Biotechnology Advances 49 (2021) 107764

Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

Research review paper

Cellular pathways of recombinant adeno-associated virus production for gene therapy

Sha Sha [a], Andrew J. Maloney [b], Georgios Katsikis [c], Tam N.T. Nguyen [b], Caleb Neufeld [a], Jacqueline Wolfrum [a], Paul W. Barone [a], Stacy L. Springs [a], Scott R. Manalis [c], Anthony J. Sinskey [a,d,*], Richard D. Braatz [a,b,*]

[a] *Center for Biomedical Innovation, Massachusetts Institute of Technology, Cambridge, MA 02142, USA*
[b] *Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02142, USA*
[c] *Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02142, USA*
[d] *Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142, USA*

A R T I C L E I N F O

Keywords:
Gene therapy
Viral vectors
Adeno-associated virus
AAV
Cellular pathways
Wild-type AAV
Recombinant AAV
Mechanistic understanding
rAAV-based gene therapy
Gene therapy manufacturing

A B S T R A C T

Recombinant adeno-associated viruses (rAAVs) are among the most important vectors for in vivo gene therapies. With the rapid development of gene therapy, current rAAV manufacturing capacity faces a challenge to meet the emerging demand for these therapies in the future. To examine the bottlenecks in rAAV production during cell culture, we focus here on an analysis of cellular pathways of rAAV production, based on an overview of assembly mechanisms first in the wild-type (wt) AAV replication and then in the common methods of rAAV production. The differences analyzed between the wild-type and recombinant systems provide insights into the mechanistic differences that may correlate with viral productivity. Based on these analyses, we identify potential barriers to high productivity of rAAV and discuss future directions for improvement to meet the emerging needs set by the growth of rAAV-based therapy and the needs of patients.

1. Introduction

Cell and gene therapies are a growing part of biopharmaceutical development pipelines, with more than 1000 treatments in clinical trials at the end of 2019 (Alliance for Regenerative Medicine 2019 Report). The manufacturing demand for these novel therapies is expected to increase with the predicted rate of approval of 10 to 20 cell and gene therapies per year by 2025 (FDA, 2019). Many of these therapies require viral vectors as either a key raw material or as the therapeutics themselves.

Recombinant adeno-associated virus (rAAV) is the most widely used viral vector for in vivo gene therapy as a replication-incompetent viral vector derived from wild-type AAV which is a small virus belonging to the *Parvoviridae* family that infects humans and some other primate species (Wang et al., 2019). AAV is non-pathogenic in humans, can transduce both dividing and non-dividing cells, and can target different tissue types by using varied serotypes, allowing for the safe, efficient, and long-term expression of therapeutic genes in patients (Nonnenmacher and Weber, 2012; Wang et al., 2019). This has led to the development of rAAV-based gene therapies for patient populations on the order of hundreds, such as Leber's congenital amaurosis, to millions, such as sickle cell anemia and hemophilia (Lillicrap, 2017; Merten, 2017). A number of rAAV-based therapies have been approved for use in patients, including the Glybera® for human lipoprotein lipase (LPL) deficiency in 2012, LUXTURNA® for Leber's congenital amaurosis in 2017, and ZOLGENSMA® for spinal muscular atrophy in 2019 (Li and Samulski, 2020). These approvals have further accelerated therapeutic development, and as of this writing, over 200 rAAV-based therapies are in clinical trials worldwide (ClinicalTrials.gov), targeting a range of diseases from neurological, ocular, muscular, cardiovascular, oncologic, autoimmune, and infectious diseases (Ginn et al., 2018; Li and Samulski, 2020; Penaud-Budloo et al., 2018). The rAAV in clinical trials have diverse capsid serotypes (natural or modified), specific vector genotypes, and two main gene structures (single or double-stranded with self-complementary (sc) sequences) (Li and Samulski, 2020; McCarty, 2008; Wang et al., 2020).

* Corresponding authors at: Center for Biomedical Innovation, Massachusetts Institute of Technology, Cambridge, MA 02142, USA.
E-mail addresses: asinskey@mit.edu (A.J. Sinskey), braatz@mit.edu (R.D. Braatz).

https://doi.org/10.1016/j.biotechadv.2021.107764
Received 4 January 2021; Received in revised form 10 April 2021; Accepted 1 May 2021
Available online 3 May 2021
0734-9750/© 2021 Published by Elsevier Inc.

Current production of rAAV is based on cell culture and a defined set of genetic elements including a transgene and structural/non-structural genes for rAAV assembly. Generally, only 5 to 30% of viral particles harvested from cells have the vector genome of interest (Adamson-Small et al., 2016; Joshi et al., 2019; Lock et al., 2010; Wang et al., 2017a) and even a smaller portion can effectively transduce cells (Joshi et al., 2019). Viral particles that are either empty or contain a truncated transgene are generally considered nonfunctional and lead to an increased likelihood of immunogenicity after administration (Flotte, 2017; Gao et al., 2014). In a typical batch cell culture, the average yield of rAAV vector genomes (vg) is 10^4–10^5 vg/cell from crude harvest (Kotin, 2011; Robert et al., 2017). Volumetric yields of optimized rAAV production in bioreactors can reach the range of 10^{14} vg per 10-layer CellSTACK® chambers in adherent cell cultures (Adamson-Small et al., 2016) or the range of 10^{14} to 10^{15} vg per liter in suspension cultures (Adamson-Small et al., 2017; Aponte-Ubillus et al., 2018; Blessing et al., 2019; Chahal et al., 2014; Durocher et al., 2007; Grieger et al., 2016; Hildinger et al., 2007; Wang et al., 2017b). At present, the generation of large fractions of empty viral particles remains a barrier to process productivity, reducing vector genome titer and adding a costly downstream processing step to remove defective rAAV (Qu et al., 2007). However, the mechanistic bottlenecks in obtaining genome-containing viral particles are not well understood.

Depending on rAAV potency and whether local or systemic administration is used for patients for a specific indication, the vector dosage per patient ranges between 10^{11} and 10^{15} vg (Merten, 2017). Thus, there is an anticipated challenge to meet the demand for rAAV therapy at sufficient scale and reasonable cost in the future. With the ongoing development of gene therapies, especially for therapies requiring whole-body treatment, such as Duchene Muscular Dystrophy, and for therapies treating patients in millions such as hemophilia and sickle-cell, improved bioprocesses for rAAV are required (Fig. 1).

Satisfying the anticipated growth of rAAV-based therapy requires improving per cell yield and increasing the production of genome-containing capsids, which would be facilitated by better understanding of the key pathways in viral production biology. For that purpose, this article reviews the cellular pathways of rAAV production and discusses the potential bottlenecks. Since the production of rAAV is derived from the biology of wtAAV replication, we begin this analysis by introducing the mechanisms involved in the genome structure, replication, and replication kinetics of wtAAV, and then introduce the mechanistic pathways involved in the main methods used in rAAV production. Via the differences analyzed between rAAV production and wtAAV replication, we examine the potential deficiency in rAAV production and discuss future strategies through molecular engineering and process optimization for rAAV production improvement.

2. Wild-type AAV genome and replication mechanisms

2.1. AAV genome

Wild-type AAV is comprised of a single-stranded genome, either positive - or negative- sensed, and a 20–25 nm diameter viral capsid. As shown in Fig. 2A, the genome is ~4.7 kb long and includes two open reading frames (ORFs) – *rep* and *cap* – which encode non-structural and structural AAV proteins, respectively. At each end of the genome is a 145-base inverted terminal repeat (ITR), which is a critical *cis*-element for AAV replication. The ITR sequence is comprised of a self-complementary region (ABB'C'CA') and a 20-nucleotide single strand (D-sequence) (Fig. 2B). The *rep* gene expresses two mRNA transcripts, each of which can be spliced into a shorter form. As a result, the gene encodes four replication-related proteins named for their size in kilodaltons, respectively, Rep78 and Rep68 from promoter p5, and Rep52 and Rep40 from promoter p19. All four Rep proteins have common regions that contain domains of helicase and ATPase, while Rep78 and Rep68 have additional domains of DNA binding and endonuclease (Cassell and Weitzman, 2004). The *cap* gene expresses one mRNA transcript from promoter p40, which is spliced in two different ways, forming three viral proteins VP1, VP2, and VP3 which are 87, 72, and 62 kilodaltons in size, respectively (Kuck et al., 2007; Lux et al., 2005; Sonntag et al., 2011; Wistuba et al., 1997). The VPs are the building blocks of viral capsid and are serotype-specific. There are more than 12 serotypes currently found for AAV (Penaud-Budloo et al., 2018). The primary tropism of each serotype is determined by variable regions of each VP on the capsid surface (Govindasamy et al., 2006; Xie et al., 2002). The *cap* gene also expresses two other regulatory proteins, i.e., assembly activating protein (AAP) encoded from promoter p40 (Maurer et al., 2019; Maurer et al., 2018; Naumer et al., 2012; Sonntag et al., 2010) and gene X encoded from promoter p81. The gene X has been reported to encode a protein in the AAV life cycle with a role in boosting AAV DNA replication (Cao et al., 2015; Cao et al., 2014).

2.2. AAV replication mechanisms

AAV replication is initiated by the co-infection of host cells with an AAV and a helper virus such as adenovirus (Ad) or herpes simplex virus (HSV) (Adamson-Small et al., 2017). After viral translocation into the nucleus, the viral genome is released from the capsid and leads to the expression of AAV proteins, assembly of viral capsids, replication of the

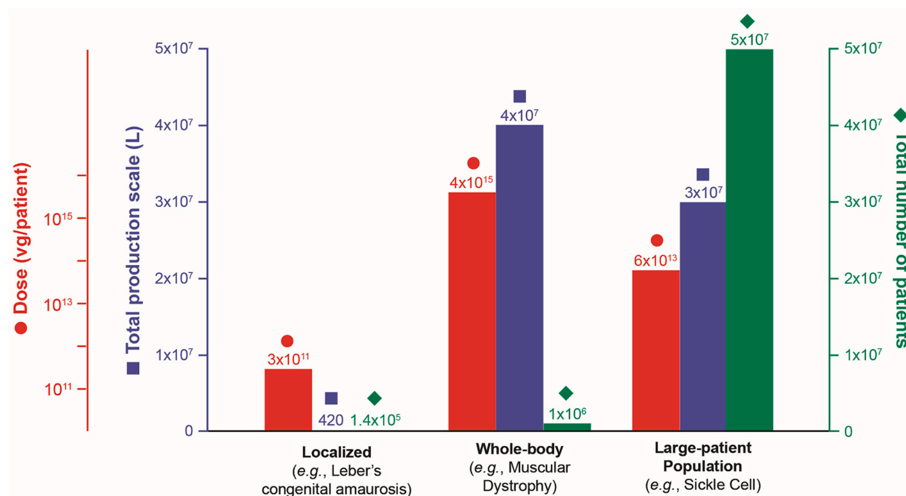

Fig. 1. The manufacturing demand of three rAAV therapies based on the dose requirement and patient prevalence. The estimation of production scale is based on a productivity of 5×10^{14} vg/L and a downstream recovery of 20%.

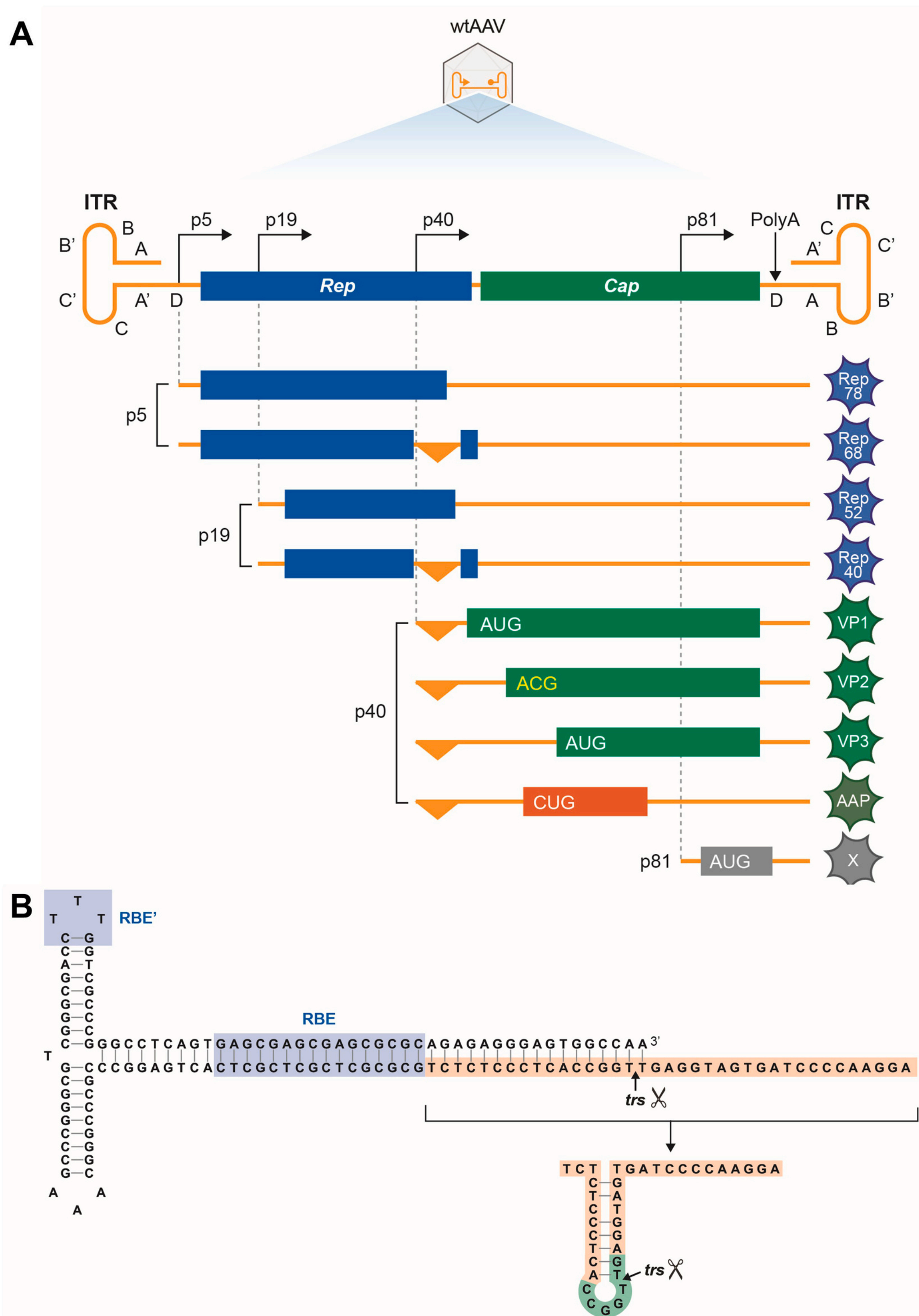

Fig. 2. The wild-type AAV genome. (A) In the wtAAV genome, Rep78 and Rep52 are transcribed under the control of promoters p5 and p19, respectively. These two transcripts can be alternatively spliced into two shorter forms, Rep68 and Rep40. The inverted triangle represents an intron that is spliced. The *cap* gene is transcribed under the control of promoter p40. The cap transcripts are spliced at the N terminus, forming three viral proteins (VPs) – VP1, VP2, and VP3 – of a successively smaller size. VP2 has an unusual initiation codon ACG which does weak translation. The ITR sequence is located at each end of the genome and contains complementary regions ABB'C'CA' (125 bp, palindrome) and a linear sequence D (25 bp). The sequence of ITR at the two ends of the AAV genome are symmetric. (B) ITR sequence of AAV2. The sequence contains a 16- base pair Rep binding element (RBE), a single tip hairpin (RBE'), and a terminal resolution site (*trs*). When Rep proteins bind at the RBE, a stem-loop is formed at the right side of the RBE and a specific cleavage is introduced at *trs*, the location pointed by the arrow.

AAV genome, and packing of the genome into a capsid (Fig. 3A). Helper virus genes play a variety of roles in AAV replication (Table 1).

2.2.1. AAV protein expression

The expression of Rep and VPs is highly regulated. The adenovirus *E1A* gene is needed to transactivate the expression of *rep* and *cap* genes (Xiao et al., 1998). P5 promoter transcription is first transactivated by *E1A* (Chang et al., 1989), and in turn, repressed by the Rep78/68 proteins (Daniel et al., 1997; Trempe and Carter, 1988). *E1A* and Rep78/68 proteins then transactivate the transcription of p19 and p40 genes (Wang et al., 2018). Other adenovirus genes such as *VA RNA* and *E2A* further support the stability of the mRNA transcripts (Qiao et al., 2002a; Xiao et al., 1998).

The expression of Rep and VPs is largely controlled by the intron size and the start codons of each spliced transcript. For Rep proteins, the unspliced form, Rep78 and Rep52, are more abundantly expressed than the spliced form, Rep68 and Rep40 (Redemann et al., 1989). On the other hand, VP transcripts are highly spliced with larger introns, thus the abundance of VP1 transcript is lower than VP2 and VP3. Because VP2 has a weak start codon ACG for initiating translation, the final expression of VP2 is also low. VP3 is formed most abundantly. After synthesis, the proteins of Rep and VPs are transported to the nucleus – the central location of AAV assembly.

2.2.2. Capsid assembly

The assembly of VPs into capsids is driven by hydrophobic, electrostatic, and hydrogen-bonding interactions, growing from small units (called capsomers) toward a final structure, the kinetics of which form a sigmoidal curve dependent on the concentrations of all three VPs. Like many other viruses, AAV capsid forms an icosahedral, symmetrical structure where free-energy reaches a minimum (Perlmutter and Hagan, 2015). A capsid of AAV is typically assembled with 5 VP1, 5 VP2, and 50 VP3, likely determined by the ratio between the VP substrates (Kondratov et al., 2017; Perlmutter and Hagan, 2015; Rapaport, 2004). Assembly-activating protein (AAP) is a critical scaffold to link VPs from their C terminus, promote viral protein stability, and facilitate VP transport into the nucleus (Maurer et al., 2019; Maurer et al., 2018; Naumer et al., 2012; Sonntag et al., 2010). Interestingly, AAP has been reported to be indispensable for capsid assembly for AAV2, but not necessarily for AAV4, 5, and 11 (Earley et al., 2017). The assembly process of a capsid is also facilitated by proteins expressed from the helper virus genome (Xiao et al., 1998).

2.2.3. Genome replication

Simultaneously with capsid formation, the AAV genome is replicated and accumulated, which occurs in a replication center in the nucleus where helper virus proteins and host cell machinery including DNA polymerase are co-localized (Weitzman et al., 1996). The steps of genome replication are outlined in Fig. 3B. As the structure of an ITR is self-primed, the ITR acts as an origin of genome replication in a DNA primase-independent manner. The synthesis of the second strand of AAV's single genome starts from the self-primed 3′ end, leading to a closed-ended, double-stranded DNA after the completion of the second strand synthesis. Rep78 or Rep68 then introduces a strand- and site-specific cleavage at a specific site named terminal resolution site (*trs*) in the ITR by binding to a 16-base pair site in the ITR composed of tetranucleotide repeats with the consensus sequence 5'-GNGC-3′, named Rep-binding element (RBE) (Im and Muryczka, 1990; Snyder et al., 1990; Urabe et al., 2002). The Rep protein tethered on the RBE uses its DNA helicase domain to unwind the RBE to result in a stem-loop at the *trs* region (Fig. 2B), and also associates with several nucleotides of RBE' at the hairpin tip in order to direct the endonuclease catalytic domain to the *trs* and finally introduces the cleavage (Brister and Muzyczka, 2000). An additional RBE is potentially present in the D sequence to stabilize the binding of Rep proteins (Wang and Srivastava, 1997). The Rep proteins of most serotypes are similar enough to interact with the ITRs of other serotypes interchangeably with the exception of Rep protein for AAV5, which has the least similarity to other serotypes and only cleaves the *trs* sequence specific for AAV5 (Chiorini et al., 1999). After the strand from the cleavage site linearizes at the 5′ end, the other strand extends to the full length by a repairing mechanism. The double-stranded (ds)DNA formed at this step is the key substrate of the single strand progeny packaged into a capsid. Alternatively, DNA monomer may concatemerize into dimers if the 3′ hairpin is not cleaved at the *trs* (Fig. 3B-b).

2.2.4. Genome packaging

A single-stranded (ss) DNA progeny is derived from the dsDNA by genome replication and packaged into a preformed capsid, a process called strand displacement synthesis (Myers and Carter, 1981). Several important observations have been made about AAV genome packaging. First, capsids and Rep52 or Rep40 proteins form stable intermediate complexes (Dubielzig et al., 1999; Wistuba et al., 1995) and ssDNA is only formed at the presence of such complexes (Dubielzig et al., 1999; Myers and Carter, 1980; Rapaport, 2004). In fact, Rep52 or Rep40 is essential for translocating a full-length genome into a capsid, as the absence of these proteins leads to a significant decrease in DNA encapsidation (King et al., 2001). Second, ssDNA is inserted into a capsid from the 3′ end to the 5′ end and packed rapidly upon its formation (Chejanovsky and Carter, 1989; Dubielzig et al., 1999; King et al., 2001; Myers and Carter, 1981). Based on these observations, a model describing ssDNA synthesis and its packing as a simultaneous process is shown in Fig. 3B-c. The Rep52 or Rep40 immobilized on capsids acts as the packing motor and its helicase domain unwinds the dsDNA from 3′ to 5′. The 3′ of one strand from the dsDNA is driven into the capsid, while the other strand re-initiates its 3′ end into the self-primed structure and starts to synthesize a complementary strand, which displaces the former strand from the dsDNA (Reddy and Sansom, 2016; Roos et al., 2007). During this process, energy generated from ATP hydrolysis is used by the Rep52 or Rep40 (King et al., 2001).

2.3. Kinetics of wtAAV replication

The approximate rates of the intermediate steps in AAV replication are shown in Table 1, based on published literature of AAV or similar viruses. Studies with AAV2 during 46 h post-infection of host cells with AAV and adenovirus have shown a progression of wtAAV replication through two distinct stages (Fig. 2C) (Redemann et al., 1989; Tyson et al., 1990). Intermediate accumulation occurs in the first stage (12–24 h after co-infection), when all four Rep proteins are formed and AAV genome is replicated. Within the first stage, the increase of *rep* and *cap* through genome replication in turn result in more expression of Rep protein and VPs. When the quantity of Rep proteins reaches a maximum 24 h after co-infection, the dsDNA accumulation also reaches the peak. In parallel, VP dynamics appear similar to that of Rep proteins, suggesting a potential complexation occurring between capsids and Rep proteins. The genome packing process is most active at the second stage (24 h after co-infection). During this stage, genome-filled capsids begin to form with concomitant accumulation of ssDNA and decrease of dsDNA.

Additional regulation occurs during the cycle of wtAAV replication. During the first stage, capsid assembly begins in the nucleolus while Rep proteins and AAV2 DNA accumulate in the nucleoplasm. When entering the second stage, the intermediates, including Rep proteins, capsids, and viral DNA, become compartmentalized and co-localized throughout the nucleoplasm, a regulation that may enable immediate access to substrates and machinery during genome packing (King et al., 2001; Wistuba et al., 1997).

3. Production of rAAV in cell culture

The process of rAAV production for therapeutic purpose aims to

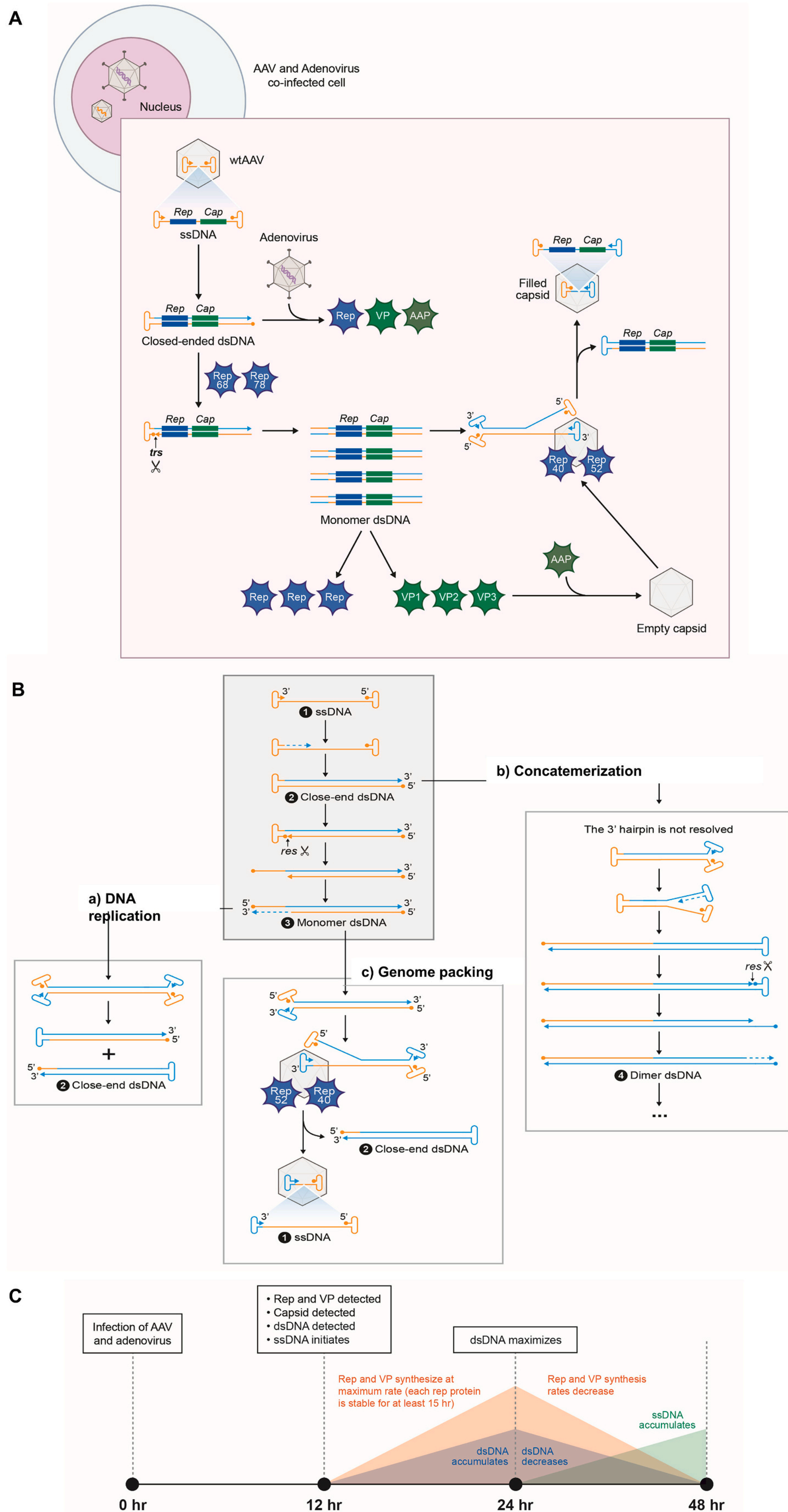

Fig. 3. Replication mechanisms and kinetics of wtAAV. (A) After an AAV particle is translocated into the nucleus, a replication cycle commences. The cycle is composed of a cascade of events including releasing the genome from the capsid, expressing the gene products, replicating the rAAV genome, assembling viral proteins, and packing the DNA components into capsids. (B) The structural formation of AAV genome during replication: first, the single stranded DNA (form 1) extends from the 5′ end at the self -primed hairpin ITR, making a progeny strand complementary against the first strand. This step forms a linear duplex replicative intermediate with one end closed (form 2). The Rep78/68 then cleaves at the *res* site and causes a linearization of the hairpin at the 5′ end of the progeny strand. Alternatively, if a nick is not made at the *res* site at the closed-end DNA intermediate, the replication process initiated at the free end of the strands produces a dimer dsDNA (form 4), which further extends to a longer concatemer if the process is repeated for more cycles (Tyson et al., 1990). Next, the parent strand extends from the nicked site via DNA repair until a complete complementary strand is reached, leaving double-stranded (ds)DNA with both ends open (form 3). Each of the strands can re-initialize the hairpin at ITR to allow formation of closed-end replicative intermediates (form 2) by following the previous cycle. Genome packing into capsids is initiated by a mechanism called single-strand displacement synthesis. During this process, a closed-ended dsDNA (form 2) and a single stranded DNA (form 1) are formed. (C) Formation of AAV intermediates observed during 48 h after AAV infection (Redemann et al., 1989).

Table 1
Intracellular machinery and kinetics of rAAV synthesis.

Regulators	Functions	Source	Kinetic characteristics of reactions
(a) Rep and VP transcription (in the nucleus)			
RNA polymerase II	Bind to promoter sequence	Host cell	Transcription: 4.6E-1–1.8E2 h^{-1} (Roldao et al., 2007; Schwake et al., 2010) or 1.44E5–1.08E6 nt/h·RNAP (Endy et al., 1996)
E1A	Transactivate and up-regulate transcription of Ad gene and AAV *rep/cap* gene (Xiao et al., 1998)	Adenovirus or E1A transduced HEK293 cells	
VA RNA	Enhance viral mRNA stability especially for *cap* (Xiao et al., 1998)	Adenovirus	RNA binding: 7.2E-1–2.34E2 $molecule^{-1}$ h^{-1} (Smeal et al., 2017)
E2A	Enhance viral mRNA stability especially for *cap* (Xiao et al., 1998)	Adenovirus	RNA elongation: 1.3E2 – 9E2 h^{-1} (Smeal et al., 2017)
E4, E1B	Required for efficient accumulation of AAV mRNA (Samulski and Shenk, 1988)	Adenovirus	RNA degradation: 4.32E1–4.32E2 h^{-1} (Smeal et al., 2017); 1.0E-1–3.3E-1 h^{-1} (Heldt et al., 2012; Lim et al., 2006; Rudiger et al., 2019; Sidorenko and Reichl, 2004)
(b) Capsid assembly (in the nucleus)			
Assembly-activating protein (AAP)	Scaffolding activity in the AAV capsid assembly reaction (Naumer et al., 2012)	AAV genome	
(c) Gene replication (in the nucleus)			
Rep68 and Rep78	Contain nickase and helicase motif; site-specific DNA nicking enzyme	AAV genome	Helicase Km: 22–180 μM (Musayev et al., 2015; Zhou et al., 1999)
E4	Facilitate AAV DNA replication (Xiao et al., 1998)	Adenovirus	Helicase Activity: 2.07E4 bp/h·enzyme (Zhou et al., 1999)
DNA dependent polymerase and ligase	Replicate DNA starting from the 3′ end of the genome	Host cell	ATPase Km: 180 μM (Zhou et al., 1999)
DNA-binding proteins	Viral DNA accumulation, nuclear translocation and the formation of viral DNA-protein complexes to facilitate virus assembly (Satkunanathan et al., 2017)	Host cell	ATP turnover: 3.0E3 ATP/h·enzyme Nuclease Km: 0.61–33 μM (Sanchez et al., 2016) DNA cleavage: 1.36E-3–3.54E-1 h^{-1}
(d) AAV genome packing into capsids (in the nucleus)			
Rep52 and Rep40	Contain helicase motif and ATPase motif; unwind DNA duplex; move nucleic acid strand in a polar fashion (King et al., 2001); catalyze energy for pumping virion gene into particle	AAV genome	DNA packing: 4.21E1 h^{-1} (Endy et al., 1996)
Adenovirus DNA-binding protein	Stabilize the displaced single strand (Ward and Linden, 2000)	Host cell	

produce a viral particle that encapsidates a therapeutic gene and avoids production of replication-competent AAV. After various breakthroughs in the last decade for rAAV production, four methods are mainly used today. These methods show different advantages and disadvantages in flexibility, productivity, and scalability (Table 2), and their usage is largely determined based on the stages of rAAV development (Ayuso et al., 2010; Naso et al., 2017; Robert et al., 2017).

The pathways used in cells to form a viral capsid and encapsidate therapeutic genome are based on the same cellular pathways of wtAAV replication. Unlike wtAAV replication that initiates upon wild-type viral infection, the production of rAAV in cells is initiated by introducing genetic components into host cells by several types of vectors.

3.1. Genetic structures needed for rAAV production

The genetic elements required for rAAV production can be divided into three groups. The first group is a genetic cassette where a transgene for therapeutic purpose and an optimized promoter are inserted between two ITRs, which are the only sequences required *in cis* for transgene replication and packaging (Li and Samulski, 2020). This cassette should be within ~5 kb to be packable into an AAV capsid. The second element is a cassette of *rep* and *cap* genes, commonly combined by an AAV2 *rep* gene and a serotype-specific *cap* gene, referred as *rep2/capX* (*X* denotes a specific serotype to be produced for rAAV). This construct is based on the capability of AAV2 Rep protein to pack an AAV2 ITR-flanked sequence into any capsid serotype (Rabinowitz et al., 2002). To avoid production of replication-competent AAV that can be caused by packaging *rep2/capX* into a viral capsid, the *rep/cap* genes for rAAV production are often supplied *in trans* by a different vector from the vector carrying the ITR. The third element is helper virus genes necessary for rAAV production and they can be introduced in one of three ways: 1) through infection with a wild-type helper virus; 2) through transduction with a recombinant viral vector to deliver genetic elements and induce AAV replication on its own; 3) through transfection with a plasmid typically containing the adenovirus genes *E1A*, *E1B*, *VA RNA*, *E4orf6,* and *E2A*. The last method enables production without any virus as a raw material and has a benefit for product safety (Matsushita et al., 1998; Xiao et al., 1998).

The *rep* and *cap* sequences for rAAV production can be both modified from the wild type. As the functions have been found redundant between Rep78 and Rep68, and between Rep52 and Rep40, rAAV can be produced in cells that have one protein out of Rep 78/68 and one protein out of Rep 52/40 (Airenne et al., 2013; Urabe et al., 2002). The expression of Rep proteins can be regulated by modifying the native promoters of *rep* gene (Emmerling et al., 2016b; Wang et al., 2018). With regard to VPs, the sequences of the *cap* gene from wild type AAV can be modified to assemble novel capsids for high potency and tissue specificity (Li and Samulski, 2020). For example, capsids with superabundant VP1 have been engineered to enhance AAV transduction efficiency given the role of the phospholipase A2 (PLA2) domain located within VP1's unique N terminus (VP1u) in facilitating AAV endosomal escape (Venkatakrishnan et al., 2013; Wang et al., 2017b). Structurally, only VP3 is required to form complete viral particles (Sonntag et al., 2011). However, care should be taken during modification of wild type sequences as disruption of regions of VPs essential for capsid assembly will lead to an inability to form capsids (Chandran et al., 2017; Lux et al., 2005).

3.2. Delivering genetic elements into production cells

The methods of delivering genetic elements are based on two main forms: stable or transient expression.

To develop a stable AAV-producing cell line from a cell line such as HeLa, the genetic elements for rAAV production are integrated into the cellular genome by stable transfection using a plasmid containing these genes and a selectable marker gene. Stably integrating genes for helper products and AAV non-structural proteins is challenging because of their cytotoxicity to cells. Therefore, only a partial set of the genetic elements are chosen to be stably expressed. For example, stable cell lines were engineered with *rep/cap* gene elements and ITR-transgene; during cell culture, rAAV production is induced by additional infection of a helper

Table 2
Comparison between common production methods for rAAV.

	Transfection only-HEK293 cells	Stable producer Hela cells	rBaculovirus-Sf9 cells	rHSV-BHK cells
Productivity (vg/L)	6E12 – 5E14	2E12 – 7E13	2E13 – 1E15	1E13 – 2E14
Process advantages	Cells are human origin; No helper virus addition; Simplicity and flexibility for different serotypes at lab scale	Cells are human origin; Only require adenovirus infection; Suitable for scale up	Non-transformed cell line; Suspension culture is well-suited to scale-up; No wild-type helper virus is needed	Cells are non-transformed; No wild-type helper virus is needed
Process disadvantages	The cell line is transformed with adenovirus genes (left arm of adenovirus 5 genome); Limited scalability to large volume and reproducibility	Cell line is transformed (HPV type 18 partial); Need to create new producer line for each transgene and rAAV serotype, Time required for cell line development and concerns with cell line stability	Cells are non-mammalian origin; Concerns with rBV stability during expansion; Ratio of VPs can be different from wild-type capsids	Cell line is non-human origin; Concerns with rHSV stability
Process-related impurities	≤ 6% vector plasmid, ≤ 0.1% rep-cap, ≤ 0.5% human genome; formation of replication competent virus via plasmid recombination (minimized by plasmid design, 0.1% to 1E-5%)	Potential contaminants include helper virus; Contamination by adenovirus, ≤ 0.4% rep-cap, ≤ 6% plasmid sequences	≤ 3% rBac, ≤ 0.1% rep-cap, ≤ 0.05% insect genome	≤ 0.05% rHSV, ≤ 0.01% rep-cap
References	(Aponte-Ubillus et al., 2018; Aucoin et al., 2008; Emmerling et al., 2016b; Lecomte et al., 2015; Louis et al., 1997; Park et al., 2006)	(Aponte-Ubillus et al., 2018; Corden et al., 1999; Emmerling et al., 2016b; Kang et al., 2009; Lecomte et al., 2015; Robert et al., 2017)	(Aponte-Ubillus et al., 2018; Kang et al., 2009; Meghrous et al., 2005; Penaud-Budloo et al., 2017; Vaughn et al., 1977)	(Aponte-Ubillus et al., 2018; Stoker and Macpherson, 1964; Ye et al., 2011)

virus (Martin et al., 2013; Qiao et al., 2002b) (Fig. 4A). Some AAV proteins may be chosen to be expressed only upon induction to reduce their lasting cytotoxic effects. The genes integrated into cellular genome can be preserved over many cell doublings (Chadeuf et al., 2000; Martin et al., 2013). Interestingly, after a stable cell is infected by helper virus, several-hundred-fold of episomal *rep/cap* genes can be replicated. One explanation is that the *rep/cap* gene is rescued by being adjacent to the *cis*-ITR or p5 sequence (Chadeuf et al., 2000; Liu et al., 2000; Martin et al., 2013) and another explanation is that a *cis*-acting replication element (CARE) is present in the *rep* gene and promotes replication in a rep-dependent manner (Nony et al., 2001).

The first method for rAAV transient production, which remains the most widely used today, is by transfecting two or three plasmids into HEK293 cells which can grow in either suspension or adherent cultures (Fig. 4B) (Chahal et al., 2014; Grieger et al., 2016). The ITR-transgene cassette, *rep/cap* cassette and helper genes are cloned on separate plasmids, except the *E1A* and *E1B* genes which are pre-transduced in HEK293 cells (Graham and Smiley, 1977). Alternatively, rAAV production can be induced by infecting cells with a wild-type helper virus such as adenovirus. A transfection step commonly uses plasmids at a ratio of 10^4–10^5 copies per cell and result in 30%–80% cells successfully transfected (Carpentier et al., 2007; Chahal et al., 2014; Durocher et al., 2007; Grieger et al., 2016; Riley and Vermerris, 2017).

The second method for rAAV transient production is in insect cells such as *Spodoptera frugiperda* (Sf9) which can be easily grown in suspension and serum-free medium (Fig. 4C). Cells are infected with two recombinant baculovirus (rBV)-based vectors that separately carry ITR-transgene and *rep/cap* genes, or three rBVs by further separating the *rep* and *cap* genes. Recently, a baculovirus system was also developed based on using Sf9 cell lines integrated with *rep* and *cap* genes; these cells are ready for rAAV production by receiving one baculovirus that contains the transgene (Joshi et al., 2019). A rBV itself is a helper virus capable of inducing rAAV genome replication in the absence of any other helper virus (Airenne et al., 2013). It is uniquely necessary in the rBV-Sf9 method for rAAV production to genetically modify the start codon to ACG for VP1 to enable the expression of VP in insect cells (Airenne et al., 2013; Bosma et al., 2018; Penaud-Budloo et al., 2018; Urabe et al., 2002) and to engineer the promoters for VPs to ensure the ratio of VPs expressed is typical for AAV capsid composition (Kondratov et al., 2017). The infection of rBVs commonly starts with a small multiplicity of infection (MOI) between 2 and 5 (Urabe et al., 2002). Then, rBVs propagate and cause Sf9 cells to be lysed. The released rBVs continue to infect uninfected cells until almost all the cells in culture are infected. Because of this feature, rBV-Sf9 production system often shows a higher volumetric yield than other methods (Kotin, 2011).

The third method for rAAV transient production is using a recombinant Herpes Simplex virus (rHSV), which is a replication-deficient viral vector, to infect mammalian cells such as HEK293 cells and baby hamster kidney (BHK) cells grown in either suspension or adherent cultures (Adamson-Small et al., 2017; Adamson-Small et al., 2016) (Fig. 4D). The pathogenic features of HSV vector are removed. Two rHSVs are usually used to separately carry ITR-transgene and *rep/cap* genes. Like rBV vector, a rHSV is capable of inducing rAAV production in the absence of a helper virus.

Unlike stable cell lines, the genes for any transient production method remain episomal and their numbers in each cell are diluted over cell doubling cycles. Understanding the kinetics of delivering genetic elements into the cell nucleus in a transient production method is important because hurdles can be present at this stage and affect the rAAV synthetic processes that follow. The genetic elements can only reach inside the nucleus after overcoming multiple biological barriers.

For plasmids (Fig. 4E), the delivery is assisted by transfection reagent such as polyethylenimine (PEI). During incubation with cells, PEI is bound on cell membrane by electrostatic interactions between the cell membrane and itself, and then internalized in a form of endosome via endocytosis. By further associating with microtubules, the endosome

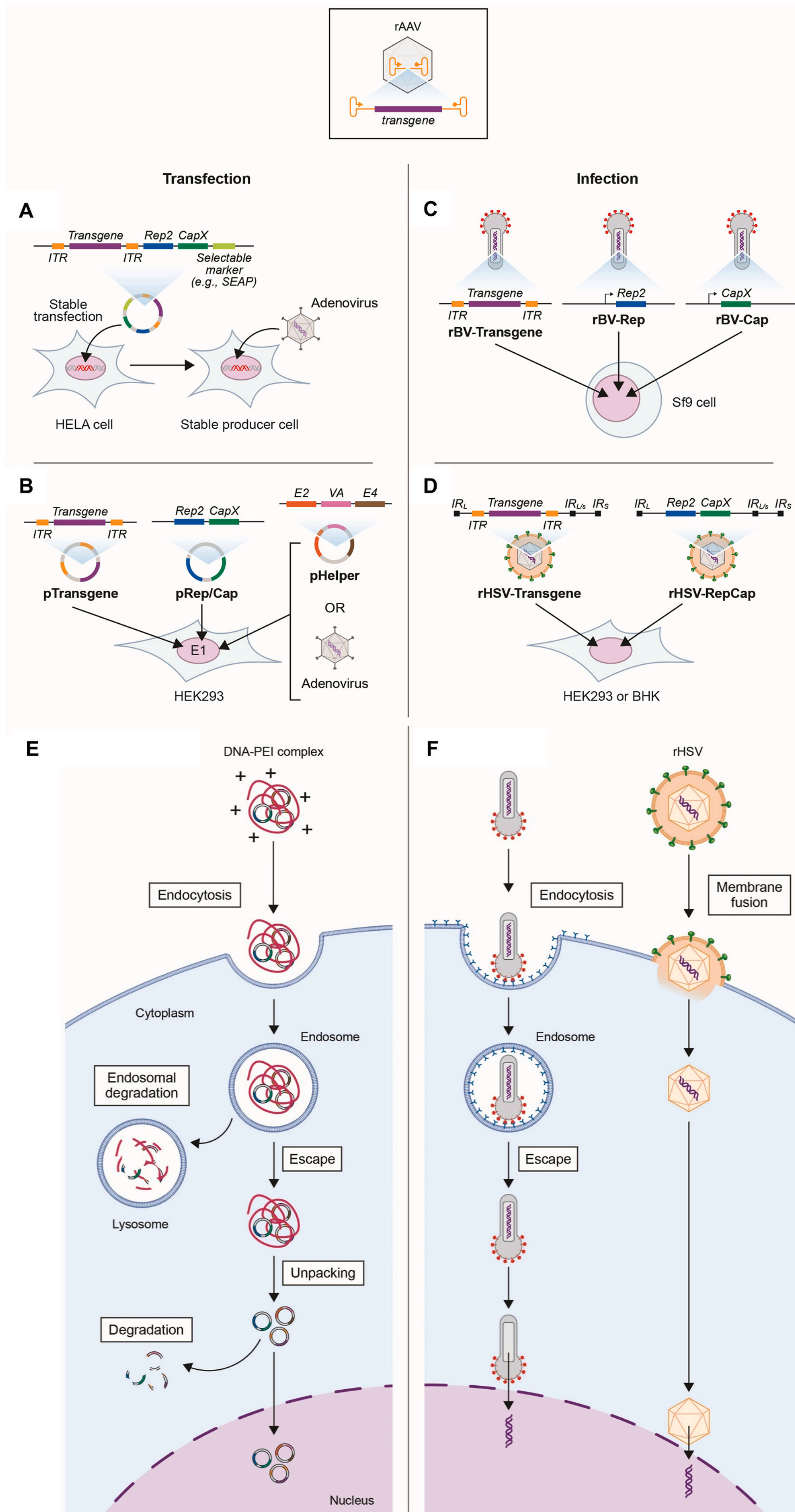

Fig. 4. Production of rAAV by different gene delivery methods. (A) Developing stable producer cell lines and induction of rAAV production by infecting adenovirus (Martin et al., 2013). (B) Transfecting cells (commonly human embryonic kidney, HEK293) with two plasmids: one with the therapeutic transgene and the other with the *rep/cap* genes. Cells are also transfected with a third plasmid that carries helper genes or are infected by a helper virus (Grieger et al., 2016). (C) Infecting *Spodoptera frugiperda* (Sf9) cells with two or three replication-deficient recombinant baculoviruses (rBV) that carry transgenes and the *rep/capX* genes (Urabe et al., 2002). (D) Infecting cells, commonly baby hamster kidney (BHK21) cells or HEK293 cells, with two replication-deficient recombinant herpes virus (rHSV) respectively carrying transgene and the *rep/capX* cassette (Adamson-Small et al., 2016). (*E*-F) The process of gene trafficking and delivery to a host cell nucleus by plasmids (E) and recombinant viral vectors (F).

traffics through the cytoskeletal network and must evade lysosome degradation until the vector "escapes" from the endosome. Free DNA is released from the vector by "unpacking" before or after the vector enters the nucleus, at a rate that is intrinsic to the type of vector used. Since the nucleases in the cytoplasm can quickly degrade naked DNA, the rate of unpacking in the cytoplasm can influence the final plasmid copy in the nucleus (Lechardeur et al., 1999). Finally, DNA associated or dissociated with the vector needs to enter the nucleus through a nuclear pore complex (NPC). The entrance of DNA into the nucleus is also dependent on cell cycle – a chronologic factor (Schwake et al., 2010). Transfection close to M phase is suggested to increase transfection efficiency because the breakdown of nuclear membrane can promote the entrance of plasmids (Brunner et al., 2000). The final step of translocating DNA has been previously described as a major limiting step. Only a small fraction of plasmids delivered inside the cells are present in the nucleus (Bieber et al., 2002; Carpentier et al., 2007; Cohen et al., 2009; Pollard et al., 1998).

For a viral vector such as rBV and rHSV (Fig. 4F), uptake to the cell cytosol can be initiated by one of the two mechanisms. For rBV, the vector is bound to the cell membrane by a cell surface receptor, followed by endocytosis that internalizes the whole viral vector (Au et al., 2013). For rHSV, the vector is internalized by cell membrane fusion and only the intact capsid enters the cytosol (Davidson and Breakefield, 2003). The viral genome is released from a viral capsid before entering the nucleus. In general, viral vector-based delivery shows higher efficiency and consistency than transfection-based delivery (Riley and Vermerris, 2017).

3.3. Post-gene delivery processes

After gene delivery, the pathway of rAAV production is similar to wtAAV replication and includes protein expression, capsid assembly, genome replication and packing (an example of the pathways after triple plasmids delivery is shown in Fig. 5).

The ITR-transgene harbored in a host cell genome or recombinant vector must be first "rescued" by Rep and helper proteins (Samulski et al., 1983; Ward et al., 2003). Subsequently, rAAV DNA is replicated in one of the following ways. To form rAAV with a single-stranded genome, the process of genome replication follows what is shown in Fig. 3B-a. To form a self-complementary (sc)AAV instead, a process resembling the pathway shown in Fig. 3B-b is used by introducing a mutation into the 3′ end ITR to convert it into an unsuitable substrate for Rep68 and Rep78. This mutation disables the termination resolution at *res* during genome replication; therefore, a dimer genome is formed that tethers the plus

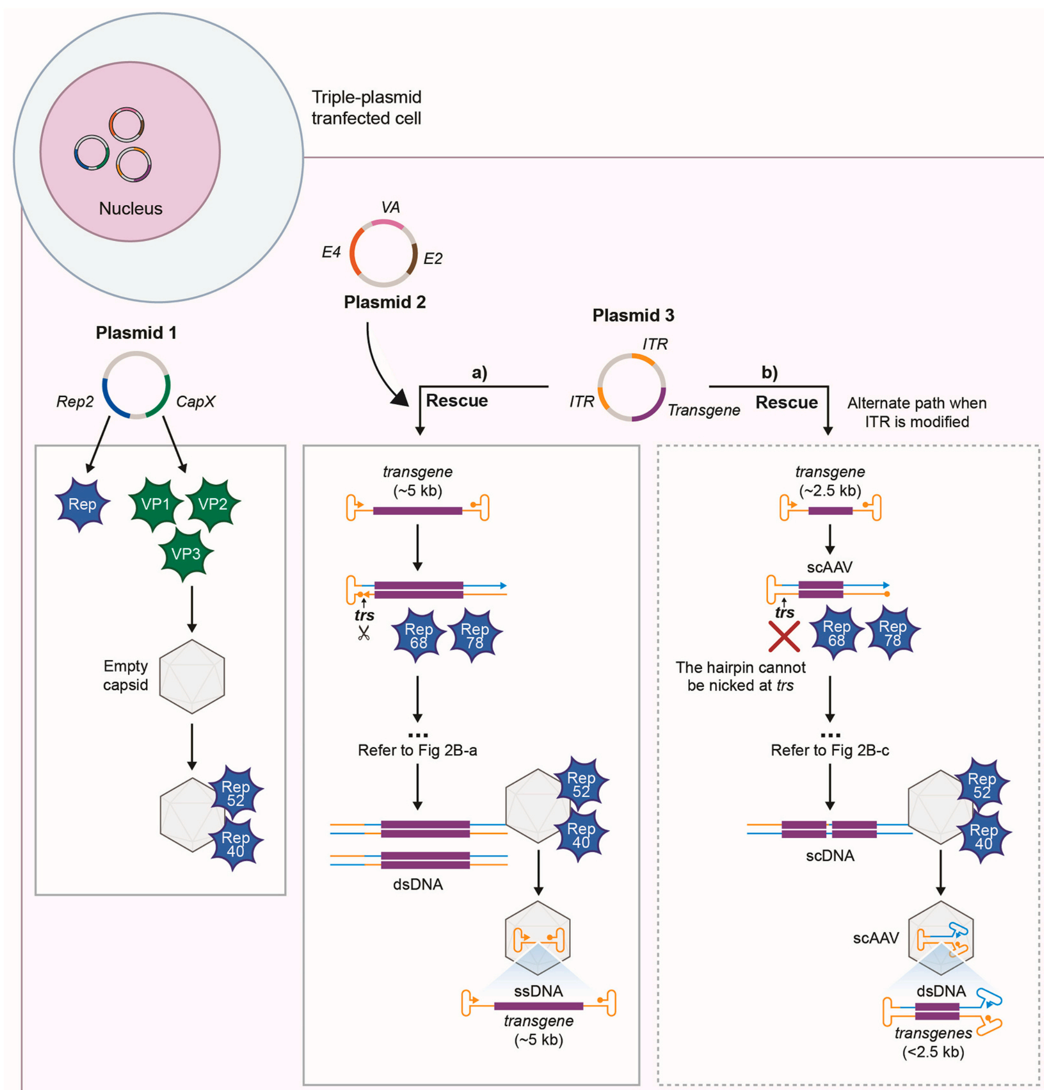

Fig. 5. Production of rAAV by triple plasmids. Post-genome activities are shown after plasmids are delivered to the nucleus. Two cases are respectively shown at the step of genome replication for monomer genome AAV (a) and scAAV (b).

and minus strand by ITR (Grieger and Samulski, 2012). However, to ensure the sc genome is packable into a capsid, the size for a transgene must be halved from a single-stranded genome. Although only the therapeutic transgene is inserted between ITRs in the initial vector for rAAV production, it has been found that a range of host cell DNA or plasmid backbone are packaged into a capsid, due to nonhomologous recombination events in cells (Lecomte et al., 2015; Schnodt and Buning, 2017). Various strategies have been developed to decrease the occurrence of non-specific DNA packaging based on the designs of plasmids (Dong et al., 2013; Halbert et al., 2011; Schnodt et al., 2016).

3.4. Kinetics of rAAV production

For current rAAV production methods, the viral vector is mainly produced during the first 48 to 72 h in either transfected or infected cultures, with productivity plateaued on the subsequent days (Chahal et al., 2014; Joshi et al., 2019). The kinetics of rAAV production can vary from wtAAV replication and depend on the type of culture, for example, cells that are either stably expressing with integrated genes or transiently-transfected with non-integrated episomal genes.

Joshi et al.'s study revealed the kinetics of rAAV production for 120 h in a Sf9 cell line integrated with *rep/cap* genes. After infecting with baculovirus, it was shown that Rep and capsid protein expression commenced immediately, reaching a peak at 48 h post infection (hpi). On the other hand, genomic particles were produced up to 96 hpi and then plateaued. The functional particles, however, were detected much later in the process and around 48 hpi. A hypothesis was that functional particles need additional conformational changes from genomic particles (Joshi et al., 2019).

The kinetics of rAAV production in a HeLa cell line integrated with rep/cap genes was investigated by Martin et al. (2013). After infecting wtAd, it was similarly found that Rep and capsid proteins reached a peak at 2 days. Differently from Joshi et al.'s studied system, the replicated rAAV DNA also peaked at 2 days, correlating with the Rep expression. The kinetics of Martin et al.'s production system resembled wtAAV replication kinetics (Martin et al., 2013).

In most bioprocesses, the rAAV yield is low with a large proportion of particles remaining empty. Studies have pointed out several limitations during rAAV production. One limitation is capsid production, with one study improving rAAV yield, with similar amounts of Rep protein, by overexpressing VP (Emmerling et al., 2016b). Another limitation is genome replication, with one study improving rAAV yield by boosting DNA replication (Wang et al., 2018). Another limitation is DNA packing, with one study identifying that only 10% of the replicated transgenes were encapsulated into capsids in a producer cell line (Martin et al., 2013). These studies demonstrate that the limitations in producing filled rAAV are multifaceted and vary widely, from insufficient expression of viral capsid proteins to a lack of effective helper function. The detailed kinetics characterization of intermediate cellular processes in most rAAV production platforms is still lacking and the mechanisms driving the limitations need to be deciphered.

4. Key factors associated with viral productivity

The yield of wtAAV replication is 10^5–10^6 copies per cell with a high ratio of filled viral particles (Weitzman and Linden, 2011). This yield shows that the efficiency of viral genome replication and packaging per cell is higher with wtAAV replication than rAAV production although the overall mechanisms used to assemble rAAV post-gene delivery are similar. Here, we aim to pinpoint several factors that could explain such differences in viral productivity between the two systems.

(1) The *rep/cap* genes continuously replicate in wtAAV replication. However, the *rep/cap* genes introduced to rAAV production cells cannot replicate and ultimately degrade in most transient production methods, which limits the number of *rep/cap* genes in rAAV synthesis as opposed to wtAAV. As a result, the amount of the intermediates Rep and VPs in the production system can be significantly lower for rAAV than wtAAV.

(2) The ITR, *rep* and *cap* sequences are linked on one cassette in wtAAV's genome. Such a spatial relationship of these elements may facilitate the *cis* regulation of ITR on the transactivation of p19 and p40 promoter genes (Wang et al., 2018) and play a critical role in controlling the temporal coordination between viral intermediates during replication. In contrast, the ITR and *rep/cap* DNA in plasmid- or recombinant viral vector- based methods are located on separate cassettes, posing a challenge in controlling their ratio.

(3) There is still a lack of understanding about how the abundance of the four Rep proteins affects the efficiency of AAV replication. However, the same ratios of different Rep proteins as wtAAV are not always reproduced in rAAV production, even under the control of wild type promoters (Martin et al., 2013). The cause of such a phenomenon has not been well understood.

(4) The role of wild-type helper virus including its recruiting of a spectrum of host cellular proteins, is considered essential in various stages of AAV replication (Satkunanathan et al., 2017). Some functions of helper virus can be lost when a plasmid with a set of helper genes is used as the substitute. For example, *rep/cap* genes can be replicated in stable producer cells with adenovirus infection, but this replication activity is disabled if adenovirus is replaced by helper plasmids, presumably because the cellular distribution for two critical adenoviral helper proteins, E4 and E1B, is altered when they are expressed by helper plasmids (Chadeuf et al., 2000; Nony et al., 2001). Although the mechanism remains largely unknown, the lack of certain helper virus genomes may lead to less effective viral assembly in the case of rAAV production.

(5) Each specific rAAV has a different sequence and size of the transgene and may carry certain capsid modifications that are different from wtAAV. The packing efficiency is affected by capsid mutations and stability (Drouin et al., 2016), genome size (Tullis and Shenk, 2000), and ITR sequence integrity (Tullis and Shenk, 2000; Wang et al., 1996a, 1996b), each of which depends on specific rAAV designs.

(6) Other than the common AAV proteins expressed in the production cells, the specific transgene for rAAV is expressed and may pose additional effects on cell physiology, which is highly dependent on the nature of the therapeutic gene used in each rAAV.

5. Optimizing rAAV production systems

Based on the characteristics of wtAAV replication, several strategies are proposed in this section to boost rAAV production using either molecular engineering or process development to improve cellular processes toward rAAV production.

5.1. Molecular engineering

Efforts in molecular engineering have focused on enabling functions that are currently non-existent in most rAAV production systems but exist in wtAAV.

For instance, in most rAAV production systems, there is no *cis* regulation of ITR on *rep* and *cap* genes. However, in some producer cell lines, this *cis* regulation may be restored. For example, in a producer cell line based on HeLa, when the *cis*-acting element ITR, transgene, *rep/cap* were integrated on the same cassette, the production efficiency was closer to that of wtAAV, with fewer empty capsids (~20%) compared to transfection culture (~80%). A varied range of *rep* and *cap* genes were detected in the rAAV produced, however, the detected replication-competent (rc)AAV was under 0.0002% of the total rAAV particles

based on a biological assay (Martin et al., 2013).

Past studies have shown a positive correlation to some extent between the copy number of *rep/cap* genes and the titer of rAAV, suggesting that increasing Rep proteins and VPs could elevate DNA replication and packing (Chadeuf et al., 2000; Martin et al., 2013). To mimic the replication capability of wtAAV to produce an abundance of *rep* and *cap* genes, several novel ideas using molecular engineering have been implemented. In one study, ITR sequences were added to the *rep* and *cap* gene cassette to enable gene replication, while adding introns to the *rep/cap* genes and an additional mutation to the ITR to prevent packing these genes into rAAV (Cao et al., 2002). In another study, *rep2/cap2* gene amplification was enabled by flanking these genes between ITR5 and introducing a plasmid for expressing Rep5 protein. In this study, the size of *rep/cap* cassette was similarly increased by inserting introns to avoid packing the genes into rAAV (Li and Samulski, 2005). In both studies, rAAV productivity was increased by 20-fold.

Despite the essential role in rAAV production, Rep proteins have a deleterious effect on cell health at the same time. Thus, careful control of Rep expression is also important to improve yield. Success has been achieved in increasing rAAV production by upregulating Rep52 alone (Wang et al., 2018) or optimizing the ratio between Rep68 and Rep52 (Emmerling et al., 2016b). Despite the essential roles of Rep proteins in viral replication, decreasing the expression of some Rep proteins is also of interest due to their adverse effects. First, the expression of rAAV regulatory gene products consumes cellular resources that would otherwise be used for cell maintenance and rAAV synthesis. Second, some Rep proteins themselves are cytotoxic, for example Rep78 (Ni et al., 1998; Wright, 2009). To reduce unnecessary toxicity to host cells, several studies downregulated the expression of Rep78 and resulted in improved rAAV productivity (Emmerling et al., 2016b; Kube et al., 1997; Li et al., 1997; Qiao et al., 2002a; Qiao et al., 2002b; Urabe et al., 2002).

Helper viral genes play various functions in rAAV production, one of which is to enhance the replication of rAAV DNA. In the interest of developing novel helper gene systems, Wang et al. (2018) applied a dual helper plasmid system using a set of human bocavirus 1 (HBoV1) genes in addition to Ad helper gene sets. This system enabled two-fold enhancement of rAAV replication, which led to two-fold greater yield than the traditional Ad helper system. The authors noted a particular importance of Ad *E2A* gene in rAAV DNA replication (Wang et al., 2018).

5.2. Process development

Each method of delivering genetic elements, based either on stable or transient expression, is associated with a series of process parameters that can be optimized in the context of process development and rAAV production.

In transient expression methods, the transfection step is critical for achieving high rAAV titer. The efficiency of gene delivery depends on cell physiology, plasmid topology, physicochemical properties of transfection reagents, and complexation conditions applied between plasmids and transfection reagents (Carpentier et al., 2007; Chahal et al., 2014; Durocher et al., 2007; Grieger et al., 2016; Riley and Vermerris, 2017). Transfection conditions are hence often optimized among process parameters that include cell density, transfection reagent, plasmid concentration, and incubation time of transfection complex (Chahal et al., 2014; Powers et al., 2016; Strobel et al., 2019; Zhao et al., 2020). In practice, a design-of-experiment (DOE) approach combining many of these parameters can be used to determine the optimal conditions (Zhao et al., 2020).

In transient transfection- or infection-based processes, the ratio between vectors such as plasmids or recombinant baculovirus for different genetic elements is often optimized for balancing the abundance of ITR, *rep*, *cap,* and helper genes (Durocher et al., 2007; Grieger et al., 2016).

Several studies have reported the effects of cell culture temperature on rAAV production. One study found that increasing the culture temperature from 24 °C to 30 °C post-infection led to better packing efficiency of rAAV production in a rBV-Sf9 system. The study has suggested that the temperature alteration might increase the expression of AAV proteins including Rep at earlier stages and subsequently lead to a quicker cascade of rAAV assembly events (Aucoin et al., 2007). Another study found that, under mild hypothermia (32 °C), upregulation of the miRNA miR-483 controlled rAAV production leading to higher titer (Emmerling et al., 2016a). Hypothermia condition (33 °C) has also been reported to improve cellular productivity for recombinant proteins from HEK293 cell cultivation systems (Lin et al., 2015).

Glucose supplementation impacts cellular metabolism and rAAV productivity. In a study by Kimura et al. (2019), glucose supply was reduced in culture to mitigate lactate accumulation and stabilize pH. The process achieved a high yield of 10^{10}–10^{11} copies of viral genome per microliter while requiring five times fewer HEK293 cells than standard protocols. The authors suggested that cells shifted to use glutaminolysis, a more efficient pathway in producing energy, when the glycolytic pathway became limited (Kimura et al., 2019).

In addition to glucose, parameters such as cell viability, pH, osmolarity, byproduct accumulation, and nutrient consumption can also broadly affect cellular processing and rAAV production, as demonstrated in a study using baculovirus-based production (Joshi et al., 2019).

Lastly, culture medium and feeding strategy are also important for rAAV production, to meet the metabolic needs of cells in producing rAAV. Studies have found that choice of culture medium affects production yields, preferably using serum at the culture stage for viral production (Durocher et al., 2002; Durocher et al., 2007). These studies suggest that it is necessary to identify metabolic requirements and energetic needs in cells to find optimal culture medium formulations for viral production.

While most studies observe a plateau in rAAV production after 48 to 72 h, in several studies, when the culture medium is replaced daily, cell specific productivity was maintained for 3 to 4 more days, increasing the culture time to about 6 days (Ansorge et al., 2009; Benskey et al., 2016; Grieger et al., 2016; Piras et al., 2016). Thus, a possible way to increase product yields is based on optimizing the metabolic conditions of production cells to increase both cell specific productivity and culture time, which can be achieved by establishing fed-batch or perfusion cultures. Adding additional medium exchange or adding nutrient by fed-batch culture was reported to increase process yields (Joshi et al., 2019; Powers et al., 2016). For some AAV serotypes such as AAV8 and AAV9, a benefit can be simultaneously gained as the products are increasingly secreted to the medium as culture progresses, thus enabling a simplified harvest of rAAV from a continuous collection of medium (Grieger et al., 2016).

Intensified culture processes have been well established for other biologics to enable high volumetric productivity (Mellahi et al., 2019; Venereo-Sanchez et al., 2017). For example, a study producing vesicular stomatitis virus (VSV) using HEK293 cells was able to increase volumetric productivity with an increased cell density of up to 20 million cells/mL (Elahi et al., 2019). One study producing recombinant protein increased efficiency by transfecting HEK293 cells at densities up to 20 million cells/mL followed by production at lower cell density (Backliwal et al., 2008). In rAAV production systems, success has been made with intensified processes using Sf9 cells. In another study, cells maintained the same specific productivity at high cell density in a fed-batch process, which made it possible to achieve a six-fold increase in volumetric productivity (Joshi et al., 2019). However, little success has been achieved with intensified processes using transient transfection for viral vector production in HEK293 cells, with common transfection cell density in the range of 0.5 to 2 million cells/mL (Zhao et al., 2020). One study observed an inverse relationship in cell specific productivity and transfection cell density, with higher cell densities (up to eight million cells/ml) leading to decreased cell specific productivity (Chahal et al.,

2014).

6. Concluding remarks

Improving the manufacturing of rAAV to meet the required volumes of clinical trials is an urgent task. The manufacturing process should provide production with speed, safety, quality control, and easy transition to a commercial-scale production. Major improvement should be made for each of the existing production platforms. This review presents a foundation for the mechanistic analysis of rAAV production system and highlights several areas to improve productivity, with a critical comparison to the mechanism of wtAAV replication.

Process development is key to improving rAAV manufacturing. While intensified culture has been established with Sf9 cells, the progress is still hindered by the low efficiency of transfecting HEK293 cells at high cell density. Biological understanding is needed to overcome the limitations for cells to enable intensified processes for higher process efficiency. Additionally, perfusion culture and continuous processing may play a potential role in accelerating the scaling of making clinical-grade material. High-yield stable producer cells are still an attractive endeavor, simplifying the process by establishing a cell bank for manufacturing clinical and commercial material. In parallel, analytical technology for product characterization is another key aspect of rAAV production and development (Gimpel et al., 2021).

We also believe that the mechanistic understanding of rAAV production pathways will enable the development and utilization of mathematical models, which are another powerful tool to unravel system complexity and generate understandings for process optimization and control (Sha et al., 2018). While mathematical models for plasmid-based DNA delivery (Ligon et al., 2014; Schwake et al., 2010), influenza replication (Sidorenko and Reichl, 2004), rotavirus infection (Roldao et al., 2007), and HIV infection (Mohammadi et al., 2013) exist, mathematical models for rAAV production just start to emerge (Nguyen et al., 2021).

A number of tools are poised to impact our understanding of the biology of rAAV production in the coming years. Systems biology approaches have been widely used in the field of Chinese hamster ovary (CHO)- based monoclonal antibody manufacturing and can likewise be applied to rAAV production and accelerate the understanding of these systems. Understanding the mechanistic limitation is helpful to bring novel strategies in molecular engineering and process development. With an example of rAAV presented here, the growing knowledge of viral production mechanisms and improved productivity will be an engine for the whole field of cell and gene therapy.

Acknowledgement

This work was supported by the U.S. Food and Drug Administration (Grant number: 1R01FD006584-02). The figure illustration was supported by Elizabeth Skrip (MIT Center for Biomedical Innovation). A.J. M. was partially supported by the National Science Foundation (NSF) Graduate Research Fellowship Program under Grant No. 1122374.

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