5) is the most widely used vector.<sup>22</sup> In the early 1990s, Ad vectors were used to express the alpha-1 antitrypsin (A1AT) gene in rat hepatocytes and lung tissue.<sup>23,24</sup> Subsequently, Ad vectors were rapidly used in clinical trials for the treatment of various monogenic diseases. Numerous clinical trials have confirmed the safety and efficacy of Ad vectors. Since Ad vectors were applied to gene therapy, they have been continuously modified to improve their accommodation capability, transduction efficiency, exogenous gene-expressing duration, and *in vivo* safety. A wide variety of Ad vectors are currently being used in clinical trials, which demonstrates the promising prospects of Ad as a vaccine vector and gene therapy tool.<sup>25–28</sup>

## CHARACTERISTICS OF AD VECTORS

Ad is a non-enveloped virus with a linear double-stranded DNA genome containing four fragments encoding early gene products (E1-E4) and five fragments encoding late gene products (L1-L5).<sup>29,30</sup> E1, E2, and E4 gene products can regulate transcription and translation of late-stage genes, which are essential for viral replication.<sup>31</sup> E3 gene products can disrupt the immune response by altering antigen presentation, inhibiting cytokine production, and cellular apoptosis, but are not necessary for viral replication. The L1-L5 proteins encoded by late genes are associated with Ad particle assembly.<sup>32–34</sup> Each end of the Ad genome is flanked by 100–150 base pair (bp) long inverted terminal repeats (ITRs), the nucleotide sequence immediately adjacent to the left ITR is the packaging signal ( $\Psi$ ) of Ad, a *cis*-acting element necessary for Ad replication (Figure 1A).<sup>35–37</sup>

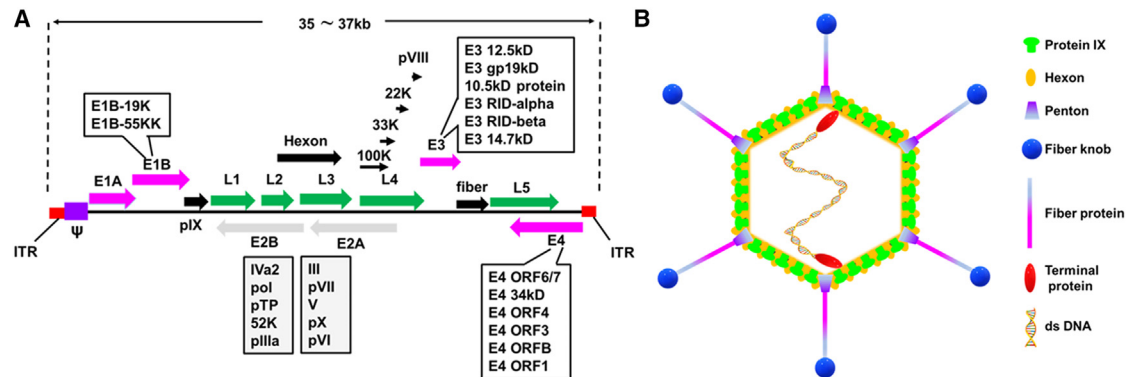
To utilize Ad vectors more safely and effectively, researchers have continuously modified Ad vectors through genetic engineering techniques. In the first-generation Ad vector with a deletion of the E1/E3 region in the Ad genome, the deleted region was used as an insertion site for exogenous genes and the capacity is approximately up to 4.5–8 kilobases (kb).<sup>38,39</sup> The construction method is simple and it is easy to obtain high titers of virus in helper cell lines.

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**Figure 1. Schematic of adenovirus genome and particle structure**

(A) The length of adenovirus genome is around 35–37 kb. Each end of the Ad genome is flanked by 100–150 bp long inverted terminal repeats (ITRs), the nucleotide sequence immediately adjacent to the left ITR is the packaging signal ( $\Psi$ ) of Ad. E1-E4 represent four fragments encoding early gene products on the adenovirus genome; L1-L5 represent five late genes on adenovirus genome. (B) The viral capsid of adenovirus has a diameter of 80–100 nm. The viral capsid has a typical icosahedral symmetry, consisting of 252 8–10 nm diameter capsid particles, including 240 hexons and 12 pentons.

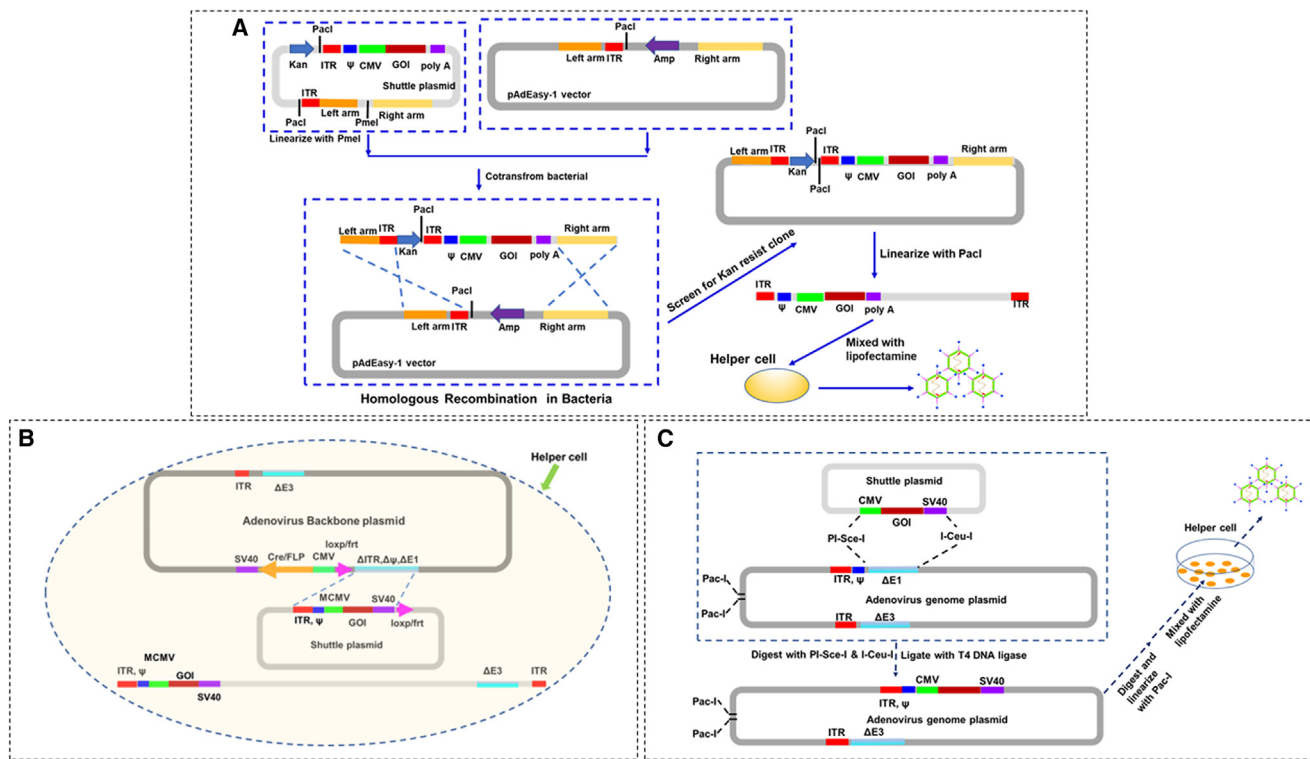
The second generation of Ad vectors, with a deletion of the E2/E4 regions in addition to deletion of the E1/E3 regions, which has increased the accommodation capacity of the vector to carry exogenous genes, improved transgenic capacity and reduced the likelihood of replication-competent adenovirus (RCA) generation.<sup>40–42</sup> However, titers of the second generation were significantly lower than those of the first generation, being only 1/1,000 to 1/10 of the latter. Meanwhile, although the host immune response against the vector was diminished, it still existed.<sup>43–46</sup>

The third generation of Ad vector removes all or most of the adenoviral genome, retaining only necessary *cis*-acting elements of ITR and packaging, hence it is also known as a gutless adenovirus vector.<sup>47</sup> Due to deletion of most of the adenoviral genome and absence of viral protein expression, the accommodation capacity for the exogenous gene has been increased substantially to 36 kb, while immune response against the vector has been reduced and safety has been significantly enhanced.<sup>48,49</sup> The absence of adenoviral protein expression *in vivo* means the protein of the vector itself will not be recognized by major histocompatibility complex class I molecules and presented to CD8<sup>+</sup> T cells,<sup>50</sup> thereby significantly reducing stimulation of the immune response and vector toxicity, resulting in prolonged expression of the transgene, which has been shown to last up to 10 years. However, construction of this kind of Ad vector is difficult, and the required DNA length must be supplemented with a stuffer sequence and a foreign transgenic sequence, and a helper virus and helper cell line are required during the packaging process. The subsequent purification process requires removal of helper virus contamination, which increases the difficulty and cost of purification and makes its clinical application difficult.

All three kinds of adenoviral vectors mentioned above are replication deficient and have their own advantages and disadvantages. Ad vec-

tors have the following significant advantages: (1) their genome can be easily edited and manipulated, and can be used as delivery vectors of the exogenous genes; (2) as the first expression system that can express multiple genes in the same cell line or tissue, their genome has high accommodation capacity, up to 8–36 kb exogenous gene fragments can be inserted, and multiple genes can be expressed simultaneously; (3) they can efficiently transduce different types of human and other mammalian cells, including some highly differentiated tissues, such as muscle, heart, lung, and brain tissue, which is not limited by whether the target cells are in the division period, and is an efficient gene transmission and expression vector; (4) they do not integrate into chromosomes in almost all known cells except oocytes, so there is no potential risk of mutagenesis by insertion into the genome; (5) the adenoviral vector itself has an adjuvant effect, inducing strong and effective humoral and cellular immunity; (6) Ads can proliferate in helper cells, making it easy to obtain high titer viruses, and can be industrialized and mass produced using suspension cell culture systems. Based on the numerous advantages mentioned above, adenoviruses have been widely used in various fields such as *in vitro* gene transduction, *in vivo* gene therapy, and vaccination. At present, first-generation adenoviral vectors are still the main ones used for gene therapy and vaccine vectors.

On the other hand, adenoviral vectors also have some limitations and shortcomings: (1) Ads can infect a variety of cells and tissues, which results in a lack of targeting of the gene transduction, (2) the immunogenicity of the adenoviral vector itself often induces a strong immune response against itself, which restricts repeated use of the vector, (3) an adenoviral vector needs to encode dozens of proteins for itself, which often results in low expression efficiency of the foreign gene, (4) due to the high affinity of the Ad capsid for receptors such as coagulation factors, adenoviral vectors rapidly accumulate in the liver after immunization and have a non-negligible impact on liver metabolism, resulting in liver damage.



**Figure 2. Packaging system for the first generation of adenoviral vectors**

(A) pAd-Easy adenovirus packaging system: homologous recombination to complete the insertion of an exogenous gene into the Ad genome in *Escherichia coli*, obtaining a circular recombinant Ad genome with the necessary components for replication in bacteria. The adenovirus packaging plasmid was linearized with PacI and then transfected into a helper cell line. (B) The pAd-Max<sup>TM</sup> packaging system: co-transfects 293 cells with a shuttle plasmid cloned with an exogenous gene and a packaging plasmid carrying the majority of the Ad genome, and uses the Cre/loxP-based system to achieve recombination and generate the recombinant adenovirus. (C) Direct-cloning strategy to construct the adenovirus vector: clones the adenoviral genome directly into a plasmid vector and then cuts the exogenous gene from the shuttle vector and inserts it into the adenovirus backbone vector. The final plasmid is linearized with PacI and transfected into the helper cell line, which can rescue the recombinant Ad after transfection into the packaging cell line.

## METHODS FOR CONSTRUCTION OF RECOMBINANT ADS

The viral capsid of Ads has a diameter of 80–100 nm and a relative molecular mass of about 175,000 Da. The viral capsid has a typical icosahedral symmetry, consisting of 252 8–10 nm diameter capsid particles, including 240 hexons and 12 pentons (Figure 1B).<sup>51</sup> The epitope on the hexon determines the serotype and can stimulate production of neutralizing antibodies (NAs) against Ad vectors in the host.<sup>52</sup> Exogenous epitopes can be inserted into hexons, penton bases, fiber proteins, and peptide IX without affecting stability and function of viral structures, and can induce specific immune responses against the inserted epitope.<sup>53,54</sup>

With development of molecular biology and genetic engineering techniques, the packaging technology for recombinant Ads has constantly been improved. In the original recombinant Ad packaging method, the exogenous gene is cloned between two homologous arms on the shuttle vector and co-transfected with the Ad genome into human embryonic kidney 293 (HEK293) cells or other cells supplied with E1 in *trans*.<sup>55</sup> In helper cell lines, the recombination that occurs

between the upper homologous arm of the shuttle vector and the nucleotide homologous region on the Ad genome replaces the E1 region with a foreign gene. However, the incidence of recombination is relatively low with this approach; obtaining recombinant Ads using this method is laborious, time consuming, and requires multiple rounds of plaque purification.

In the second method, homologous recombination occurs in *Escherichia coli*. The commonly used pAdEasy Ad packaging system uses homologous recombination to complete insertion of an exogenous gene into the Ad genome in *E. coli*, obtaining a circular recombinant Ad genome with the necessary components for replication in bacteria (Figure 2A).<sup>56</sup> This method is efficient but more complex and requires three steps of transformation involving the use of two different competent strains of *E. coli*, including specific *E. coli* that express RecA recombinase.<sup>57</sup> In addition, because recombination occurs in bacteria, small genomic changes are not easily detected; therefore, to obtain high-quality recombinant Ad strain (high viral yield, high target gene expression), multiple recombinant Ad plasmids packaging DNA should be selected and

transfected separately in 293 cells to rescue multiple recombinant Ad viruses for screening.

The third method was developed by Ng and co-workers and is commercially available as an AdMax packaging system. This method co-transfects 293 cells with a shuttle plasmid cloned with an exogenous gene and a packaging plasmid carrying the majority of the Ad genome, and uses the Cre/*loxP*-based system to achieve recombination and generate the recombinant Ad (Figure 2B). Frequency of Ad vector rescue by Cre-mediated site-specific recombination is about 30-fold higher than traditional homologous recombination.<sup>58,59</sup> This process takes place in 293 cells, thus avoiding recombination in bacteria and greatly simplifies the process of molecular cloning during the construction of recombinant Ad vector. In this system, stronger mCMV promoters are often used to obtain higher expression levels of the exogenous gene.

The fourth approach is to clone the Ad genome directly into a plasmid vector and then insert the exogenous gene into the vector, which can rescue the recombinant Ad after transfection into the packaging cell line (Figure 2C). This approach is technically demanding for molecular cloning, as the Ad genome is large (~36 kb) and there are few restriction sites available, which makes it very difficult to clone the whole Ad genome into a plasmid vector.<sup>60</sup> However, the great advantage of this approach is that it allows for the direct cloning and assembly of the adenoviral genome, completely eliminating potential risks in the original Ad genome and avoiding the risk of uncertainty that may exist in the homologous recombination process, which can affect the clinical application of Ad vectors.

What should be particularly introduced is the packaging method for third-generation Ad vectors. Third-generation Ad vectors, also known as gutless Ad vectors, remove all viral sequences except the ITR and packaging signals. These vectors, also known as "high-capacity" adenoviral vectors (HCAds), can accommodate approximately 36 kb of exogenous genes.<sup>49</sup> Parks et al. developed a helper adenovirus-dependent packaging system for production of gutless Ads. In the genome of the helper virus, the packaging signal is flanked by *loxP* sites; so, in 293 cells stably expressing Cre recombinase (293Cre), the packaging signal is effectively excised, preventing the helper virus genome from being packaged into the viral particle.<sup>44</sup> However, the genomic DNA of the helper virus replicates in a normal manner and can therefore be expressed in *trans* to provide elements required for gutless Ad replication and packaging (Figure 3A). The gutless Ad can be separated from the residual helper virus fraction by cesium chloride buoyant density centrifugation. Cheshenko et al. developed an alternative system for gutless Ad production using a baculovirus gene transduction system, in which genes essential for Ad vector replication and packaging are delivered to production cells via a baculovirus delivery vector.<sup>61</sup> They constructed a hybrid baculovirus, Bac-B4, carrying the packaging signal for the Ad genome that can be cleaved and excised by Cre recombinase. The gutless Ad obtained using this method is completely free of helper Ad and is capable of transducing cultured 293 cells (Figure 3B). This gutless Ad packaging

method based on baculovirus delivery systems is also a very useful alternative method. Lee and co-workers reported the production of gutless Ad with a helper plasmid in the absence of helper Ad.<sup>62,63</sup> With the helper plasmid, they successfully produced large amounts of recombinant gutless Ad. Importantly, the helper plasmid-based system produced only gutless Ads, but not Ads and RCA derived from the helper plasmid (Figure 3C). The generated gutless Ad was effective in delivering the transgene and showed therapeutic potential for Huntington's chorea (HD) and Duchenne muscular dystrophy. Their data suggest the helper plasmid-based gutless Ad-producing system may become a new platform for gene therapy.

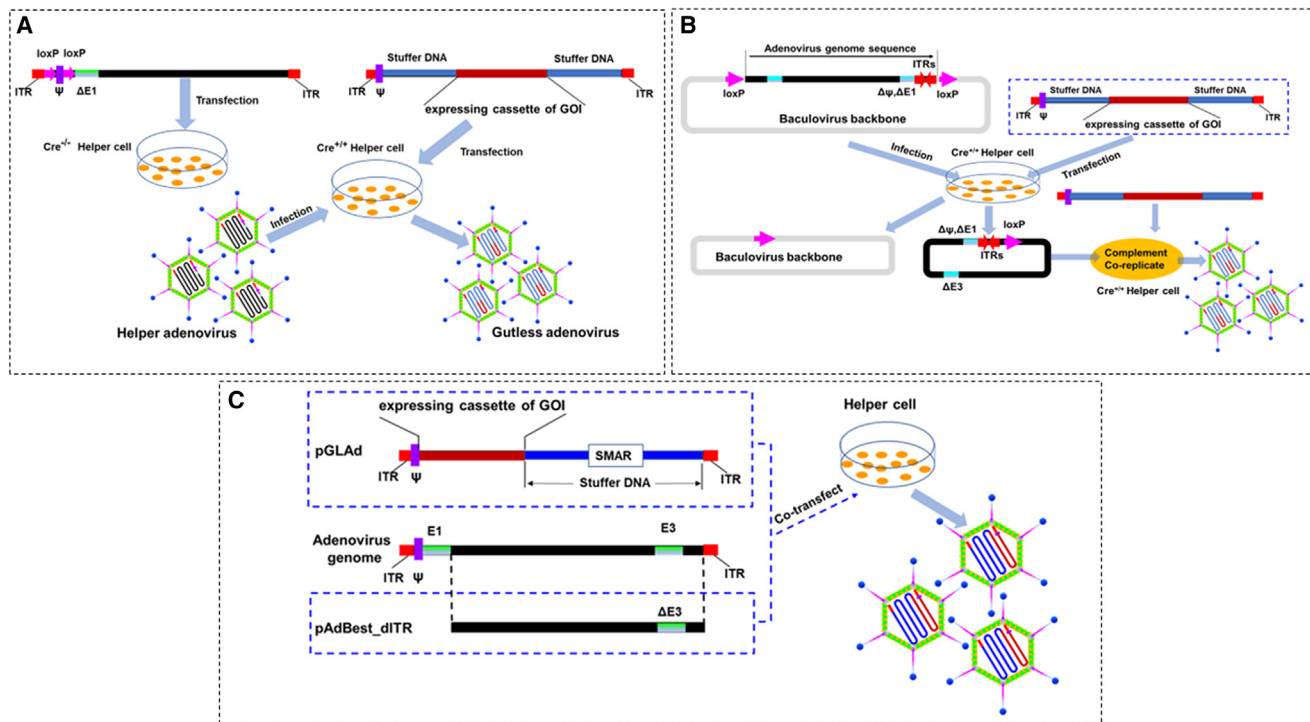
#### DISTRIBUTION OF RECEPTORS AND CONSTRUCTION OF NOVEL ADS FOR RECEPTOR NEGATIVE CELLS

Receptors on the host cell surface mediate initial contact between the virus and the cell, which in turn triggers a signaling pathway for the virus to enter the cell. Ads can transduce cells efficiently because their receptors are widely distributed on various cell types and there is a very high affinity between Ads and their receptors. In fact, Ads enter the cell using a variety of co-receptors and attachment factors of the host cell. Co-receptors and adhesion factors help Ads adhere to cells and facilitate their entry and replication.<sup>64–66</sup> Other kinds of host factors, such as intracytoplasmic Ca<sup>2+</sup> ion channel proteins and intranuclear somatic maturation factors or intracellular transport factors, such as actin and microtubule filaments, can also enhance infection (e.g., by increasing receptor expression levels on the surface of the host cell), although these kinds of factors do not directly bind to viral particles.

Coxsackievirus belongs to the enterovirus family and is made up of a single strand of ribonucleic acid (RNA). Ads belong to adenoviridae, which is a kind of double-stranded DNA virus. However, Ads and coxsackieviruses share the same receptor, the coxsackie-adenovirus receptor (CAR), to enter cells. CAR is a kind of transmembrane glycoprotein that belongs to the immunoglobulin superfamily. A variety of Ads, including HAd5, recognize CAR that is expressed on the surface of many types of cells, including hepatocytes, epithelial cells, and cardiomyocytes as its receptors.<sup>67</sup> Published results showed that, on the cellular membrane of some malignant tumor cells, expression of CAR was downregulated to varying degrees. In addition to this, the higher the degree of malignancy of the tumor, the lower the expression of CAR.

CAR exists in the tight junctions and adherent junctions of polarized epithelial cells. It interacts with the fiber knob domain of all HAds except for those in subgenus B, and mediates virus attachment.<sup>68</sup> Some chimpanzee adenoviruses (ChAds) also can bind to CAR to initiate the cell entry process. Ads of subgenus B mainly use CD46 and desmoglein 2 as receptors, and can also bind to CD80 or CD86, some immune molecules expressed on the surface of dendritic cells and mature B cells.<sup>69–71</sup> Subgenus C can also use acetyl heparin sulfate, major histocompatibility complex class I $\alpha$ 2, etc., as adhesion receptors.<sup>72–75</sup> Serotypes 8, 19, 26, and 37 human adenoviruses belong to subgenus D and recognize sialic acid receptors.<sup>76–79</sup> Integrins are





**Figure 3. Gutless packaging system**

(A) Helper adenovirus-dependent gutless packaging system: in the genome of the helper virus, the packaging signal is flanked by *loxP* sites, so that in 293 cells stably expressing Cre recombinase (293Cre) the packaging signal is effectively excised, preventing the helper virus genome from being packaged into the viral particle. The genomic DNA of the helper virus replicates in a normal manner and can therefore be expressed *in trans* to provide the elements required for gutless Ad replication and packaging. (B) Helper baculovirus-dependent gutless packaging system: a hybrid baculovirus, Bac-B4, carrying the packaging signal for the Ad genome can be cleaved and excised by Cre recombinase. The gutless Ad obtained using this method is completely free of helper Ad and is capable of transducing cultured 293 cell. (C) Helper plasmid-based system produced gutless adenovirus: the adenoviral proteins that are essential for packaging and replication are provided *in trans* by the helper plasmid, gutless adenovirus, but not wild-type adenovirus and replication-competent adenovirus (RCA) derived from the helper plasmid.

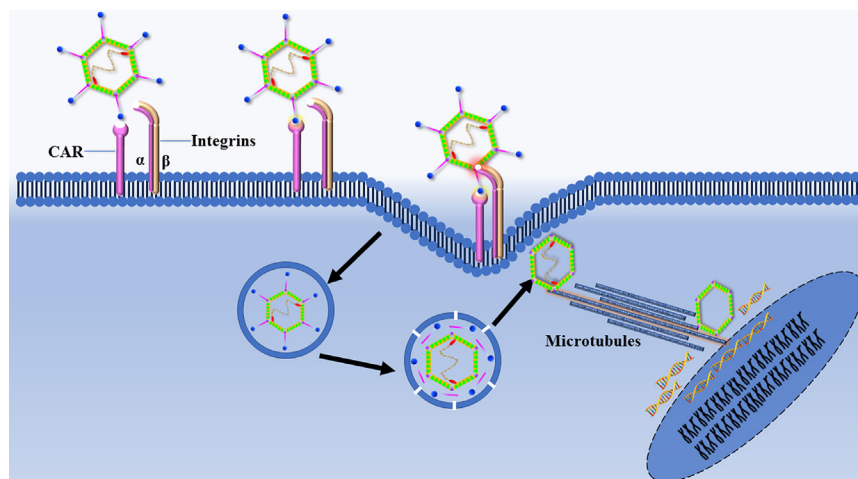
secondary receptors on the surface of HAdV-invading cells, such as integrins  $\alpha v\beta 3$  and  $\alpha v\beta 5$ , which bind to the RGD motif of the penton and mediate internalization of the Ads.<sup>80–84</sup>

Next, take HAd5 as an example to review the process of Ads entering cells. At the initial stage of infection, the fiber protein on the surface of the Ad virion binds to the CAR and then, through the endocytosis of clathrin, the virion is internalized into the cytoplasm, and there endosomal acidification causes conformational changes of the capsid protein.<sup>85</sup> After conformational changes, Ad virions travel through microtubules to the nuclear pore complex, where the viral genome is released into the nucleus to initiate transcription and translation (Figure 4).

Unlike HAd5, which utilizes the CAR as the main receptor, the fiber knob proteins of several other serotypes of Ads (including HAd3, HAd11, HAd26, HAd35, HAd37, and HAd52) can bind to CD46, desmoglein 2, GD1aglycan, and/or polysialic acid receptors on the cell surface for cell entry (Figure 5).<sup>76,86–92</sup> Based on this feature of Ads, the fiber knob coding genes from Ads utilizing different receptors can be used to replace the corresponding genes in HAd5, constructing a novel chimeric Ad vector that can efficiently deliver genes

to CAR-negative target cells. For example, the HAd5/3 chimeric Ad was constructed by replacing the fiber knob domain of the HAd5 with the corresponding domain of the HAd3.<sup>93,94</sup> Since the receptor of HAd3 has a higher expression on the surface of tumor cells, HAd5/3 chimeric vectors can enter the tumor cells through the Ad3 receptor.<sup>94,95</sup> DNX-2401 is a selectively replicated chimeric adenoviral vector. The ACDCRGDCFCG peptide was inserted into the fiber protein of the Ad, which causes the modified fiber protein to be able to bind to integrin  $\alpha v\beta 3$  and  $\alpha v\beta 5$  and mediate Ad invasion into cells without the need for CAR receptors.<sup>96</sup> The vector has been clinically tested in patients with recurrent malignant glioma. The RGDK amino acid sequence in the fiber protein of Ad vector VCN-01 was replaced by the heparin sulfate glycosaminoglycan binding site (KKTK), which causes the chimeric Ad to enter the cell through the glycosaminoglycan receptor on the cell surface.<sup>97,98</sup>

Transduction efficiency of HAd5 mainly depends on the expression level of CAR on the surface of target cells. However, in some cell types, such as primary tumor cells, stem cells, dendritic cells, etc., expression level of CAR on the surface is low.<sup>99–101</sup> Therefore, the transduction efficiency of HAd5 on such cells is low, thus limiting its application.



**Figure 4. Cell attachment and entry of human serotype 5 adenovirus**

The fiber protein on the surface of the Ad virion binds to the CAR, and then, through the endocytosis of clathrin, the virion is internalized into the cytoplasm, and there endosomal acidification causes conformational changes of the capsid protein. After conformational changes, adenovirus virions travel through microtubules to the nuclear pore complex, where the viral genome is released into the nucleus to initiate transcription and translation.

HAd35 recognizes CD46, which is highly expressed on human CD3+ T cells as its receptor. Replacing the fiber gene of HAd5 with that of HAd35 to generate a chimeric Ad vector, Ad5/F35, enhances the transduction efficiency of the Ad to T cells, resulting in broader application.<sup>102</sup> However, modification of the fiber protein cannot completely avoid the immune response against the hexon and penton. In fact, in addition to modification of the fiber and fiber knob, other proteins on the surface of the Ad virion such as hexon and PIX can also be modified to construct chimeric Ads (Figure 6).

It should be pointed out that the role of receptors in cell entry of Ads remains unclear.<sup>64,66,103</sup> For example, skeletal muscle cells are the main target cells for the adenoviral vector-based vaccines when inoculated using the intramuscular pathway. However, the expression level of CAR on the skeletal muscle is significantly lower than in other tissues.<sup>104,105</sup> Published results showed that expression levels of receptors in target cells had no significant impact on the immune effect of the vaccine. CD46, another important receptor for HAd35, is only expressed in the testes of wild-type mice, but in CD46 transgenic mice this protein can be widely expressed in various tissues throughout the body. Nevertheless, immunizing wild-type mice and CD46 transgenic mice with Ad35-based vaccine intramuscularly caused no significant difference in the expression level of transgene or the protective effect of the vaccine between the two groups.<sup>106,107</sup> In addition, CAR is mainly expressed on the basal lateral surface and the Ad has difficulty accessing CAR by intranasal or intratracheal immunization.<sup>108–110</sup> In fact, adenoviral vector-based vaccines administered by nasal spray has achieved very good results.<sup>111–114</sup> To further improve gene delivery efficiency and extend application of adenoviral vectors, it is of great significance to study the underlying mechanism of receptors in the process of adenovirus entry into cells.

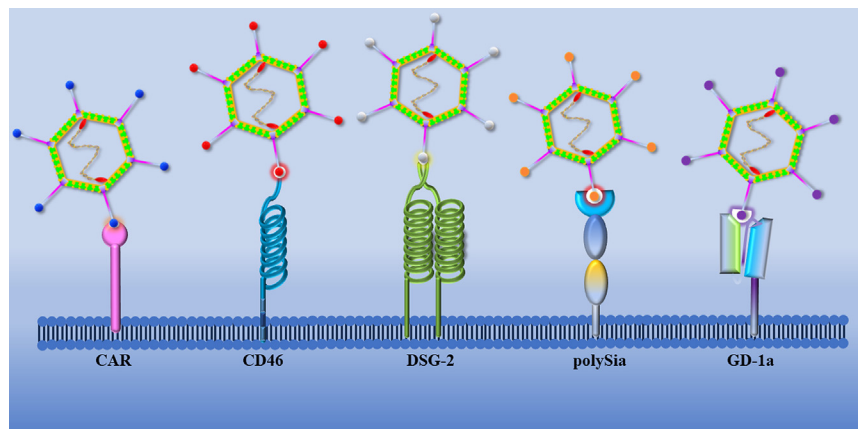
#### STRATEGIES TO CIRCUMVENT PRE-EXISTING ANTIBODIES AGAINST AD VECTORS

The biggest obstacle limiting repeated use of the adenoviral vector is the strong immunogenicity of the vector itself, which often induces

the body to produce NAs against the Ad. NAs against multiple serotypes of Ads are commonly found in the population, resulting in rapid clearance of the adenoviral vector by the host immune system, affecting transduction efficiency and expression intensity and duration of the exogenous gene.<sup>115,116</sup> How to circumvent the inhibitory effect of NAs against the adenoviral vector is currently one of the main limiting factors for its clinical application. Although increasing the immune dose of Ad can overcome antagonism of pre-existing antibodies *in vivo* to some extent, increasing the immune dose will also increase related side effects and economic costs, and is therefore unlikely to be a realistic choice for adenoviral vector-based vaccine application.

Since the first discovery and successful isolation of Ad in the 1950s, more than 100 serotypes have been isolated to date, of which 52 different serotypes have been identified in humans and can be divided into 6 subgroups (A, B, C, D, E, and F).<sup>117</sup> Human adenovirus serotype 2 (HAd2) and HAd5 are the two Ads most commonly used as gene therapy tools and vaccine delivery vectors, both belonging to subgroup C, and sharing 95% homology in DNA sequence. In fact, HAd2 and 5 infections are very common in the population, with some studies showing the pediatric population was 59.6% and 43.3% seropositive for rAd2 and rAd5, respectively. Some reports demonstrated that about 80% of the population has NAs against HAd5.<sup>118</sup> Development of new serotypes of Ads as vectors for vaccines and gene therapy is becoming increasingly important, especially to develop rare serotypes of Ads with low prevalence in the population and Ads from other species like chimpanzees.

Tatsis and co-workers from the Wistar Institute at the University of Pennsylvania constructed multiple replication-deficient ChAd vectors with E1 deficiency including ChAd68, ChAd7, and ChAd6.<sup>119–121</sup> These ChAd vectors can effectively replicate in HEK293 cells, indicating that ChAd can utilize the E1 gene of HAd5 to complete the replication cycle. Another advantage of using E1 motifs from different hosts to provide *trans* complementarity is that it can reduce the probability of producing RCA, as the nucleotide fragments on both sides of the E1 gene from different host sources have very low homology. Zhou and co-workers validated the gene delivery ability of these ChAds to different cells and tissues, as well as



**Figure 5. Different knob receptors on the cellular membrane for adenovirus entry**

Human serotype 5 adenovirus utilizes the coxsackievirus and adenovirus receptor (CAR) as the main receptor, the fiber knob proteins of several other serotypes of adenovirus (including HAd3, HAd11, HAd26, HAd35, HAd37, and HAd52) can bind to CD46, desmoglein 2 (DSG-2), polysialic acid (polySia), and/or GD1aglycan (GD1a) on the cell surface for cell entry.

their ability to induce humoral and cellular immunity *in vivo*.<sup>119,122–127</sup> Their results indicated that adenoviral vectors derived from chimpanzees have the same transport capacity and immunogenicity as HAd5.

In fact, the use of ChAd as a vaccine vector has some distinct advantages.<sup>128</sup> First, ChAds can be cultured in human cell lines, such as HEK293 cells, which have been approved for production of multiple vaccines. In addition, ChAds have low seroprevalence in the population because they rarely circulate in humans.<sup>129,130</sup> Its amino acid sequence in the hypervariable region of hexons, which contains most of the neutralizing antigenic determinants, differs from common serotype HAd, so ChAd can efficiently evade the NA against HAd. According to some published reports, NAs against ChAds are rare in European and US populations, with prevalence ranging from 0% to 4%, compared with up to 20% in people in sub-Saharan African countries.<sup>131</sup> This difference may be attributed to differences in lifestyle and level of interaction with wildlife. However, 20% seroprevalence is still significantly lower than of HAd5. There is a general lack of NAs against ChAds in the population, and its powerful gene delivery ability and the ability to induce humoral and cellular immunity, make ChAd vectors another powerful weapon for humans to fight against emerging infectious diseases. In addition to HAds and ChAds, a number of other mammalian Ads and avian Ads, including porcine Ads, bovine Ads, sheep Ads, avian Ads, and canine Ads, have also been used as tools for gene therapy and vaccine research.<sup>132–137</sup>

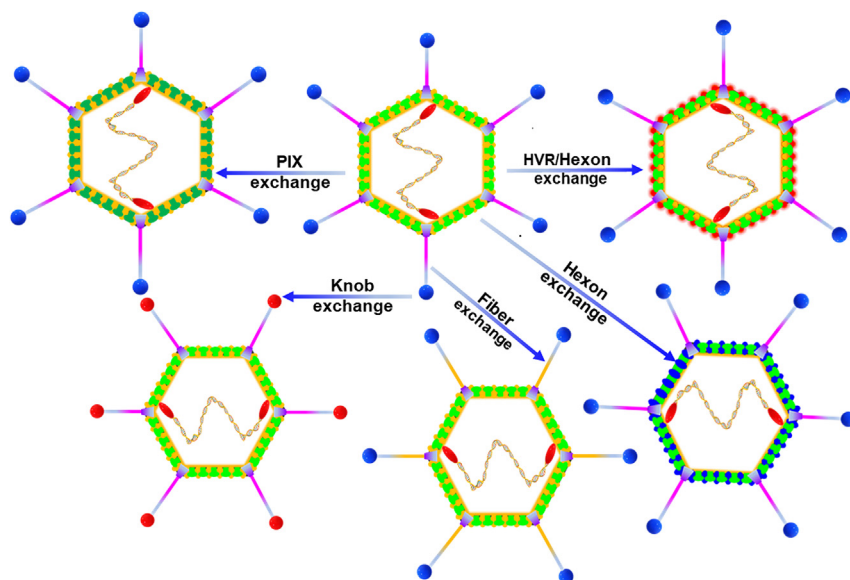
Studies have shown that the strong immune response of the host against the adenoviral vector itself will reduce the feasibility of using the same vector for booster immunization.<sup>116</sup> However, there is little cross-immune response between Ads of different serotypes, but they share similar genomic structures and functional proteins, which provides more possibilities and options for development of chimeric adenoviral vectors using Ads of different serotypes.<sup>54,138</sup>

In the past few decades, the vast majority of research on adenoviral vector-based vaccines or gene therapy have been based on HAd5,

as its pathogenesis and replication mechanisms have been well understood. To expand the application range of adenoviral vectors, fiber protein from other serotypes (such as HAd3, HAd11, HAd35, HAd37, HAd40, etc.) are commonly used for chimerism with HAd5 fiber proteins to improve transduction efficiency and circumvent pre-existing immune responses against HAd5.<sup>139,140</sup>

Modification of the viral capsid protein structure is currently the most common targeted modification strategy.<sup>141</sup> However, these modification strategies are not perfect. For example, modification of the fiber protein will significantly change the Ad's tissue tropism, which may bring unknown safety risks; modification of the hexon often affects the Ad's assembly, making it difficult for packaging of the recombinant Ad, or significantly reducing the Ad's proliferation ability.<sup>53</sup> Therefore, there is still great room for improvement in targeted modification of Ad.

To circumvent the inhibitory effect of pre-existing NAs on adenoviral vectors (mainly against HAd5), it is also an intentional exploration to modify the surface of the viral particle's surface by physical or chemical means. Polymers such as polyethylene glycol (PEG) and multivalent hydrophilic polymers were used to mask certain antigenic epitopes and reduce the immune response, which can significantly prolong Ad-mediated gene expression.<sup>142–144</sup> Encapsulation of HAd5 with PEG can block binding of anti-HAd5 antibodies to viral particles and avoid the inhibitory effect of anti-Ad5 NA. Covalent attachment of polymers to Ad capsid proteins can reduce the inhibitory effect of NAs on Ad transduction efficiency. Ad vectors containing poly-lysine peptide-modified fibers can reduce hepatotoxicity by decreasing IL-6 production.<sup>145,146</sup> In addition, liposome-encapsulated HAd5 vectors can also escape the inhibitory effect of NAs, and both cationic and anionic liposomes can be used for encapsulation of HAd vectors.<sup>147,148</sup> In fact, since Ad virions have a negative charge on their surface, they can be easily encapsulated by cationic liposomes simply by mixing Ad carriers and cationic liposomes together. Zhong et al. successfully formulated HAd5 with anionic liposomes. Their results showed that anionic liposome-formulated HAd5 could escape from the effect of NAs, while improving its transduction efficiency in CAR-deficient cells.<sup>149</sup> In addition, recent studies have shown that Ad particles can be encapsulated by extracellular vesicles, such as exosomes, but it is



**Figure 6. Construction of chimeric adenovirus**

The modification of fiber and fiber knob; other proteins on the surface of the Ad virion such as HVR/Hexon and PIX can also be modified to construct a chimeric Ad.

unclear whether extracellular vesicles containing HAd5 can bypass anti-HAd5 NAs.<sup>150</sup>

Primary immunization with a low dose of adenoviral vector-based vaccine in the immunization protocol and boost with a high dose of Ad can induce a stronger specific immune response. In addition to this, intranasal immunization is significantly better than intramuscular injection in preventing respiratory infections, which means the immunization method also can affect the effectiveness of the adenoviral vector-based vaccine. In the study of the COVID-19 vaccine, it was found that intranasal administration significantly reduced side effects of the vaccine while achieving better protection.<sup>151</sup> Studies have shown that nebulized vaccines with intranasal immunization are more effective in stimulating mucosal immunity and reducing adverse reactions and side effects of the vaccine.<sup>152,153</sup>

#### ADENOVIRAL VECTOR-BASED VACCINE FOR INFECTIOUS PATHOGENS

Due to their characteristics, Ads have become the preferred vaccine vectors against infectious disease and been considered as the most promising vaccine vectors against infectious diseases. Currently, adenoviral vector-based vaccines have been developed to cover a wide range of infectious agents from malaria to HIV-1. As of March 2023, a total of 739 clinical trials of vaccines and gene therapy have used Ads as vectors (Table 1), and this number is still on the rise. Among them, adenoviral vector-based vaccines against SARS-CoV-2 and Ebola have been successfully used in clinical practice.

In vaccine research, Ad is often used as delivery vectors for antigens, where protective antigen genes are inserted into the Ad genome, and a recombinant Ad is generated as a potential candidate vaccine. After immunization, the recombinant Ad will activate the body's immune

system, resulting in massive production of protective antibodies. However, the main factors limiting the prospect of adenoviral vector-based vaccine applications are the widespread presence of pre-existing NAs against the vector itself in the population, as well as robust immune-related adverse events induced by adenoviral proteins, all of which must be taken into account and solved in clinical application.

The majority of adenoviral vectors currently used as prophylactic vaccines against infectious diseases are still first-generation adenoviral vectors, i.e., those deleted in the E1 and E3 regions. Gene encoding the antigenic protein that induces protective antibodies is often inserted into the E1 region.

#### ADENOVIRAL VECTOR-BASED EBOLA VACCINE

Ebola is a deadly disease caused by the Ebola virus, formerly known as Ebola hemorrhagic fever. After Ebola virus infection in the human body, it can quickly spread to tissues and organs throughout the body, destroying connective tissue, inducing a severe inflammatory cytokine storm and causing apoptosis of the immune system, and eventually infected people will die due to multiple organ failure and extensive hemorrhage.<sup>154,155</sup>

The lyophilized EBO vaccine jointly developed by the Institute of Bioengineering of the Academy of Military Medical Sciences (CAS) and CanSino Biologics (CanSino) uses the AdMax packaging system to construct Ad5 vectors with E1 and E3 deletion, and uses HEK293SF-3F6 cell lines for mass production. Antibody titers decreased gradually 6 months after primary immunization, and antibody titers increased rapidly on day 28 after booster immunization with the same recombinant adenovirus. Another research team from China has developed a novel Ebola virus vaccine based on human serotype 2 adenovirus (HAd2).

The EBO vaccine developed by Janssen Pharmaceuticals is based on HAd26 and the vaccinia Ankara strain is used for booster immunization. In the phase I clinical study, NAs were stimulated and generated in 87%–100% of volunteers after receiving the second dose of vaccine. The ChAd3 vector-based EBO vaccine (ChAd3-EBO-Z), developed in collaboration with the US National Institute of Allergy and Infectious Diseases and GSK, has also shown good immunogenicity and safety.

#### ADENOVIRAL VECTOR-BASED COVID-19 VACCINE

In the global war against COVID-19, messenger RNA (mRNA) vaccine saved millions of lives, while the adenoviral vector vaccine also



**Table 1. Adenoviral vector vaccines against pathogens**

Serotype of Ad	Pathogens	Status	NCT no.
Ad5	SARS-CoV-2	phase I	NCT05526183
Ad5 and ChAd68	SARS-CoV-2	phase 1	NCT05094609
AdCLD (Chimeric 5/35)	SARS-CoV-2	active	NCT04666012
ChAdOx1	SARS-CoV-2	phase 2	NCT05049226
ChAdV68	SARS-CoV-2	phase 1	NCT04776317
Ad 4	HIV	phase 1	NCT02771730
Ad5 or Ad35	HIV	phase 1	NCT00472719
Ad26	HIV	phase 1	NCT00618605
AdC6 and AdC7	HIV	phase 1	NCT05182125
Ad3	Ebola	phase 1	NCT04041570
Ad5	Ebola	phase I/II	NCT04840992
Ad26	Ebola	phase 3	NCT02509494
Ad26	hemorrhagic fever, Ebola	phase 2	NCT02564523
Unknown	influenza (H1N1)	phase 2	NCT02918006
Ad4	H5N1	phase 1	NCT01443936
Unknown	norovirus	phase 1	NCT03125473
Unknown	norovirus	phase 1	NCT02868073
ChAd3	Marburg virus disease	phase 2	NCT05817422
ChAd63	malaria <i>Plasmodium vivax</i>	phase Ia	NCT01816113
Ad35	malaria	phase 2	NCT01366534
Ad5	malaria	phase 2	NCT00870987
ChAdOx2	Crohn disease, <i>Mycobacterium avium</i> subspecies <i>paratuberculosis</i>	phase 1	NCT03027193
ChAdOx1	MERS	phase 1	NCT03399578
Ad26	RSV	phase 2	NCT03303625
Ad35	<i>Plasmodium falciparum</i>	phase 1	NCT00371189
ChAd155-	hepatitis B	phase 2	NCT03866187
ChAd3	hepatitis C	phase 1	NCT03688061
AdCh3 and Ad6	hepatitis C	phase 1	NCT01094873
ChAdOx1	chikungunya fever	phase 1	NCT03590392
Ad4	anthrax infection	phase 1	NCT01979406

played an extremely important role.<sup>156</sup> Adenoviral vector vaccine and mRNA vaccine have their own advantages and disadvantages. However, adenoviral vector vaccine has advantages on storage conditions. Liquid formulations of adenoviral vector vaccine could maintain its effectiveness for more than 1 year at 2°C–8°C and its lyophilized formulation can be stored longer under the above conditions, and even can be stored for several days at 30°C, without any significant loss of vitality.<sup>157</sup> Different from the adenoviral vector vaccine, the mRNA vaccines BNT162b2 and mRNA-1273 need to be kept at –80°C and –20°C, respectively.<sup>156</sup> This storage condition is a challenge for the mRNA vaccine. Strict dependence on low-temperature storage and transportation of the mRNA vaccine can be attributed to instability of the LNP system. Safety concerns for mRNA vaccines, especial for LNP particles, remain.<sup>158</sup> After many years of clinical research, adenoviral vector vaccines have proved their safety, while

long-term safety of mRNA vaccine is not well understood. Meanwhile, an increasing number of studies have shown that immunization with mRNA vaccines increases the risk of vascular closure.<sup>159</sup> In the long run, adenoviral vector vaccines are also better than mRNA vaccines in induced immune duration. BNT162b2 and mRNA-1273 vaccines can induce excellent short-term NA response and protective efficacy. However, high initial serum NA titer induced by the mRNA vaccine declines in 3–6 months, with a further decline in 8 months. Different from BNT162b2 and mRNA-1273, the initial NA titer induced by Ad26.Cov-2.S was low, but the NA response and clinical effect were quite durable, lasting at least 8 months.<sup>156</sup> In addition, compared with the mature industrial-scale automated production of adenoviruses, production of mRNA vaccines has not been continuous and automated, which greatly limits the production capacity and increases the cost of mRNA vaccines. Therefore, although

adenoviral vector vaccines also have some side effects and risks, they are still the first-line vaccines in many developing countries.

Since the outbreak of COVID-19, four kinds of adenoviral vector-based COVID-19 vaccines have been approved and widely applied among the human population. Among the four adenoviral vector-based vaccines, Ad5-nCoV was developed by the Academy of Military Sciences/CanSino in China,<sup>160</sup> AZD1222 was developed by Oxford University/AstraZeneca in the UK,<sup>161</sup> Ad26.CoV-2.S was developed by Johnson & Johnson in the US,<sup>162</sup> and Sputnik V was developed by the Gamaleya Research Institute in Russia.<sup>163</sup> All four vaccines used replication-deficient Ads to express full-length spike (S) protein, but the antigen design and vaccination dose were different. The codon of the S gene in AZD1222 has been optimized, and a tPA signal peptide has been added at the N-terminal. Its vector ChAdOx1 is a replication-deficient version of ChAdY25, and the pre-existing immunity to this vector is very low among the human population. The antigen design of Ad5 nCoV is similar to AZD1222. The optimized S gene with the tPA signal peptide is cloned into the Ad5 vector that lacks E1 and E3, and the recombinant Ad is constructed using the AdMax system. Sputnik V, also known as Gam-COVID-Vac, expresses the full-length S protein using Ad26 (first dose) and Ad5 vectors (second dose), respectively, which can bypass the inhibitory effect of anti-vector NA induced by the first-dose vaccination.

In addition to the EBO and COVID-19 vaccines, which have been approved for clinical application in the human population, there are a number of adenoviral vector-based vaccines against infectious diseases that are currently in various stages of clinical trials.

#### ADENOVIRAL VECTOR-BASED INFLUENZA VACCINE

Compared with current influenza vaccine technology, the Ad carrier platform has its own advantages and can serve as a necessary supplement to current influenza vaccine technology. In response to the global influenza pandemic, the adenoviral vector-based vaccine platform can produce a large quantity of adenoviral vector-based vaccines at a low cost in a short period of time, which is crucial to suppress virus transmission and reduce infection-related morbidity and mortality at the early stage of the pandemic. Several human and non-human Ad vector systems have already been used to develop seasonal and universal influenza vaccines. Clinical studies have shown that human avian influenza vaccines based on HAd4 and HAd5 expressing H5N1 HA have good immunogenicity and safety.<sup>164</sup> Adenoviral vector-based vaccine ChAdOx1 NP + M1, expressing NP and matrix protein 1 (M1), was constructed based on a ChAd Y25 isolate, which had protective effect against different subtypes of influenza viruses.<sup>165</sup> Ad vector-based influenza vaccines that co-expressed the main influenza virus antigens (e.g., HA, NP, and M2) have been tested in clinical trials.

We constructed a novel adenovirus vector ChAdC7 utilizing the direct clone strategy and inserted the H5N1 hemagglutinin (HA) into the E1 region on its genome. Our previous study showed that

a ChAd vector carrying influenza virus HA gene could be a promising vaccine candidate of multiple HA subtypes influenza virus.<sup>166</sup>

#### ONCOLYTIC AD

Oncolytic viruses (OVs) only selectively replicate within cancer cells without affecting normal cells.<sup>167-171</sup> In recent years, OV therapy has been extensively studied as an emerging anti-tumor biological therapy, which can induce the body's innate and adaptive immune response to attack tumor cells.<sup>168,172,173</sup> As a member of the of OV family, oncolytic Ads are in a leading position in the field of research and development both in the laboratory and in clinical practice.<sup>174</sup> Unlike replication-deficient Ad vectors that require a helper cell line that provides replication-essential gene E1 *in trans* to complete the replication cycle, oncolytic Ad, also known as selectively replicating recombinant Ad, which can complete the replication cycle only in cancer cells is harmless to normal cells. Oncolytic Ad replicates inside tumor cells and then releases large quantities of offspring viruses, which go on to infect surrounding tumor cells, inhibiting tumor growth, and eventually eliminating tumors. Construction of selective or conditional replication recombinant Ad mainly involves placing the E1A or E1B genes, which are essential for Ad replication, under the control of tumor tissue-specific promoters, so they are only expressed in specific tumor tissues.<sup>85</sup>

Tumor-specific promoters can be divided into two categories, one is broad-spectrum tumor-specific promoters: these promoters initiate transcription and expression of downstream genes in all tumor cells, such as survivin promoters, cyclooxygenase (COX-2) promoters, hTERT promoters, and telomerase promoter, among which the most studied is the telomerase promoter, because the vast majority of malignant tumor cells are telomerase positive; the other type of promoter is active and functional only in a specific tumor cell, such as prostate-specific antigen promoter, alpha-fetoprotein liver cancer promoter, carcinoembryonic antigen epithelial cancer promoter, and breast cancer cell-specific promoters.<sup>175,176</sup> To further improve the effect of oncolytic Ads, anti-tumor genes and immunomodulatory genes are often inserted in its genome to enhance the ability of the OV to kill tumor cells while improving the body's anti-tumor immune response.

Hypoxia is a characteristic feature of solid tumors that occurs during tumor development and growth and can lead to cell-cycle arrest.<sup>177</sup> Taking advantage of this property of tumor cells, Hernandez-Alcoceba et al. designed a tumor-lysing Ad in which expression of the E1A gene is controlled by a promoter containing a hypoxia response element, and the modified oncolytic adenovirus can fully replicate under hypoxic conditions to achieve the purpose of oncolysis.<sup>178</sup>

The use of OVs to express certain cytokines with immunomodulatory function is beneficial to improve the body's anti-tumor immune response.<sup>179-184</sup> At present, the most studied is granulocyte macrophage colony-stimulating factor (GM-CSF). Oncolytic Ads express GM-CSF to help infiltrate DC precursor cells such as monocytes in tumors to

**Table 2. Oncolytic adenovirus armed with immunomodulation**

Immunomodulatory molecules	Mechanism	Cancer type	References
IL-2	stimulates NK and T cells	melanoma, renal cancer, prostate cancer, glioblastoma, other solid tumors	O'Malley et al. <sup>227</sup> ; Yao et al. <sup>228</sup> ; Toloza et al. <sup>229</sup> ; Mulders et al. <sup>230</sup>
IL-12	regulates the immune response and the differentiation of T cells	melanoma, head and neck squamous cell carcinoma	Bortolanza et al. <sup>231</sup> ; He et al. <sup>232</sup> ; Nasu et al. <sup>233</sup> ; Sangro et al. <sup>234</sup>
IL-15	activates NK and T cells	glioblastoma	Isvoranu et al. <sup>235</sup> ; Morris et al. <sup>236</sup>
IL-18	promotes the development of Th1 and memory CD8 T cells	malignant melanoma	Yang et al. <sup>237</sup> ; Zheng et al. <sup>238</sup> ; Zheng et al. <sup>239</sup>
TNF- $\alpha$	stimulates the T cell compartment in tumor microenvironment	pancreatic ductal, adenocarcinoma	Sato-Dahlman et al. <sup>240</sup> ; Cervera-Carrascon et al. <sup>241</sup> ; Cervera-Carrascon et al. <sup>242</sup>
IFN- $\alpha/\beta$	inhibits tumor growth directly and indirectly by acting upon tumor and immune cells	hepatocellular and pancreatic carcinomas	Huang et al. <sup>243</sup> ; Borden et al. <sup>244</sup> ; Dinney et al. <sup>245</sup>
GM-CSF	promotes differentiation, maturation, expansion, and enhances the function of DCs and macrophages	melanoma, thymic carcinoma, lung esophageal cancer	Lv et al. <sup>194</sup> ; Sato-Dahlman et al. <sup>240</sup> ; Tian et al. <sup>246</sup> ; Wang et al. <sup>247</sup>
CXCL 10 and 12	promotes T cell activation	advanced solid tumors	Li et al. <sup>248</sup> ; O'Bryan et al. <sup>249</sup>
IL-24	induces cancer cell apoptosis	lung cancer, breast cancer	Ramesh et al. <sup>128</sup> ; Mao et al. <sup>250</sup>
RANTES	enhances tumor infiltration and maturation of APCs <i>in vivo</i>	hepatocellular carcinoma	Ang et al. <sup>251</sup> ; Li et al. <sup>252</sup>
Bispecific T cell engager	T cell activation, upregulation of proinflammatory cytokines	prostate cancer	Li et al. <sup>252</sup> ; Freedman et al. <sup>253</sup> ; Huang et al. <sup>254</sup>

differentiate into DC, and oncolytic Ads release a large number of tumor-associated antigens after dissolving tumor cells to provide DC with abundant tumor antigens,<sup>185–194</sup> which stimulate the body's cytotoxic T lymphocyte response specific to tumor antigens. In addition to GM-CSF, immune regulator genes, such as IL-12, IL-18, CD40L, 4-1BBL, and B7 are expressed by oncolytic adenovirus alone or jointly, which can also enhance the ability of anti-tumor immune response of the body to a certain extent (Table 2).<sup>167,169,172,173,179,180,182,187,195,196</sup>

#### APPLICATION OF ADENOVIRAL VECTORS FOR GENE EDITING

Clustered, regularly interspaced, short palindromic repeats (CRISPR)-related protein 9 (Cas9), and guiding RNA (gRNA) gene editing technology, also called CRISPR-Cas9, has opened up new avenues for treatment of a wide range of genetic defects, and several pre-clinical studies and clinical results have demonstrated its powerful therapeutic effects.<sup>197,198</sup> However, how to safely and effectively deliver genome editing components is still a major challenge for *in vivo* genome editing. Adenoviral vector is considered to be a promising delivery vehicle for *in vivo* gene editing. Palmer et al. developed a single helper-dependent Ad (HDAd), which is used to deliver all the components of CRISPR-Cas9 for efficient gene editing in transduced cells. This all-in-one HDAd provides an important platform for achieving and expanding CRISPR-Cas9 utility, particularly for difficult-to-transfect cells and *in vivo* applications.<sup>199</sup> Palmer and co-workers have shown that HDAds can efficiently deliver donor DNA for homology-directed repair via spontaneous homologous recombination.<sup>200,201</sup>

Human papillomavirus (HPV) causes malignant epithelial cancers, including cervical cancer, non-melanoma skin cancer, and head and neck cancer. They drive development of tumors through expression of oncoproteins E6 and E7.<sup>202</sup> Ehrke-Schulz and co-workers inserted the complete CRISPR-Cas9-gRNA genome-editing module into a HCAd. After the cells are transduced by this HCAd, HPV18-E6 or HPV16-E6 genes can be specifically knocked out, causing a decrease in HPV-positive viability, while HPV-negative normal cells are not affected.<sup>203,204</sup> This indicates that the HCAd can be used for HPV-specific cancer gene therapy when combined with HPV-specific CRISPR-Cas9-gRNA.

Cystic fibrosis (CF) is caused by mutations in the CF transmembrane conduction regulatory factor gene.<sup>205</sup> Despite the success of CRISPR-Cas9 genome editing *in vitro*, there are still challenges when using CRISPR-Cas9 *in vivo* to treat CF lung disease. HD Ad has the advantages of large capacity and low immunogenicity, coupled with the versatility of the CRISPR-Cas9 system, delivering CRISPR-Cas9 together with HD Ad to the airway for lung gene therapy and shows great potential.<sup>206</sup>

More than 240 million people worldwide are chronically infected with hepatitis B virus (HBV), leading to chronic hepatitis, cirrhosis, and hepatocellular carcinoma.<sup>207</sup> Genome editing using CRISPR-Cas9 could offer new therapeutic approaches for chronic HBV infection as it can directly disrupt the HBV genome. Kato et al. constructed an Ad carrying eight multiplex gRNA of CRISPR-Cas9 targeting the HBV X gene. With the treatment of this Ad, the HBV X gene

integrated into the chromosomes of HepG2 cells can be cleaved and destroyed multiple times. The Ad containing eight multiple gRNA units not only ensures that the target gene is knocked out multiple times, but also solves the problem of off-target effects and escape mutants in genome editing therapy.<sup>208</sup>

#### APPLICATION OF ADS TO RNAi THERAPY

RNA interference (RNAi) is a phenomenon of silencing gene expression through specific degradation of homologous mRNA sequences, which exists in almost all eukaryotic cells.<sup>209</sup> Due to its high specificity and efficiency, RNAi has been widely used in basic research on gene function and human diseases, as well as antiviral research. In recent years, Ad-mediated small hairpin RNA (shRNA) has been used as a potential method for antiviral research on dengue fever virus, porcine circovirus type 2, and foot-and-mouth disease virus, as well as other kinds viruses.<sup>210</sup>

Motegi et al. developed an effective Ad vector-mediated RNAi system, in which the Ad vector carries four shRNA expression cassettes (Ad multi-shRNA vectors), which can enhance the silencing effect to the same gene by carrying four identical shRNA sequences, or effectively silence multiple target genes simultaneously, by carrying four different shRNA sequences.<sup>211</sup> Ad multi-shRNA vectors not only have potential practicality in basic research but also in clinical gene therapy.<sup>212</sup>

In mammals, microRNAs (miRNAs) are involved in regulation of many genes. More than 1,000 human miRNAs have been identified and are estimated to regulate expression of at least 30% of human genes.<sup>213</sup> RNAi has already been widely used as a tool for gene knock-down in basic research or clinical medicine. Ad vectors are often used to deliver shRNA or miRNA expression cassettes into target cells *in vitro*. In addition, because Ad vectors have a relatively large packaging capacity, they are often used to transcribe or co-express protein and shRNAs or miRNA in the same expression cassette.<sup>214–217</sup>

Our previous study suggested that artificial miRNAs targeting the inside conserved regions of influenza A virus delivered by ChAd68 could provide cross-protection against multiple subtypes of IAV challenging in ICR mice.<sup>189,218</sup> We also found that delivering the artificial miRNA of the HA gene on the H5N1 avian influenza virus (AIV) by ChAd68 could also provide 40%–70% immediate protection against a lethal H5N1 AIV challenge in mice.<sup>219</sup>

Ad vectors are often used to deliver shRNA or miRNA expression cassettes into target cells *in vitro*. By co-expressing miRNAs targeting p21, the anticancer effect of Ad expressing p53 can be enhanced.<sup>214</sup> Another beneficial exploration is to combine oncolytic Ads with RNAi expression boxes. Gürlevik et al. inserted multiple miRNAs targeting essential genes for Ad replication into the oncolytic Ad genome and controlled by the p53 promoter. The replication of Ad would be inhibited in normal cells expressing p53, while its replication would not be restricted in tumor cells with p53 dysfunction.<sup>220</sup>

Hepatitis C virus (HCV) is a hepatophilic human virus, and its infection causes chronic liver inflammation and fibrosis, which can finally lead to hepatocellular carcinoma.<sup>221</sup> Currently, 170 million people worldwide are infected with HCV. Sakurai and co-workers developed an adenoviral vector expressing tough decoy RNA (TuD-122a) targeting miR-122a to inhibit HCV replication in hepatocytes, and their results suggested that adenoviral vector-mediated expression of TuD-122a would be a promising tool for the treatment of HCV infection.<sup>222,223</sup>

#### CONCLUSIONS

The outbreak of new infectious diseases such as SARS-CoV-2 and mpox has accelerated the research pace of new vaccines.<sup>224,225</sup> The adenoviral vector-based COVID-19 vaccine has been approved and clinically applied worldwide, and shows great potential and the promising prospect of the adenoviral vector platform in dealing with emerging infectious diseases.<sup>226</sup> In addition, recombinant Ad has also been widely used in clinical research as a delivery tool for gene therapy or been modified as an oncolytic Ad.<sup>47,174</sup> Although there are some obstacles to application of adenoviral vectors, such as how to circumvent antagonism of the NA against the Ad vector, and how to improve targeting of adenoviral vectors, it is believed that with in-depth understanding of the biological characteristics of Ad, researchers can find ways to solve these problems, further improve immunogenicity, therapeutic effects, and safety of adenoviral vectors, and reduce their side effects.

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#### AUTHOR CONTRIBUTIONS

Z.C. and Y.A. conceptualized the outline of this manuscript. H.Z. and H.W. wrote and revised the manuscript. All authors made substantial, direct, and intellectual contributions to this article and approved it for publication.

#### DECLARATION OF INTERESTS

The authors declare no competing interests.

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