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Review

Role of homologous recombination/recombineering on human adenovirus genome engineering: Not the only but the most competent solution

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ABSTRACT

Adenoviruses typically cause mild illnesses, but severe diseases may occur primarily in immunodeficient individuals, particularly children. Recently, adenoviruses have garnered significant interest as a versatile tool in gene therapy, tumor treatment, and vaccine vector development. Over the past two decades, the advent of recombineering, a method based on homologous recombination, has notably enhanced the utility of adenoviral vectors in therapeutic applications. This review summarizes recent advancements in the use of human adenoviral vectors in medicine and discusses the pivotal role of recombineering in the development of these vectors. Additionally, it highlights the current achievements and potential future impact of therapeutic adenoviral vectors.

1. Introduction

Adenoviruses, discovered in 1953 from human adenoid tissues [1], have been a focal point in virology for over seven decades. To date, more than 100 human adenovirus types, classified into seven subgroups (A–G), have been isolated and characterized. These medium-sized (70–100 nm in diameter), non-enveloped, icosahedral viruses consist of a protein capsid encasing a double-stranded linear DNA genome, typically 33–36 kgbases (Kb) in length. Although human adenoviruses are known for causing respiratory, ocular, and gastrointestinal diseases, the most severe cases occur predominantly in immunocompromised individuals and transplant patients [2–4]. In the general population, these infections are usually resolved quickly and confer lifelong immunity. Adenoviruses have historically served as a model for understanding various aspects of virus biology, including viral entry, DNA transcription and replication, mRNA splicing, viral assembly, cellular transformation *in vitro*, and tumorigenesis [5].

Adenoviruses have gained prominence not primarily as pathogens, but as potent tools in therapy. Over the past few decades, adenoviruses have been extensively used as vectors in gene therapy clinical trials (Fig. 1A). These trials have predominantly focused on cancer treatment, with the first oncolytic adenovirus (OAd) approved in China in 2005 [6,7]. Additionally, adenoviral vectors have been employed in targeting monogenetic diseases, particularly for direct *in vivo* delivery of therapeutic genes (Fig. 1B). In the realm of infectious disease prevention, adenovirus-based vaccines have been developed for several life-threatening diseases, most notably coronavirus disease 2019 (COVID-19) [8,9].

The development of human adenoviral vectors, especially in their initial vectorization, experienced a significant boost following the discovery of recombineering (Fig. 2). Prior to this discovery [10], only a limited number of adenovirus types were vectorized from 1953 to 2002. The landscape changed dramatically from 2003 to 2010, with 10 new types of adenoviral vectors being developed [11]. The most rapid ad-

Abbreviation: AAV, adeno-associated virus; Ad5, adenovirus serotype 5; AdV, adenoviral vector; AFP, α-Fetoprotein; AIDS, acquired immunodeficiency syndrome; AMA1, apical membrane antigen 1; ChAd155, chimpanzee adenovirus 155; Cox2, cyclo-oxygenase 2 promoter; DSG2, desmoglein 2; Env, envelope; FIX, factor IX; FVIII, factor VIII; GFP, green fluorescent protein; gp, glycoprotein; GMCSF, granulocyte–macrophage colony-stimulating factor; HCAdVs, high-capacity adenoviral vectors; HDAdV, helper-dependent adenoviral vector; HK2, human glandular kallikrein; HSPC, hematopoietic stem cell; HV, helper virus; ITRs, inverted terminal repeat sites; LDL, low-density lipoproteins; MAYV, Mayaro virus; NHPs, non-human primates; OAds, oncolytic adenoviruses; pIX, protein IX; prM, premembrane; Rb, retinoblastoma protein; RhAd52, rhesus monkey adenovirus vector 52; RSV, respiratory syncytial virus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SEBOV, Sudan-Ebola; SIV, simian immunodeficiency virus; TB, tuberculosis; vp, viral particles; ZEBOV, Zaire-Ebola; ZGP, Zaire ebolavirus glycoprotein.

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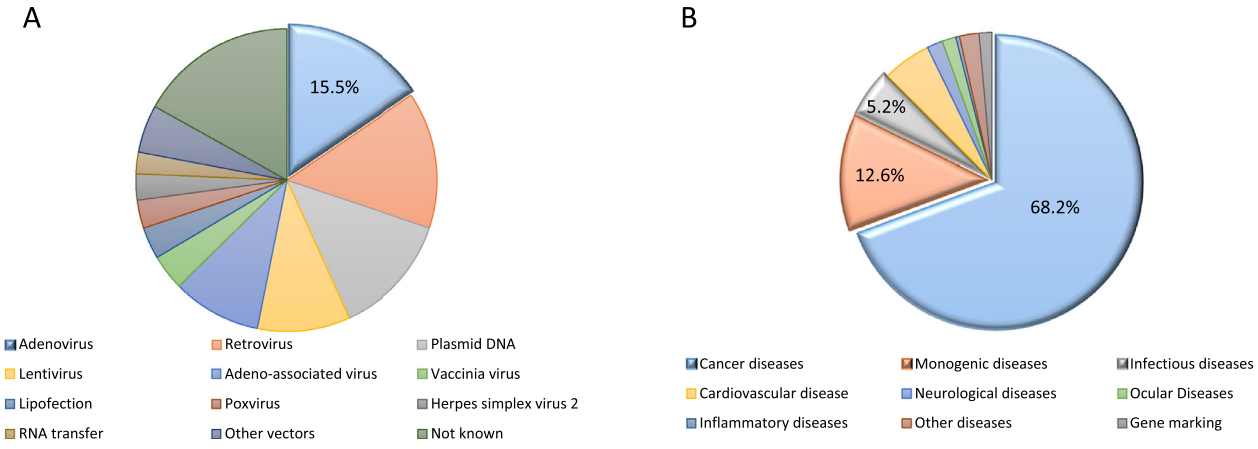

Fig. 1. Overview of used vectors and targeted diseases in gene therapy clinical trials worldwide. As of May 2023, over four thousand gene therapy trials have been reported. (A) Within these trials, adenoviral (n = 573) and retroviral (n = 538) vectors were the most commonly used vectors in gene therapy clinical trials, whereas non-viral vectors, mainly naked/plasmid DNA (n = 483), lipofection (n = 126), and RNA transfer (n = 85), represent one fifth of all trials. (B) In terms of the disease indication, a majority (n = 2513) of trials were involved in the treatment of cancer, followed by inherited monogenic diseases (n = 463) and infectious diseases (n = 191). Modified from the Journal of Gene Medicine. (https://a873679.fmphost.com/fmi/webd/GTCT).

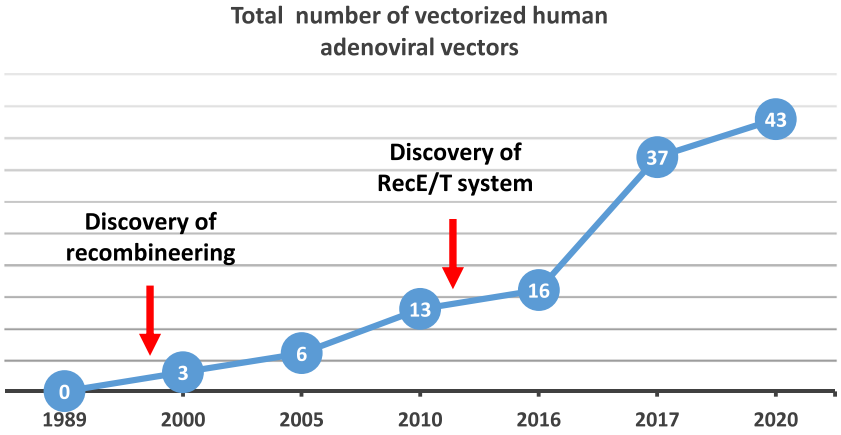

Fig. 2. Timeline on the vectorization of human adenoviral vectors. The figure shows the number of available vectors derived from different human adenovirus types. The discovery of recombineering and the RecE/T system are marked to show their impact on the increasing variety of vectorized human adenoviruses.

vancements occurred with the introduction of the Rec E/T system into adenovirus genome engineering [12–14]. In this review, we summarize the latest essential achievements in human adenoviral vector development, focusing on their applications as vaccine vectors and in gene delivery and tumor therapy. We also highlight the role of various methods and techniques in vector construction

2. Adenoviruses as vaccine vectors

Vaccines are the most effective strategy against infection-related diseases. Compared with traditional vaccines, such as attenuated or inactivated virus-based vaccines and recombinant protein subunits, recombinant viral vector vaccines offer advantages in development speed, safety, and specificity. Adenoviral vectors, in particular, are efficient carriers for encoded recombinant antigens, with a high transgene capacity and a low risk of side effects (Fig. 3). Research on adenovirus-based vaccine vectors spans various diseases, including malaria [15], acquired immunodeficiency syndrome (AIDS) [16], COVID-19, and even cancers (against tumors-associated antigens) [17].

Adenoviral vector-based vaccines are categorized into two types: replication-competent viruses, which pose higher application risks, and replication-deficient viruses, which feature deletions of the early regions E1 and E3 [18]. The E1 gene is essential for viral replication, whereas E3 proteins exhibit immunosuppressive properties. An example includes controlling the E3 gene with an NF-κB inducible promoter [19,17,17], where E3 protein downregulation enhances the NF-κB pathway. Deletion of E1 and E3 genes in adenovirus vectors increases the capacity for transgene insertion up to 8 kB.

The prevalent immunogenicity against the commonly used human adenovirus serotype 5 (Ad5) has led to investigations into alternative human adenovirus types and non-human adenoviruses. For instance, chimpanzee adenoviruses have been successfully developed as vectors for vaccines against diseases such as malaria or COVID-19 [20]. In subsequent chapters and Table 1 (along with Supplementary Table 1), we will discuss all the adenovirus types utilized as vaccine vectors, focusing on features, disease targeting, and achievements. A particular emphasis will be placed on vector construction/modification methods/strategies, highlighting the crucial role of recombineering in the development of adenovirus-based vaccine vectors.

2.1. Ad5-based vaccines: effective yet with notable side effects

Ad5 is the most extensively studied vector owing to its pathogenicity and widespread exposure in humans during childhood. Considering its research prominence, we first provide an overview of Ad5 as a vaccine vector. Ad5 has been used to target severe infectious diseases such as HIV [16,21–23], Ebola [24–28], and Zika virus [29,30], providing valuable insights for future vaccine development.

In a former STEP study [21], an HIV vaccine was developed using a replication-deficient Ad5 vector with deletions in the E1 and E3 regions

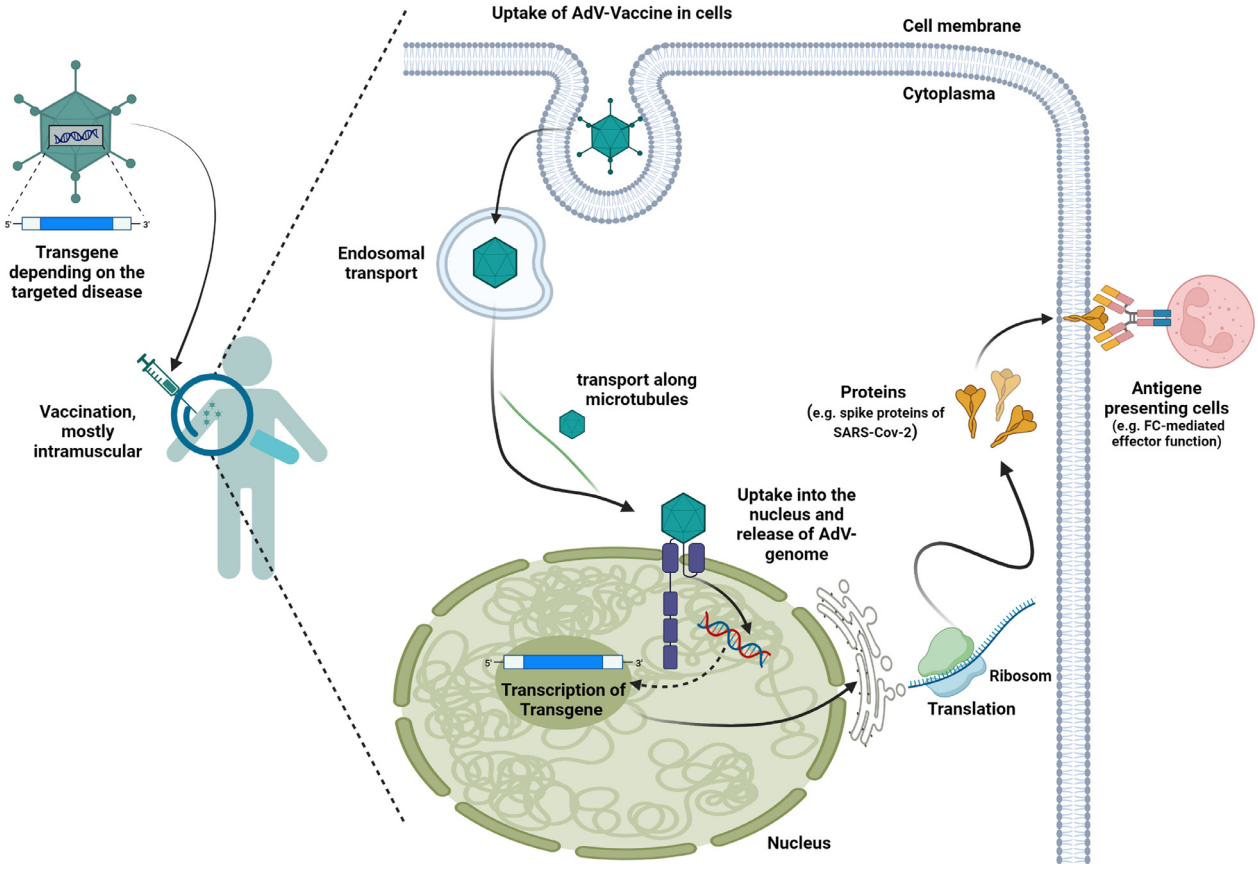

Fig. 3. Principle of using adenoviruses as vaccine vectors. The adenoviral vector (AdV) is modified with a transgene of interest and used for vaccination, typically intramuscularly. Endosomal uptake occurs through the cell membrane. Upon nucleus entry, the AdV genome is released, and transcription of the transgene takes place. The mRNA is then transported out of the nucleus, and ribosomal translation produces the intended protein. After presentation of the protein, antigen-presenting cells take up the protein, and signaling pathways alerts immune cells, including $CD4^+$ and $CD8^+$ (illustration was modified after L. Coughlan et al. 2022 [30]; created with BioRender.com).

[16]. The vector construction involved recombining the shuttle vector pHCMVIBGHpA1, containing the SIV gag transgene, with the adenoviral backbone pAd-ΔE1E3 via the homologous recombination. The resulting pre-adenoviral vector, pAd5-SIV gag, was linearized by PacI digest and transfected into HEK293 cells for virus production. However, despite its efficacy in animal models, this vaccine showed limited effectiveness in humans against HIV infection [21], likely due to preexisting immunogenicity against wild-type Ad5 [21].

Another significant research focus is on the vaccine development against Ebola virus, a lethal disease prevalent in sub-Saharan Africa. Many groups worked on new vaccinations for people living in risk regions. An Ad5-based bivalent EBOV vaccine, first published in 2006 [21], targeted Zaire-Ebola (ZEBOV) and Sudan-Ebola (SEBOV) strains, expressing glycoproteins (GP) from both. The construction involved E1, E3 and partial E4 deletion in Ad5 genome. A shuttle-vector was used to recombine the transgene for SEBOV and ZEBOV GP into the Ad5 vector. Although a study in 2010 on non-human primates (NHPs) [21] showed promising results, the preexisting immunity against Ad5 in humans may have hindered further clinical development.

More recently, in 2021, Ad5 was used to design a vaccine against the Mayaro virus (MAYV) expressing MAYV structural proteins [31]. This vector (containing E1 and E3 deletions) expressing the MAYV structural polyprotein (Capsid, E3, E2, 6 K/TF, E1) was generated using the AdMax HilQ-System [31]. In another novel application, Ad5 served as a transport vehicle for the cocaine analog GNE [32–34]. Here, the cocaine analog GNE was attached to the capsid of a disrupted replication-incompetent Ad5 by EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) cross linker, aiming to aid in cocaine addiction treatment. It is of note, since the first report of this design, the dAd5GNE was continually studied in different animal models with therapeutic effect, and current it is on the way to clinical trial [35–37] .

2.2. Human adenovirus types 2 and 4 (Ad2 and Ad4) as early alternative vaccine vectors

Ad2 has been the focus of two notable vaccine developments tested in animal studies [38]. The Ad2-prM-NS1 vaccine, developed by Liu and colleagues, expresses the premembrane (prM) and nonstructural protein-1 (NS1) of the Zika virus led to robust immune response in neonatal mice born from immunized mothers [38,39]. Another vaccine, rAd2-ZGP, targets Ebola by expressing Zaire ebolavirus glycoprotein (ZGP). Constructed through homologous recombination of the transgene with the Ad2 genome, this vaccine showed promising results in rhesus macaque studies. As Ad2 wild-type is less prevalent than Ad5, these vaccines present a potential advantage in immunogenicity. However, until today there are no clinical trials published concerning Ad2 as a vaccine vector.

Ad4, initially utilized in a military context for oral influenza vaccination in the 1950s [40], has evolved in its applications. One such development is Ad4-H5-Vtn, a recombinant Ad4 vector with a partial deletion in the E3 region, allowing for viral replication [41]. This vector includes the hemagglutinin (HA) influenza gene, coding for the HA surface protein of the influenza virus, aiming to protect against influenza [41,42]. Recombination in bacteria was used to construct the Ad4 vector for vaccination. The shuttle vector was generated with Ad4 homologous regions to shuttle the gene into the ΔE3 region. With the partial deletion of the E3 region, the endogenous splice acceptor remained in the final construct, and the HA expression cassette was inserted into the

Table 1
Summary of adenovirus-based vaccines.

Types	Vaccine	Disease targeting	Vector construction method	Year and Reference
Human adenoviruses				
2	Ad2-prME-NS1	Zika virus disease	Homologous recombination in *Escherichia coli (E. coli)* BJ5183 and *E. coli* XLI blue strain	2015, 2018 [38,86]
	rAd2-ZGP	Ebola virus disease	Homologous recombination with replication-incompetent Ad2 in *E. coli* BJ5183 and *E. coli* XLI blue strain	2014, 2018 [39,87]
4	Ad4-H5-Vtn	Influenza (H5N1 strain; avian influenza)	Homologous recombination in *E. coli* BJ5183	2012, 2021 [41,88]
	rAd4-Clade C rAd4-Env rAd4-SIVgagp55 rAd4-GBV-C E2	Acquired immunodeficiency syndrome (AIDS)	Homologous recombination of adenoviral plasmid and shuttle plasmid containing the transgene that was inserted in the E4 gap in *E. coli* BJ5183	2012, 2021 [43,41]
	Ad4-prM-E	Zika virus disease	Homologous recombination with AdEasy adenoviral vector system in *E. coli* BJ5183	2018, 2020 [29,44]
5	Ad-CAGoptZGP	Ebola virus disease	First generation Ad5 with deletion of the E1/E3-region with pCAGα-optZPG-expression cassette. The researchers used a shuttle plasmid and inserted it into the former E1 region [26]	2009, 2015 [26,28]
	CAdVax ($GP_{S/Z}$) CAdVax EBO7	Ebola virus disease	Deletion of parts of the E4 region for complex transgene insertion; shuttle vectors pLAd and PRAd were used	2002, 2006, 2010 [25,24,27]
	Display of three rAdVs 5	Malaria	Homologous recombination in *E. coli* BJ5183	1996, 2017 [89,90]
	pAd5-SIV *gag*	AIDS	Restriction digest and homologous recombination with unknown bacterial strain	2002, 2010, 2010 [16,23,22]
	rAdCHIKV-E2, rAd-CHIKV-E1, rAd-CHIKV-E2-K6-E1	Chikungunya virus disease	HEK293 cells were co-infected with shuttle plasmid and backbone plasmid in the presence of X-tremeGene HP DNA transfection reagent	2022 [91]
	Ad5.ZIKA-Efl/-rEfl	Zika virus disease	LoxP homologous recombination with pAd shuttle vector	2016 [92]
	Ad5-prM-E	Zika Virus disease	Homologous recombination with AdEasy adenoviral vector system in *E. coli* BJ5183 and XLI blue strain	2020 [29]
	Sputnik V (boost) /Gam-COVID-Vac)	Coronavirus disease (COVID-19)	Method unpublished	2020 [84]
	Ad5-nCOV (CanSino)	COVID-19	The transgene was inserted into shuttle plasmid pGA1 via homologous recombination with the pAd5-ΔE1-ΔE3-backbone	2020, 2021, 2023 [93–95]
	rAd5-MAYV	Mayaro virus disease	AdMax HilQ-System (Microbix)	2021 [31]
	dAD5GNE	Cocaine dependence	Deletion of E1a, partial E1b, and E3 in the Ad5 genome with the addition of LacZ. Disruption of the genome via incubation in 0.5 % sodium dodecyl sulfate. Activated GNE cocaine analogs were than attached to the capsid.	2020 [32]
26	Ad26.Cov2.S (JnJ)	COVID-19	Plasmid/cosmid system for construct building (pAdApt26 and pWE.Ad26ΔE.5orf6)	2007, 2019, 2022 [54,55,96]
	Sputnik V/Gam-Covid Vac	COVID-19	Homologous recombination	2020 [84]
	Ad26.RSV.FA2	Respiratory syncytial virus (RSV) disease	AdVac® adapter plasmid/cosmid combination	2012, 2015, 2019 [46,56,55]
	Ad26.RSV.preF	RSV disease	AdVac® adapter plasmid/cosmid combination	2023, 2020 [57,58]
	rAd26 EBOV	Marburg virus and Ebola virus disease	AdVac® Cloning the expression cassette into E1 deleted adapter plasmid, and transfection with a cosmid into Ad26 adenoviral sequence.	2018, 2012 [45,46]
	Ad26.ZEBOV + MVA-BN-Filo (modified vaccina Ankara, MVA)	Ebola virus disease	Crucell Holland homologous recombination	2023, 2016 [47,48]
	Ad26-EnvA vaccine	AIDS	Blunt end DNA Ligation	2013, 2016 [49,97]
	Ad26.Mos4.HIV	AIDS	Unknown	2020, 2018 [50,51]
	Ad26.ZIKV.001	Zika virus disease	Homologous recombination, plasmid/cosmid system with pAdApt adaptor plasmid containing the left end of Ad genome and a transgene cassette as well as a deletion of E1 and pWE cosmid with most of the Ad genome but with deletion of E3 and modification of E4	2007, 2018, 2021 [52–54]
35	rAd35 EBOV	Marburg virus and Ebola virus disease	Cloning the expression cassette into E1 deleted adapter plasmid; co-transfection with an Ad26-cosmid	2018, 2012 [45,46]
	Ad35.Env	AIDS	Transgene in the E3 region under the influence of an E3 promoter. Blunt end ligation with pCR-TOPOblunt-4 vector (Invitrogen)	2007, 2016 [97,98]
	Ad35.RSV.FA2	RSV disease	AdVac® adapter plasmid/cosmid combination	2019, 2015, 2012 [55,56,46]
	HAdV35 plX with mammalian codon optimized *P. falciparum* CS protein	*P. falciparum* malaria	Cosmid clone/adapter plasmid with pIX deleted pWE.Ad35.ΔpiX. EcoRV (left), pBR.Ad35 PRdE3 5Eorf6/7, and pAdapt35.Bsu.pIX-mod (right)	2003; 2017 [67,99]
	AERAS-402 live	Tuberculosis	Homologous recombination manufactured by Crucell, Netherlands	2017 [100]

(*continued on next page*)

Table 1 (*continued*)

Types	Vaccine	Disease targeting	Vector construction method	Year and Reference
48	Ad48/MVA + TLR-7 stimulation	Simian immunodeficiency virus infection	Plasmid/cosmid system with a pAdApt adaptor plasmid containing the left end of the Ad genome and a transgene cassette as the deletion of E1 and pWE cosmid with most of the Ad genome; deletion of E3 and modification of the E4 region	2007, 2020 [54,60]
	AD48-CMV-ASP-2 Ad48-pIX-ASP-C Ad48-pIX-gp83	Chagas disease	Adapter plasmid pWE.Ad48.ΔE3.orf6 plus PCR-amplified transgenes of interest	2016 [101]
Chimpanzee AdVs				
1	ChAdOx1-MVA	5T4-positive tumors	Gateway® recombination technology (Thermo Fisher Scientific; PCR based, with pENTR4 vector as shuttle plasmid), [79,82]	2017, 2012 [73,81]
	ChAdOx1 NP+M1	Influenza	GalK recombineering [59]	2019, 2014 [74,20]
	ChAdOx1 (ChAd-E2Δ12) + E2Δ12$_{HMW}$ Protein boost	Hepatitis C virus disease	Gateway® recombination technology (Thermo Fisher Scientific; PCR based, with pENTR4 vector as shuttle plasmid) [79,82]	2012, 2018, 2022 [75,81,102]
	ChAdOx1 Chik	Chikungunya virus disease	Gateway®recombination (LR-Clonase II system, Invitrogen) [79,82]	2021, 2019, 2012 [81,76,80]
	ChAdOx1-ZIKV	Zika virus disease	Gateway®recombination technology (Thermo Fisher Scientific) [79]	2018 [78]
	ChAdOx1 nCov-19 (AstraZeneca)	COVID-19	Gateway®recombination technology (Thermo Fisher Scientific) [79,82]	2020, 2012 [81,77]
3	ChAd 3 EBO Zaire ChAd 3 EBO Zaire Sudan + Boost with MVA-BN Filo	Ebola virus disease	Homologous recombination (bacterial strain unknown [103])	2014, 2017 [104,105]
7	AdC7-M/E	Zika virus disease	Replication incompetent Ad molecular clones using unique restriction sites and cloning the transgene part by part into a plasmid. Plasmid and Ad genome have PI-SceI and I-CeuI restriction sites to shuttle the gene of interest into the AdV genome	2010, 2018 [106,107]
63	ChAd63-SUDV GP	Ebola virus disease	Homologous recombineering in *E. coli* BJ5183	2014 [104]
	ChAd63-EBOV GP	Ebola virus disease	Homologous recombineering in *E. coli* BJ5183	2014 [104]
	ChAd63-MVA MSP-1 ChAd63-AMA1	*P. falciparum* malaria	Homologous recombineering, manufactured by Clinical Biomanufacturing Facility, University of Oxford IDT Biologika, Rosslau, Germany	2011, 2012, 2014 [108,109,15,69,68]
	ChAd63-Me Trap MVA Me-TRAP boost	*P. falciparum* malaria	Same as above in ChAd63-MVA MSP-1 and ChAd63-AMA1	2011, 2012 [108,15]
	ChAd63-PvDBPII, MVA	*P. vivax* malaria	Homologous recombineering	2022 [110]
	ChAd63-Me Trap	Leishmaniasis	Homologous recombineering (Vector Biolabs)	2012, 2017, 2021 [15,111–113]
155	ChAd155-RSV	RSV disease	Homologous recombineering Strain and manufacturing unknown	2020, 2023 [114,65]
RhAd52	RhAd52-ZIKV.M-Env	Zika virus infection	Gibson Assembly [63]. The transgene for membrane and envelope structural protein (M-Env) form ZIKV strain BeH815744 that was cloned into RhAd52	2016, 2017 [61,62]

gap. The Ad4 plasmid was linearized through restriction digestion in the E3 region, and the HA transgene was recombined with the linearized vector and transformed into BJ5138 recombinant competent bacteria cells. The published clinical trials showed good immunity after single-shot vaccination in healthy adults aged 18–49 years (clinical trial numbers: NCT01443936 and NCT01806909). Moreover, Ad4 is also being explored for vaccination against HIV-1 [43] and Zika virus disease [29]. Out of 10 vaccinated rhesus macaques, 7 showed no viral cell load after an HIV-1 challenge. Recently, Ad4-prM-E (pre-membrane envelope protein) was tested head-to-head with Ad5-prM-E. Interestingly, increased T-cell response was observed only for Ad5-prM-E, but not for Ad4-prM-E. However, the authors suggested that the transgene sequences might not be compatible with mouse mechanisms [44].

2.3. Intensive research on other human adenovirus types 26, 35, and 48 (Ad26, Ad35, and Ad48) and non-human adenoviruses as vaccine vectors

Human adenovirus type Ad26 has been well-established in the field of vaccine research. Several Ad26-based vaccines are currently in clinical trials, with notable efforts directed against the latest pandemic virus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Ad26 is being explored for vaccines against several severe viral diseases, including Ebola [45–48], HIV-1 [49–51], Zika [52–54], and respiratory syncytial virus (RSV) [46,55–58]. For the Ad26.ENVA.01 vaccine targeting HIV-1, a replication-deficient Ad26 was recombined with an HIV-1 clade A envelope glycoprotein (Env) gene encoding the gp140 protein [20]. The clade A Env gene was inserted into the pAdapt26 adapter plasmid (Crucell Holland, Netherlands) via restriction enzyme digest, followed by blunt end ligation. This construct, containing the Env gene, was then transfected into HER96 cells for virus production [59]. Another construct, Ad26.Mos.HIV [51], was developed in 2018. This vector expresses optimized mosaic antigens of HIV-1 Env and Gag-Pol. This Ad26-based vaccine demonstrated promising results in both clinical phase I/II studies in humans and in rhesus monkeys. Additionally, in 2020, Ad48 was published [60] for vaccination against simian immunodeficiency virus (SIV) in rhesus macaques, aiming to combat the infection in NHPs.

In 2021, a clinical phase I study was conducted for Ad26.ZIKV.001 to evaluate the humoral and cellular immune responses in healthy adults [52]. This study aimed to develop a vaccine against Zika virus disease following the epidemic outbreak in 2015 [53]. The vaccine vector was constructed through homologous recombination using a plasmid/cosmid system. This involved the use of pAdApt, as an adap-

tor plasmid containing homologous regions to the Ad26 genome, and the transgene expression cassette that replaces the E1 region [54]. The second game player was pWE cosmid. It contains most of the Ad genome, including the inverted terminal repeat sites (ITRs), with deletion in the E3 region and modifications to the E4 region. Another vaccine targeting Zika virus disease utilized the rhesus monkey adenovirus vector 52 (RhAd52) [61,62]. This vector was modified with the membrane Env transgene and cloned using Gibson Assembly [63]. Initial animal studies have shown encouraging results. Compared to human adenovirus vectors, RhAd52 exhibits low seroprevalence, but comprehensive studies on potential side effects in humans are still needed.

RSV causes severe lower respiratory tract infections, particularly in children, older adults, and immunosuppressed individuals [64]. In 2015, preliminary mouse studies on Ad26 and Ad35 vectors expressing the RSV fusion protein were conducted in mice [56]. The vectors were generated through recombination using the AdVac® system for [55]. More recent trials for children aged 12–34 months (clinical trial number NCT02927873) involved a modified chimpanzee adenovirus 155 (ChAd155) with transgenes coding RSV fusion proteins and nucleoproteins [65]. Clinical phase I/II trials in young children (12–36 months old; clinical trial number NCT02927873) and a phase I clinical trial in adult humans (clinical trial number NCT02491463) have shown promising initial results.

To combat filoviruses such as Ebola and Marburg, Ad26 and Ad35 have been adapted to express GPs from both viruses. For Ebola, the vectors incorporate transgenes coding for Mayinga gp, SUDV Gulu GP, and TAFV GP whereas for the Marburg virus, MARV Angola G was used. These vectors were tested in rhesus macaques, with Ad26 serving as the primary vaccine and Ad35 as a booster administered 2 and 3 weeks later [46,66]. These vectors were modified in the deleted E1 region, using the AdVac® system [46]. The cloning process involved recombination between an adapter plasmid containing the expression cassette and a cosmid carrying the actual Ad26/Ad35 genome. [66].

Protecting against malaria, particularly *Plasmodium falciparum* malaria is associated with challenges. However, in 2017, an Ad35 vector modified with protein IX (pIX) by Salisch et al. demonstrated promising results in *in vitro* experiments. The group employed homologous recombination using a plasmid/cosmid concept to construct this new vaccine design [67]. Additionally, as outlined in Table 1, chimpanzee adenoviruses have also been used as vectors against malaria infection. Modified ChAd63, expressing merozoite surface protein 1 and apical membrane antigen 1 (AMA1) [68,69], is currently under investigation in phase I/II clinical trials [70].

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* [71], continues to be a significant health threat [72], as evidenced by the increase in TB-related deaths in 2021. A notable development was the 2017 publication by van Zyl-Smit et al. of a phase II trial for an Ad35 tuberculosis vaccine modified with three antigens targeting TB (clinical trial number NCT02414828). This study observed immune responses in participants with past or current pulmonary disease and found the vaccine to be safe and capable of inducing a robust immune response.

Considering the challenge of seroprevalence associated with human adenovirus vectors, researchers have been exploring non-human adenoviruses for vaccine development. A prominent example is the ChAdOx-1 vaccine, based on chimpanzee adenovirus 1, initially developed at Oxford University [73–78]. The ChAdOx-1 vector was recombined using the Gateway®recombination technology [79]. This vector has demonstrated impressive results in various clinical trials, such as in 2021 with ChAdOx-1 Chik against Chikungunya disease (clinical trial number NCT03590392), which expressed CHIKV structural proteins that can form virus-like particles [80] in healthy adults. The same technology was employed for the ChAdOx1-HCV vaccine, incorporating strongly conserved HCV immunogenes [81] inserted in the ChAdOx-V vector to address a broad range of HCV variants. In 2016, ChAdOx-1-ZIKV was developed following the Zika virus epidemic in South America, South Africa, and Asia [78], using the prM-E transgene with an Asian consensus sequence. Additionally, ChAdOx-1 NP+M1, aimed at combating influenza [20] was tested in ferrets in 2019 [74], with the vector being recombined using the GalK recombineering system. Another noteworthy ChAdOx-1 based vaccine, the application of which is not associated with infectious diseases but with the ongoing fight against cancer [73,81], is ChAdOx1-MVA, developed in 2012 [73]. It contains a 5T4 antigen-expressing cassette to generate immunogenicity and features a modification linking to MHCII-associated invariant chains. This vector, recombined using the Gateway® method, was tested in mice, which showed a decrease in tumor growth [79,82].

2.4. Contribution of adenovirus vaccine to combat COVID-19

The COVID-19 pandemic has brought unprecedented attention to adenoviral vector vaccines, particularly with the development of the COVID-19 vaccine, also known as the AstraZeneca vaccine. This vaccine uses the ChAdOx1, an adenoviral vector, which was modified using Gateway® recombination technology [79,82] against the pandemic outbreak of SARS-CoV-2 in 2020. In Gateway® recombination, the gene of interest is inserted into an entry plasmid with specific attL1/L2 sites, which then recombine with attR1/R2 sites in the destination plasmid. This recombination is facilitated by LR-Clonase II enzyme activity, with different antibiotic resistances in the destination plasmid and the entry plasmid serving as selection markers.

The onset of the SARS-CoV-2 pandemic in 2020 put significant pressure on researchers and pharmaceutical companies to develop efficient vaccines against COVID-19 rapidly. This urgency was to prevent severe infections, lung damage, and other consequences of the coronavirus [83]. Consequently, various adenovirus vector vaccines advanced to phase III clinical trials in a matter of months. Jacob-Dolan and Barouch [9] reviewed four distinct adenoviral vector vaccines: Ad26.COV2.S vaccine (Johnson & Johnson, New Brunswick, USA), ChAdOx-nCov19 (AstraZeneca, Cambridge, UK), Ad5-nCov (CanSino, Tianjin, China), and Gam-COVID-Vac/Sputnik V (Gamaleya Research Institute, Moscow, Russia) [84].

Despite rare cases of vaccine-induced immune thrombotic thrombocytopenia [85] observed with some adenovirus-based COVID-19 vaccines, these vaccines have played a crucial role in curbing the spread of SARS-CoV-2 [9]. With an expanding understanding of adenovirus-cell/protein interactions and adenovirus immunology, future adenovirus vectors are anticipated to have reduced adverse side effects and improved safety profiles. Considering their efficient production and remarkable efficacy, adenovirus vectors are expected to maintain a key platform for managing infectious diseases in the future.

3. Adenovirus as a gene therapy vector: advances in helper-dependent adenoviral vector (HDAdV) development

3.1. Advantages of HDAdV

Adenoviral vectors are among the most utilized vector types for gene delivery both *in vitro* and *in vivo*, effective in targeting both dividing and quiescent cells. Since the initial concept of adenoviral vector in the 1990s, several generations of adenoviral vectors have been developed. The first and second generations, featuring deletions of early genes E1 and E3 (first generation) and additional deletions of E2 or E4 (second generation), gained popularity because of their straightforward production, particularly for Ad5 using commercial kits [115]. However, in *in vivo* gene therapy, the presence of viral proteins expressed by the residual virus coding gene can lead to the elimination of transduced cells by the immune system, thereby limiting the duration of transgene expression *in vivo* [116]. To circumvent the potential toxic effects of these adenoviral proteins, HDAdVs have emerged as the most suitable option. These vectors, also known as “gutless adenoviral vectors,” are characterized by the absence of all viral coding

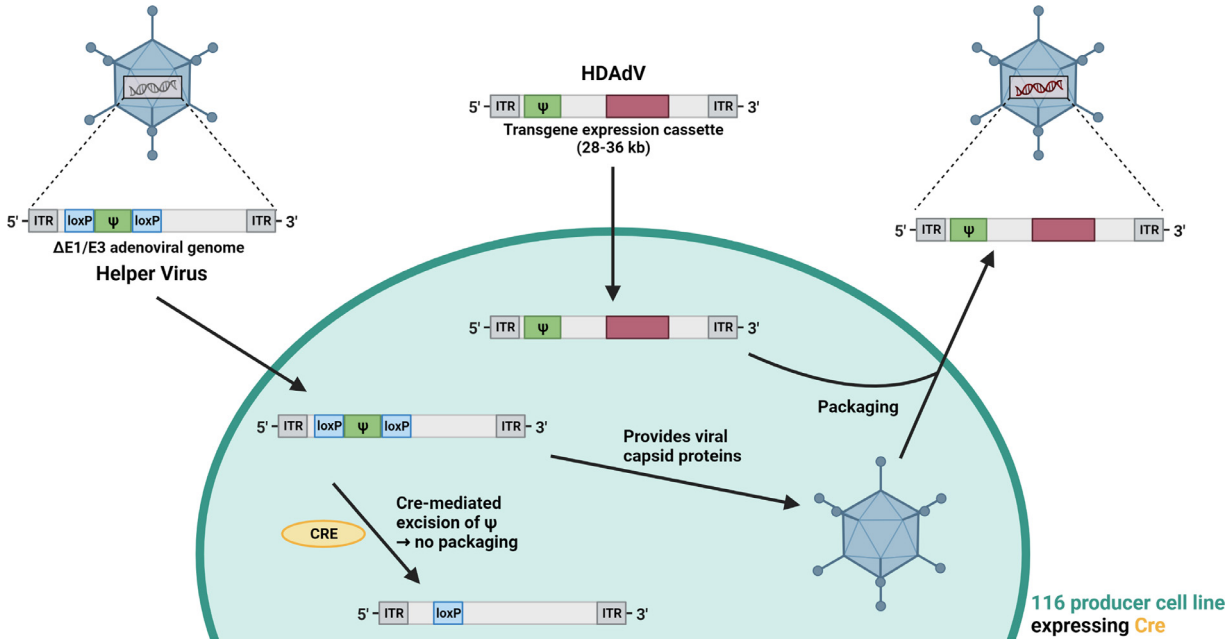

Fig. 4. Helper-dependent adenovirus vector (HDAdV) production. Graphical representation of the method for the production of HDAdV. The linearized HDAdV genome containing the gene of interest is transfected into the HDAdV producer cell line (116 cells) followed by helper virus transduction providing all necessary proteins for HDAdV packaging in trans. The red bar represents the gene of interest (created with BioRender.com).

sequences. The only adenoviral sequences retained, which are essential for genome packing, are noncoding: the ITRs at both ends and the packaging signal at the 5′ end. This extensive deletion of viral sequences allows for the transfer of up to 36 kb of foreign DNA [117,118], earning HDAdVs the alternate name of high-capacity adenoviral vectors (HCAdVs).

3.2. HDAdV production

The production process of HDAdVs, as illustrated in Fig. 4, involves critical steps for virus rescue and amplification. A key aspect of this process is the efficient removal of the packaging signal from the helper virus (HV) genome, which is typically achieved through Cre/loxP recombination. This strategy results in a remarkably low HV contamination rate of only 0.1 % [119]. An alternative approach employs FLPe recombinase in producer cells to accomplish similar excision of the HV packaging signal [120]. Another noteworthy strategy involves maintaining the E3 while removing the E2 regions from the HV. This modification improves efficacy and safety. However, it requires special packaging cells to supplement the missing E1 and E2 genes [121]. Additionally, a self-inactivating HV has been developed, which incorporates the recombinase in its own genome. This innovative design addresses the challenge of efficiently eliminating the packaging signal [119]. To achieve helper-free HDAdV production, all the necessary adenovirus genes for complementation the virus replication are added via transfection of an adenoviral genome containing plasmid that lacks a packaging signal and ITR. This is coupled with the co-transfection of another plasmid encoding the adenovirus pre-terminal protein, which serves to enhance vector yields [122,123].

HDAdVs can be purified using procedures similar to those employed for other adenoviral vectors. This process typically involves CsCl density ultracentrifugation, followed by desalting through size exclusion chromatography [124,125]. The purified HDAdV can then be quantified. The physical genome titer, virus particles (vp), can be determined by measuring the absorbance at 260 nm [126,127]. The quantity of infectious unit contained in the final vector preparation can be accurately measured using a vector genome-specific quantitative polymerase chain reaction [128]. Both methods are reproducible in practice.

3.3. Factors playing a role for HDAdV used in gene therapy

Upon entering a cell, HDAdVs efficiently deliver their genetic payload into the nucleus [129]. However, cell defense mechanisms, such as Toll-like receptors and nucleic acid sensors, can detect the vector components and DNA, triggering an immune response. This response typically involves the release of type I interferons, tumor necrosis factor α, and other cytokines [130]. Although the non-replication characteristic of the HDAdV genome minimizes the activation of antiviral defenses, the absence of E3 and E4 genes in these vectors indicates the absence of the natural adenoviral mechanisms for counteracting these immune responses [131]. Consequently, predicting the impact of antiviral pathways on transgene expression efficiency becomes challenging.

After cell entry, the HDAdV genome exhibits better stability compared with dsDNA introduced via non-viral methods [132]. Within an hour of infection, the adenovirus genomes are still associated with core proteins, while histones begin to attach to the adenovirus DNA. In the absence of replication, histone H3.3 primarily associates with the vector genomes, progressively replacing protein VII [133].

The lack of viral coding sequences in HDAdV allows for a substantial capacity of up to 36 Kb for foreign DNA delivery. In addition to the transgene, non-coding human DNA, called stuffer DNA, is normally used to fill up the adenovirus genome to maintain the vector stability. Such “stuffer” DNA, which may be homologous to specific chromosomal regions, can influence both the duration of transgene expression and the maintenance of the HCAdV genome within the cell [134]. The persistence of the genome at the cellular level is crucial, but also other cellular- and tissue-related factors within the targeting organ can significantly impact the stability of the transgene-related phenotypic effect *in vivo*.

The effects of the transgene product itself are also pivotal. Variations have been observed when the vectors are administered to different-aged rodents, dogs, macaques, and baboons. Notably, prolonged stability has been documented in adult NHPs when the transgene encodes an en-

Table 2
Summary of gene delivery with helper-dependent adenoviral vectors (HDAdV).

Types	Transgene/disease	Vector construction method	Year and Reference
Ad2	AdSTK109, hemophilia B	Recombination *E. coli* strains HBIOI or HMS174	1999 [142,164]
Ad5/ AdSTK109	α1-antitrypsin (hAAT) gene	Homologous recombination *E. coli* strains HBIOI or HMS174	1999 [142]
Ad5	human 1-antitrypsin (hAAT),	Homologous recombination *E. coli* strains HBIOI or HMS174	2000 [165]
Ad5	FG-Ad-cE, the apolipoprotein (apo) E gene	Homologous recombination *E. coli* strains HBIOI or HMS174	2001 [144]
Ad5	HD-CMV-cFVIII, HD-HNF-cFVIII, hemophilia A	Homologous recombination *E. coli* strain BJ5183	2003 [166]
Ad5	Ad5 OTC vector, ornithine transcarbamylase deficient	Information not found	2003 [167]
Ad5	Coagulation factor IX	Information was not found	2005 [141]
Ad5	bAFP	Homologous recombination *E. coli* strain BJ5183	2006 [168]
Ad5	FDAdhGAA, glycogen storage disease type II	Cre-lox recombinant technology	2006 [169]
Ad5	Aapolipoprotein A-I (APOA1) gene	Homologous recombination *E. coli* strain BJ5183	2007 [145,146]
Ad5	pPN-myc-mFLdys	*E. coli* LacZ	2008 [170]
Ad5	A1ATD	Homologous recombination *E. coli* strain BJ5183	2009 [143]
Ad5F3	HDAd5/3-luc and HDAd5-luc	Homologous recombination *E. coli* strain JM110	2012 [159]
Ad5	(hApoA-I), low-density lipoprotein-receptor-deficient	Homologous recombination *E. coli* strain BJ5183	2013, 2004, 2003 [146,148,171]
Ad5	hPBGD HDA-hPBGD	Homologous recombination *E. coli* strain BJ5183	2013 [172]
Ad5 (HD-RIGIE)	*GUSB* cDNA	Information not found	2014 [173]
Ad5	(HDAd-AGT) HDAd vectors for liver-directed gene therapy of PH1	Homologous recombination *E. coli* strain JM110	2016 [174]
HAd5-CB-CFTR	CFTR	Restriction digestion and homologous recombineering, *E. coli* strain BJ5183	2018, 2003, 2002 [175–177]
Ad5F35++	HDAd5F35++	Unknown	2016 [161]
Ad5F3++	HDAd5F3+	Homologous recombination *E. coli* strain JM110	2022, 2003 [163,178]
Ad6F35++	HDAd5F35++	Homologous recombination *E. coli* strain GB05-dir *E. coli* strain GBRed-GyrA462	2023 [179]

dogenous protein, with the protein detectable in serum for over 7 years post-vector administration [135].

In the upcoming sections and in Table 2, as well as Supplementary Table 2, we will delve into the current HDAdVs used in gene therapy. This will include their development path, disease targeting, major features, and achievements. Additionally, we will underscore the role of recombineering in vector development and its potential importance for future vector innovations.

3.4. Implementation of HDAdVs into practice

HDAdVs have been increasingly used for therapeutic purposes, particularly targeting liver diseases and metabolic disorders. One significant application of HDAdVs involved the use of vectors carrying up to 7 Kb of cDNA for coagulation factor VIII (FVIII), under the regulation of the albumin promoter [136]. This approach successfully restored FVIII circulation, as evidenced in studies involving mice and dogs [137]. A clinical trial ensued with patients receiving an intravenous injection of 4.3×10^{11} vp/kg showed initial efficacy as indicated by an increase in FVIII levels from <1 % to 3 % in serum. However, the trial was terminated because of side effects such as acute thrombocytopenia, elevated liver transaminases, and increased IL-6 levels [138]. In the context of hemophilia B, characterized by coagulation factor IX (FIX) deficiency, significant progress has been achieved using HDAdV therapy in mice and dogs [139,140]. Subsequent research from another group also succeeded in maintaining therapeutic levels of FIX in a canine model for hemophilia B [141]. Additionally, studies in NHPs have focused on the stability and efficacy of transgene expression involving α−1 anti-trypsin and α-fetoprotein [142,143].

HDAdV has also shown effectiveness in treating dyslipidemia models. For instance, a single intravenous injection of HDAdV in Apo E-deficient mice could permanently regulate cholesterol levels. This was achieved by combining the Apo E gene and its regulatory sequences within the same genome [144]. Furthermore, vectors containing Apo AI could increase high-density lipoproteins and reduce low-density lipoproteins (LDLs), and even mitigate atherosclerotic lesions in Apo AI-deficient mice [145,146]. In contrast, cardiovascular issues decreased in Apo E and LDL receptor-deficient mice models [147,148]. In rabbits on a high-fat diet, HDAdV encoding Apo lipoprotein A-I delivered via arteries led to enduring expression from endothelial cells and reduced atheromatous plaques [147]. Moreover, co-expressing Apo AI and ABCA1 using HDAdV increases cholesterol efflux from endothelial cells [149]. These studies underscore the need to optimize delivery routes and expression cassettes, especially for diseases requiring high levels of transgene expression.

In the realm of using gene therapy for neurological diseases, a technique named Trio (tracing the relationship between input and output) stands out as an innovative approach. It combines three different vectors: canine adenovirus type 2, adeno-associated virus (AAV), and rabies

virus vectors. This technique is used to map input–output connections and identify information pathways in the brain [150]. In another interesting research, Ad5-based vectors can be directed in a controlled, retrograde manner, making them potentially useful in similar applications [151–153]. These studies have employed Cre knock-in mice to decipher the roles of neurons and their connections using opto- and chemogenetic techniques. Employing HDAdV vectors expressing Cre recombinase, detailed cellular-level investigations can be conducted. Additionally, HDAdvs have been used in models mimicking neurodegenerative diseases. In studies involving mice and NHPs, HDAdVs expressing truncated forms of the protein LRRK2 were used to simulate inflammation relevant to these illnesses [154,155]. This approach was also adapted to develop a continuous *in vitro* model of Huntington's disease using basic neuron collections [156].

The application of HDAdVs in treating muscular dystrophies, such as Duchenne muscular dystrophy, has shown promising results. By delivering full-length cDNA (14 Kb) under the control of a potent CAG promoter, HDAdVs have been instrumental in restoring dystrophin expression [157]. Studies using mouse models have demonstrated that intraperitoneal injection of these vectors can restore respiratory function [158]. Additionally, the local administration of Ad5-based chimeric vector with fiber knob from Ad3 (creating a Ad5F3 chimera) has been effective in increasing muscle infection and sustaining dystrophin expression [159,160].

The complexity and genotoxic risks associated with cell-based gene therapies have led to the development of a new gene therapy concept for the direct *in vivo* transduction of hematopoietic stem cells (HSPCs). This innovative approach involves mobilizing HSPCs from the bone marrow into the peripheral bloodstream, followed by intravenous injection of viral vectors for safe and efficient stem cell transduction. Specifically, a chimeric Ad5 vector equipped with an optimized Ad35 fiber knob from species B (Ad5F35++), which exhibits increased CD46 binding, is employed. In experiments involving hCD46 transgenic mice, this method facilitated the sustained transduction of primitive HSPCs, as evidenced by green fluorescent protein (GFP) marking in bone marrow HSPCs (Lin-Sca1+Kit cells) in most mice. This indicates successful transduction and expansion of long-term surviving HSPCs [161].

To mitigate the anti-Ad5 antibody reaction associated with HDAd5/35++ vectors, a new vector, HDAd6/35++, derived from Ad6, has been developed. This vector efficiently transduces human and rhesus $CD34^+$ cells *in vivo*. The transduction efficiency of HSCs in both the bone marrow and spleen was comparable when either HDAd5/35++-GFP or HDAd6/35++-GFP vectors were injected intravenously in mice with established human hematopoiesis or human CD46 transgenic mice following G-CSF/AMD3100 mobilization. In long-term studies, at least 75 % of peripheral blood mononuclear cells showed stable GFP expression with both vectors. Notably, HDAd6/35++ demonstrated a lower rate of unintended transduction of hepatocytes compared to HDAd5/35++. Furthermore, HDAd6/35++ enabled efficient *in vivo* HSC transduction even in Ad5 pre-immune mice [162].

Another significant development in*in vivo* HSPC transduction involves the use of desmoglein 2 (DSG2)-targeted Ad3 fiber. The HDAd5/3+ chimeric vectors, based on Ad5 but incorporating fibers from Ad3, were designed to utilize DSG2 as a high-affinity attachment receptor. The safety and efficacy of *in vivo* HSPC transduction were evaluated following the intravenous injection of HDAd5/3+ vectors expressing GFP in granulocyte colony-stimulating factor/AMD3100 (plerixafor)-mobilized rhesus macaques. To enhance the reintegration of transduced mobilized HSCs into the bone marrow, transient expression of CXCR4 was induced using the HDAd5/3+ vector from mobilized HSPCs. Remarkably, up to 7 % of GFP-positive CD34+/CD45RA-/CD90+ cells were observed in the bone marrow following *in vivo* transduction with an HDAd5/3+GFP/cxcr4 vector at a low dose of $0.4 \times 10\hat{}12$ viral particles/kg. This conversion rate lays a promising foundation for the use of base or prime editing *in vivo*, as well as for the natural or drug-induced expansion of modified HSCs [163].

Collectively, preclinical studies have substantiated the capability of HDAdVs in effectively delivering genetic material to intended target cells in living organisms, primarily focusing on hepatocytes, neurons, and endothelial cells. Notably, in these cells, the HDAdV genomes are stably maintained as episomes, facilitating continuous transgene expression. This phenomenon has been observed in rodent models and in more complex animal models, such as NHPs.

Despite the wealth of data demonstrating the success of these vectors in treating a variety of diseases, their translation into clinical settings has been limited. This is partly due to the focus on other vector systems, such as AAVs, which have shown comparable pre-clinical efficacy within the 5 Kb size limit of their therapeutic cassettes. AAVs also tend to elicit fewer inflammatory responses and are easier to manufacture under good manufacturing practice conditions. Nevertheless, progress in the development of HDAdVs is ongoing, particularly in refining delivery pathways, devising techniques to prevent and manage side effects, and establishing protocols for large-scale production. HDAdVs are expected to play a crucial role in the field of gene therapy, particularly for delivering large transgenes, owing to their capability to load transgenes up to 36 KB. Beyond being an alternative to AAVs and lentiviruses, HDAdVs are critical in meeting the demands of advanced gene editing and the transportation of lengthy genetic sequences.

3.5. Adenoviruses as oncolytic agents

Adenoviruses have emerged as the most prevalent type of vectors used in clinical trials for oncolytic therapy, constituting 31 % of total trials [180] (https://clinicaltrials.gov/). Among the 114 known types of human adenoviruses (http://hadvwg.gmu.edu/), Ad5 from species C is the most extensively studied. Researchers have transformed wild-type adenoviruses into OAds by genetically modifying the E1A, E1B, and E3 regions and the knob domain of the virus [180,181]. These modifications are often combined to enhance tumor specificity, lysis function, and safety profiles of the OAds. Additional, immunomodulators are often expressed from OAds to enhance the host antitumor immunity, such as the recent research on the combination of OAds with immune checkpoint inhibitors for cancer treatment. This approach aims to further stimulate the host's immune system, leading to more efficient anticancer therapy [180,181]. The generation of OAds over the past decade has primarily used a combination of restriction enzyme-based cloning and homologous recombination. For comprehensive information on all generated OAds developed to date, refer to Table 3 and Supplementary Table 3. More detailed information about OAds approved and in clinical trials is not the focus of this review and summarized elsewhere [182–184].

3.6. Knob exchange and chimeric adenoviruses

The first modification of the adenovirus genome to enhance tumor targeting was performed by replacing the Ad5 fiber knob with that of Ad3 in 1996 through homologous recombination [185]. Subsequently, the Ad5 knob was replaced with knobs from Ad35 [186–189] and Ad37 [190]. This strategy aimed to overcome the limitation of Ad5's reliance on the coxsackievirus and adenovirus receptor (CAR), which is often downregulated in tumor cells. More recently, a chimera combining Ad3 and Ad11p, specifically in the E2B region, has demonstrated high oncolytic potential [191–193].

3.7. Modifying the E1 region essential for virus replication

The E1 replication region of the adenovirus genome is critically modified to ensure that oncolytic adenoviruses replicate exclusively in cancer cells, not in healthy ones (Fig. 5). A common modification involves controlling E1A gene expression under a tumor-specific promoter. This allows adenovirus replication only in cancer cells expressing the corresponding protein of that promoter. Tumor-specific

Table 3
Summary of adenoviruses as oncolytic viruses.

OAd	Modification	Generation	Year and Reference
Chimerics			
Ad5/3	Chimeric fibers, Ad5 with Ad3 knob	Homologous recombination (Dam-*E. coli* strain JM110)	2021, 2019, 1996 [225,226,185]
Ad5/35 (SG635-p53)	Chimeric fibers, Ad5 with Ad35 knob	Homologous recombination (*E. coli* BJ5183)	2018, 2013, 2011 [188,187,186]
Ad3/11p (ColoAd1)	Ad11p ΔE3, ΔE4, chimeric Ad3/Ad11p E2B region	Directed evolution	2019, 2008 [192,191]
OAd1, OAd2	Chimeras of ColoAd1 and Ad3	Directed evolution	2017 [193]
Ad5/37	Ad5 temporally carrying Ad37 knob	HEK293 expressing Ad37 fiber and miRNA for knock down of Ad5 fiber	2020 [190]
Promoters			
Ad3-hTERT-E1A	E1A gene under the control of hTERT promoter	Restriction cloning and homologous recombination (bacteria unknown)	2012, 2011 [227,194]
KH901 (Ad5-hTERT)	E1A promoter replaced by hTERT (modified with two E2F-binding sites), E3gp19k replaced by GMCSF	Conventional molecular cloning techniques	2009, 2009, 2007 [195,221,228]
Ad35-hTERT-E1A	E1A gene under the control of hTERT promoter	Homologous recombination (*E. coli* BJ5183)	2023, 2021 [229,196]
Ad6-hT-GMCSF	hTERT promoter drives E1A gene and GMSCF instead of E3 6.7 K and gp19K	Homologous recombination (in-fusion kit)	2023 [199]
Ad3-hTERT-CMV-CD40L	E1A gene under the control of hTERT promoter and hCD40L inserted in the E3 region under the CMV promoter	Homologous recombination (in-fusion kit)	2021, 2018, 2017 [222,197,223]
SG600-p53 (Ad5)	hTERT promoter drives E1A Δ24; E1B is directed by HRE; p53 is controlled by the CMV promoter	Homologous recombination (packaging plasmid in HEk293 cells)	2008 [198]
Ad5/3-E2F-Δ24-GMCSF (CGTG-602)	E2F-1 promoter driving E1A Δ24, fiber knob3, E3gp19K, and 6.7 K replaced with GMCSF	Homologous recombination (*E. coli*)	2015 [200]
CG0070 (Ad5)	E2F-1 promoter drives E1A expression, GMCSF under the control of viral E3 promoter	Homologous recombination (*E. coli* BJ5183)	2022, 2006 [230,201]
ONYX-411 (Ad5)	ΔE1A-CR2 region (dl922–947) and E1A and E4 under the control of human E2F1 promoter [202]	Unknown	2000 [202]
Ad5/3cox2	Ad3 knob, Cox2 driving E1A with Δ24	Restriction cloning	2006 [189]
AvE1a04i (TSRRA)	AFP promoter drives Ad5-E1A, ΔE3	Homologous recombination in HEK293 cells	1999 [203]
YKL-1001	ΔE1B-55 K, AFP (alpha-fetoprotein) promoter drives E1A and E1B19K	Restriction cloning	2002 [204]
CV706 Cell Genesys (Ad5)	Human PSA drives E1A	Unknown	2001 1997 [231,205]
CV787 (CG7870) Cell Genesys	Prostate-specific rat probasin promoter driving Ad5 E1A and E1B	Unknown	2006, 1999 [232,206]
CV763	hK2 promoter for Ad5-E1A expression	Unknown	1999 [207]
CV764 (Prostate)	hK2 promoter for Ad5-E1A and E1B expression	Unknown	1999 [207]
Ad.DF3-E1	DF3/MUC1 promoter drives E1A expression	Restriction cloning and homologous recombination (shuttle plasmid in HEk293 cells)	2000 [208]
AdSurp-P53	Ad5-CMV-P53-survivin promoter-E1A	Restriction cloning and *in vivo* recombination	2011 [209]
Deletions			
Ad5-Δ24	Δ24 in E1A Rb protein	Homologous recombination in producing cell line	2000 [210]
Ad2&5-dl1520 (Onyx-015)	ΔE1B-55K	*In vivo* overlap recombination	2001, 2000, 1998, 1997, 1996, 1987 [233–238,216]
Oncorine H101	Ad5, ΔE1B55K, ΔE3	Unknown	2018, 2006 [7,6]
ORCA-010	Ad5-Δ24RGDΔE3gp19k	Homologous recombination (yeast strain YPH-857)	2014 [211]
AdΔΔ	Ad5-Δ24-ΔE1B19k	Homologous recombination (*E. coli* BJ5183)	2010 [212]
Ad2-dl250	ΔE1B-19K	Restriction cloning	2004, 1984 [239,217]
Ad5-dl309	ΔE3B-10.4/14.5 kDa-RID and 14.7 K deletion	Restriction cloning	2003, 1979 [219,218]
Ad5-dl704	ΔE3gp19K	Restriction cloning	2003 [219]

(*continued on next page*)

Table 3 (*continued*)

OAd	Modification	Generation	Year and Reference
Ad5-dl922–947	ΔE1A of amino acids 122 to 129 in CR-2	Restriction cloning	2000, 1989 [214,240]
Ad5-dl1101	Deletion in E1A of amino acids 4 to 25 (CR-1, p300-binding mutant)	Restriction cloning	2000 [214]
KD1	dl1101/1107 E1A and overexpression ADP, ΔE3	Restriction cloning	2000 [215]
KD3	dl1101/1107 E1A and overexpression ADP, ΔE3 except for 12.5 K	Restriction cloning	2000 [215]
Ad5-CD/tk-rep (FGR)	Onyx-015 and cytosine deaminase/HSV-1 thymidine kinase fusion gene	Restriction cloning	1998 [241]
01/PEME	PRP and E2F-Rb inserted to the E3 region RIDab and 14.7 K in reverse	Homologous recombination (*E. coli* BJ5183)	2001 [224]
Ad5-Δ24-RGD	Δ24-RGD	Homologous recombination in HEK 293 cells	2018, 2003, 2000 [242,213,210]

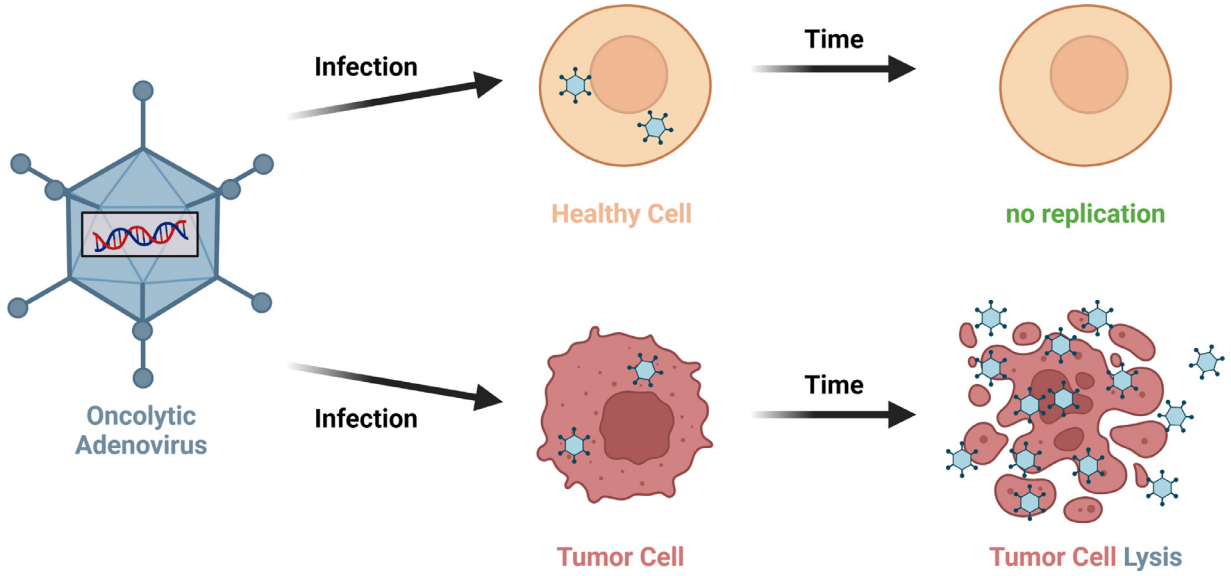

Fig. 5. Oncolytic adenovirus infection of healthy and tumor cells: the oncolytic adenovirus (OAd, shown in light blue) may infect both healthy (light yellow) and tumor (brown) cells. Over time, efficient replication of OAds occurs in tumor cells, leading to tumor cell lysis, but no replication or lysis occurs in healthy cells (created with BioRender.com).

promoters used for this purpose include the human telomerase reverse transcriptase promoter [194–199], human E2F-1 promoter [200–202], cyclooxygenase 2 promoter (Cox2) [189], α-fetoprotein (AFP) promoter [203,204], prostate-specific promoters [205,206], human glandular kallikrein (hK2) promoter [207], mucin-like glycoprotein promoter (DF3/MUC-1) [208], and the survivin promoter [209]. Another common E1A modification is the introduction of a 24 bp deletion in the CR-2 gene region. This alteration disable the competition of E1A with the tumor suppressor retinoblastoma protein (Rb) to E2F, leading to virus replication exclusively in cancer cells, where the Rb pathway is often dysfunctional [210–214,202]. An additional E1A mutation involves a deletion in the CR-1 region, preventing E1A binding to the tumor suppressor protein p300. However, this mutation is typically used in conjunction with the 24 bp deletion of the Rb binding site [214,215]. For the E1B region, modifications include deletions of the E1B-55 K [7,216,204] and E1B-19 K [217,204], and control of E1B expression under prostate-specific, hK2, and hypoxia response element promoters [206,207,198].

3.8. Influencing the host´s immune system by modifying the E3 region

The E3 region in adenoviruses plays a critical role in protecting infected cells from the host´s immune system. However, in the context of OAds, this protective function is counterproductive, as the goal is to ensure that the infected cells are eliminated. To address this, various modifications have been made to the E3 region of the OAds. These modifications often involve either a complete deletion [203,7,215] or a partial deletion of the E3 region [218,219,215]. Alternatively, gene replacements have been employed, such as the insertion of the granulocyte–macrophage colony-stimulating factor (GMCSF) [201,199,220,195,221], human CD40 ligand under the control of the CMV promoter [197,222,223], or the insertion of a p53-responsive promoter combined with the E2F-Rb pathway [224].

4. Conclusions and outlooks

With over 100 identified types in humans, adenoviruses hold immense potential as a vector toolkit for therapeutic applications. The evolution of adenoviral vector development has been remarkable, especially when comparing the earlier advancements in the first half century following their discovery to the rapid progress after the introduction of recombineering techniques into adenovirus genome engineering (Fig. 2). As discussed in Sections 2–4, this has led to the accelerated development of adenoviral vectors as therapeutic agents for gene delivery, tumor treatment, and infectious disease prevention.

Besides recombineering, various other methods have been employed and continue to be employed in adenovirus genome engineering. These

include traditional molecular cloning with restriction enzymes, cosmid-based methods with customized module selection and modification, homologous recombination in eukaryotic cells, and the more recently described Gibson Assembly. We have summarized these different strategies and the adenoviral vectors derived from each method in our previous review [11].

However, recombineering stands out as the most used and actively applied approach. Its high efficiency, flexibility, and accuracy make it a powerful tool in the advancement of adenoviral vector development, for both research purposes and as advanced medicine.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Wenli Zhang reports financial support was provided by the Internal Research Funding from Witten Herdecke University (UWH). Anja Ehrhardt reports financial support was provided by the German Research Foundation (DFG). Wenli Zhang, Anja Ehrhardt has patent ADENOVIRAL VECTORS issued to GenArc Directions GmbH. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Lisa-Marie Dawson: Writing – original draft, Resources, Formal analysis, Writing – review & editing. **Montaha Alshawabkeh:** Writing – review & editing, Writing – original draft, Resources, Formal analysis. **Katrin Schröer:** Writing – original draft, Resources, Formal analysis, Writing – review & editing. **Fatima Arakrak:** Validation. **Anja Ehrhardt:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Wenli Zhang:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Conceptualization, Funding acquisition.

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Supplementary Materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.engmic.2024.100140.

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