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Research review paper

Decoding cellular mechanism of recombinant adeno-associated virus (rAAV) and engineering host-cell factories toward intensified viral vector manufacturing

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ABSTRACT

Recombinant adeno-associated virus (rAAV) is one of the prominent gene delivery vehicles that has opened promising opportunities for novel gene therapeutic approaches. However, the current major viral vector production platform, triple transfection in mammalian cells, may not meet the increasing demand. Thus, it is highly required to understand production bottlenecks from the host cell perspective and engineer the cells to be more favorable and tolerant to viral vector production, thereby effectively enhancing rAAV manufacturing. In this review, we provided a comprehensive exploration of the intricate cellular process involved in rAAV production, encompassing various stages such as plasmid entry to the cytoplasm, plasmid trafficking and nuclear delivery, rAAV structural/non-structural protein expression, viral capsid assembly, genome replication, genome packaging, and rAAV release/secretion. The knowledge in the fundamental biology of host cells supporting viral replication as manufacturing factories or exhibiting defending behaviors against viral production is summarized

Abbreviation: AAP, Assembly Activating Protein; AAV, Adeno-associated Virus; AP1/2/3, Activator Protein 1/2/3; ARNT, Aryl hydrocarbon Receptor Nuclear Translocator; ATF6, Activating Transcription Factor 6; ATM, Ataxia-Telangiectasia Mutated; ATR, Ataxia Telangiectasia and Rad3-related; BCl2, B-cell Leukemia/Lymphoma 2 protein; CD9, Tetraspanin-29; Cdc25A, Cell Division Cycle 25 Homolog A; cGAS, Cyclic GMP-AMP Synthase; Chk1, Checkpoint Kinase 1; CHO, Chinese Hamster Ovary; CMV, Cytomegalovirus; CREB, cAMP-Response Element Binding protein; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; c-Rel, Rel proto-oncogene, NF-kB subunit; DDR, DNA Damage Reponse; DNA-Pkcs, DNA-dependent Protein Kinase, catalytic subunit; DTS, DNA Targeting Sequences; E4F, E4F Transcription Factor 1; E4orf4, Adenovirus (Ad) early region 4 open reading frame 4; E4orf6, Adenovirus (Ad) early region 4 open reading frame 6; ER, Endoplasmic Reticulum; ERAD, Endoplasmic Reticulum–Associated protein Degradation; FANCC, Fanconi anemia complementation group C; GOI, Gene Of Interest; GQRS, G-quadruplex DNA Sequence; H2AX, H2A histone family member X; HEK, Human Embryonic Kidney; Hsp70, Heat Shock Protein 70; HSPA5, Heat shock protein family A (hsp70) member 5; HSPA6, Heat shock protein family A (hsp70) member 6; HSV, Herpes Simplex Virus; IFIT2, Interferon-induced protein with Tetratricopeptide repeats 2; IFITM, Interferon-induced transmembrane; IFN, Interferon; IFN-β, Interferon Beta-1a; IRE1α, Inositol-Requiring Enzyme type 1; IRF7, Interferon Regulatory Factor 7; ISG, Interferon-Stimulated Genes; ITPRIP, Inositol 1,4,5-Trisphosphate Receptor Interacting Protein; ITR, Inverted Terminal Repeats; JunB, Transcription Factor AP-1 Subunit JunB; Ku70/86, Lupus Ku autoantigen protein p70/86; LDLR, Low Density Lipoprotein Receptor; MRN, MRE11-RAD50-NBS1; NLS, Nuclear Localization Signal; NF-kB, Nuclear Factor kB; NPM1, Nucleophosmin; OAS, Oligo Adenylate Synthetase; OASL, 2′-5′-Oligoadenylate Synthetase Like; Oct-1, Octamer Binding transcription factor; PAMP, Pathogen Associated Molecular Patterns; PCNA, Proliferating Cell Nuclear Antigen; PDIA2, Protein Disulfide Isomerase-Associated 2; PEI, Polyethylenimine; PERK, Protein kinase R like Endoplasmic Reticulum Kinase; PI3KK, Phosphatidylinositol 3-kinase-related kinases; Pol, Polymerase; PKR, Protein Kinase R; RAN, RAs-related Nuclear protein; RBE, Rep Binding Elements; RBP-Jk, Recombining Binding Protein Suppressor Of Hairless; RIG, Retinoic acid Inducible Gene; RNF121, Ring Finger protein 121; RPA, Replication Protein A; RPA32, Replication Protein A 32; RSAD2, Radical S-adenosyl methionine Domain containing 2; SKA2, Spindle And Kinetochore Associated Complex Subunit 2; SMURF1, SMAD specific E3 ubiquitin protein ligase 1; Sp1, Specificity Protein 1; SPOP, Speckle Type POZ Protein; TEF-1, Transcription Enhancer Factor 1; TLR, Toll Like Receptor; TRIM19, Tripartite Motif containing 19; Ubc9, Ubiquitin-conjugating Enzyme 9; UPR, Unfold Protein Response; UPS, Ubiquitin–Proteasome System; VSVG, Vesicular Stomatitis Virus Glycoprotein; XBP1, X-box Binding Protein 1; XRCC4, X-ray repair cross-complementing protein 4; YB1, Y box Binding Protein 1; YY1, Yin And Yang 1; ZBED1, Zinc Finger BED Domain-Containing Protein 1; ZF5, Zinc Finger Protein 5 Homolog.

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for each stage. The control strategies from the perspectives of host cell and materials (e.g., AAV plasmids) are proposed as our insights based on the characterization of molecular features and our existing knowledge of the AAV viral life cycle, rAAV and other viral vector production in the Human embryonic kidney (HEK) cells.

1. Introduction

rAAV is one of the most promising gene delivery vectors in the gene therapy field (Athanasopoulos et al., 2000; Patel et al., 2019). Yet the broad applicability of rAAV gene therapy has been limited by manufacturing difficulties and high costs associated with the production process. The high expenses of gene therapy drugs are attributed to the low productivity and high dosage requirements of rAAV products (D. Wang et al., 2019). Also, only 5 to 30% of viral particles harvested from cells contain the transgene vectors (Adamson-Small et al., 2017; Q. Wang et al., 2017), and even a smaller portion of these GOI-containing vectors can effectively transduce cells depending on potency and routes of administration (Joshi et al., 2019). The presence of process impurities including empty and partially-filled viral particles remains as one of the major difficulties in the viral vector manufacturing process. This requires the needs of expensive and specialized purification equipment and reagents and further reduces the final vector genome titer (Qu et al., 2007). The detailed discussion on critical challenges and advances in rAAV manufacturing can be found in the recently published review paper (Fu et al., 2023b).

Despite a lot of efforts have been dedicated to establishing the stable producer/packaging cell lines for rAAV production (Escandell et al., 2023; Fu et al., 2023b; Jalšić et al., 2023; Lee et al., 2022), the triple plasmid-mediated transient transfection in mammalian cells is still a predominant workflow for rAAV production (Clément and Grieger, 2016; Fu et al., 2023b). Each step involved in the rAAV production process, including plasmid entry mediated with transfection reagent, plasmid trafficking, rAAV protein synthesis, capsid assembly, genome replication, genome packaging, and rAAV release/secretion, heavily relies on host cell machinery mediated by host cell factors. Moreover, they simultaneously trigger a series of uncharacterized host cell responses, which can represent as bottlenecks in improving viral vector production capability in the host cell factory, thereby hindering the rational modification of the host cells, restricting the upstream manufacturing capacity, and impacting the upstream process.

Extensive efforts, such as parameters optimization of transient process and media supplementation, have been aimed to increase productivity and quality (Fu and Lee et al., 2023a; Grieger et al., 2016; Scarrott et al., 2022; Zhao et al., 2020). However, the lack of a comprehensive and systematic understanding of the host cell and input materials (e.g., AAV plasmids) limits the manufacturing capacity of host cells to support the expression of viral vectors. Understanding the bottlenecks in the current production process from the host cell perspective and altering the cellular process that favors vector production can address manufacturing problems, decreasing the production cost, and increasing affordability.

In this review, individual steps of rAAV transient production process are explored. Along with the review on the fundamental biology of host cells supporting their uses as cell factories, host cell factors involved in viral production and host cell responses associated with both AAV life cycle and rAAV production will be focused. A comprehensive and fundamental understanding of production mechanisms from the host cell and input materials perspectives will help to investigate the root causes of limited productivity and suboptimal quality. These will be used as a guide to identify potential barriers to high productivity and a reference to provide more diverse directions for future manufacturing capability improvement. In addition, we propose rational strategies corresponding to each step in the cell factory and input materials in order to maximize productivity and obtain optimal quality attributes.

2. Overview of the triple plasmid transient transfection process for rAAV production and the role of host cells in rAAV production

Plasmid delivery to the cytoplasm is facilitated by transfection reagents, such as polyethyleneimine (PEI) or lipofectamine. During the incubation, positively charged transfection reagent and negatively charged plasmids form a complex by electrostatic interactions and get internalized together by endocytosis. The internalized plasmids complex will either undergo endosomal degradation or get released from the endosome. The free plasmids in the cytoplasm have to travel through the cytoskeletal transport network and avoid nuclease degradation to enter the nucleus. It has been reported that nuclear translocation of plasmids DNA is one of the major bottlenecks in the transfection process and only a small fraction of plasmids entered into cytoplasm has been detected in the nucleus (Carpentier et al., 2007; Cohen et al., 2009). Those that have successfully entered the nucleus are transcribed and utilized for later vector production. The detailed process information is illustrated in Fig. 1.

Rep gene expression is initiated by p5/p19 promoter transcription and *cap* gene expression is initiated by p40 promoter transcription. E1A is required to activate the p5 promoter and large non-structural Rep proteins (Rep78/68) are produced (Chang et al., 1989; X. Xiao et al., 1998). Similarly, small non-structural Rep proteins (Rep52/40) are under the control of the p19 promoter. After being translated in the cytoplasm, Rep proteins will be imported to the nucleus for later steps. E1A and large Rep protein will together activate p40 to initiate *cap* gene transcription and start structural protein synthesis of the Viral Proteins (VP) (Z. Wang et al., 2018). Viral Proteins (VP1/2/3) are serotype-specific and can form preassembled capsids with the assistance of Assembly-Activating Protein (AAP). Those preassembled capsids will be transferred into the nucleus for the genome packaging step. Additionally, the helper proteins E2A, and E4orf6 are expressed for rAAV genome replication. E2A can facilitate AAV viral replication by interacting with Rep protein and getting recruited to viral replication center (Stracker et al., 2004). It also participates in a range of additional functions that enhance replication, including mRNA processing and capsid formation (Carter et al., 1992; Ward et al., 1998). E4orf6 is involved in stimulating AAV mRNA export from the nucleus (Pilder et al., 1986) and boosting AAV replication by facilitating second-strand synthesis (Fisher et al., 1996).

Plasmids containing gene of interest (GOI) are flanked by inverted terminal repeats (ITRs), which behave as the origin of genome replication. The genome replication can be activated once plasmids containing GOI enter the nucleus. The host cell replication machinery facilitates the replication of single-stranded DNA to form a double-stranded DNA (Meier et al., 2020). After the second strand synthesis under the assistance of helper genes, Rep78/68 proteins will introduce the cleavage by binding to the Rep binding elements (RBE) inside ITR (Snyder et al., 1990). Rep52/40 binds to preassembled capsids with high affinity, forming a stable intermediate complex (King et al., 2001); the helicase domain in Rep52/40 unwinds dsDNA, and a single strand DNA will be drawn into the preassembled capsid and packaged rapidly (Chejanovsky and Carter, 1989; King et al., 2001). The genome replication, capsid assembly, and packaging process details are illustrated in Fig. 2.

Both empty and filled AAV capsids in the nucleus will be exported to the cytoplasm. Vector secretion capability from the host cells is serotype dependent: the majority of produced rAAV2 remains inside the cells and must be harvested upon cell lysis; the vectors, including AAV1, AAV8, and AAV9, can be directly secreted from the cells and detected in great abundance in the culture medium (Vandenberghe et al., 2010).

Additionally, it has been reported that rAAV can be secreted into the supernatant after being packaged in exosomes (Schiller et al., 2018). Recently discovered component membrane-associated accessary protein (MAAP) was reported as a novel viral egress factor, critical for AAV secretion (Elmore et al., 2021). The rAAV release/ secretion process can be found in Fig. 1.

Each step for recombinant viral vector production described above involves numerous virus-host interactions that together determine the destiny of the viral genome and the efficiency of vector production. Due to the limited fundamental research on transient transfection mediated rAAV production, the existing understanding of AAV life cycle, coinfection mediated AAV production and its viral genome interaction with host cells will be able to provide the improvement guide on rAAV manufacturing capacity and vector quality. Fig. 3 provides an overview of the interactions between virus and host cell and host cell responses during rAAV vector production. The production of rAAV can trigger various cellular responses, such as antiviral response (Chung et al., 2023; Wang and Fu et al., 2024), unfolded protein response (UPR) (Balakrishnan et al., 2013; Sen et al., 2014), DNA damage response (DDR) (Collaco et al., 2009; Schwartz et al., 2009), cell cycle arrest (Saudan et al., 2000; Winocour et al., 1988), and cell apoptosis (Schmidt et al., 2000; Berthet et al., 2005).

3. Cellular pathways and host factors involved in AAV life cycle and rAAV production

The following sections will summarize the activated host cell responses, and the corresponding host cell factors involved in each step and discuss potential modification strategies that could improve viral production.

3.1. Cytoplasmic entering of plasmids, plasmid trafficking and nuclear entry

PEI-mediated transient rAAV production is currently one of the most common approaches for rAAV production. Though the transient transfection reagent typically causes an inimical impact on host cells and leads to significant changes in cell physiology, HEK293 is tolerant to optimized concentrations of transfection reagents. Lavado et al. has shown the overall disruption of cellular homeostasis along with the decrease of cell viability when comparing the empty plasmid transfected conditions to non-transfected conditions (Lavado-García et al., 2020). Future transfection reagents, such as many synthetic gene delivery materials (Lostalé-Seijo and Montenegro, 2018), will aim to achieve high transfection efficiency while minimizing any negative impact on normal cell physiology.

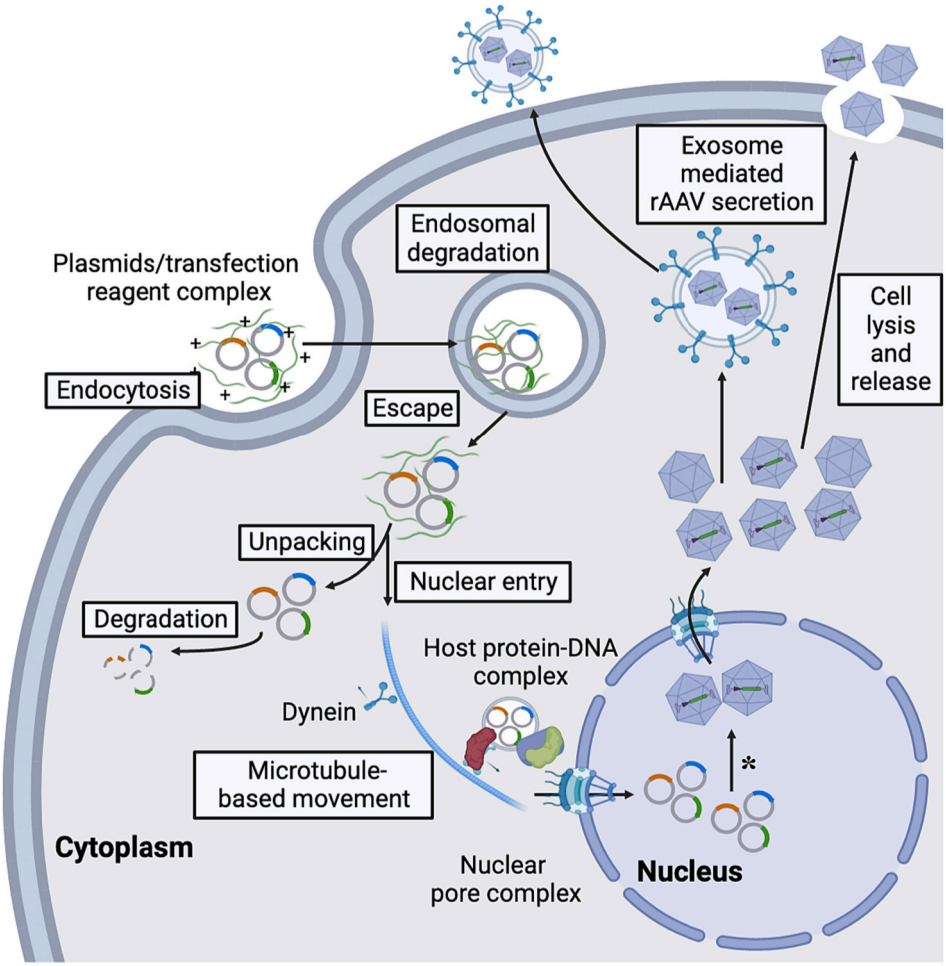

Fig. 1. Transfection reagent mediated cytoplasmic entering of plasmids, plasmid trafficking and nuclear entry via microtubule-based movement, and final vector release/secretion from host cells in rAAV production. Internalized plasmids that get released from the endosome will travel through the cytoplasm with cytoskeleton transport system and enter nucleus through the nuclear pore complex. Finally, empty/filled viral capsids will be exported from the nucleus, either remaining in the cytoplasm or releasing via cell lysis or exosome associated vesicles. "*" The animation simplified the steps during the rAAV production process, from the nucleus entry of internalized plasmids to the formation of packaged capsids. Specifics, such as transcription occurring inside the nucleus, translation in the cytoplasm, transport of the capsids back into the nucleus for assembly, and packaging, were elaborated in Fig. 2.

Once plasmids cross the cell membrane, they must travel through the cytoplasm and enter the nucleus for gene expression (Fig. 1). Trafficking across the cytoplasm represents the first barrier for efficient gene expression, because 1) free DNAs are subject to cytoplasmic nuclease degradation (Lechardeur et al., 1999) and 2) large plasmids with a size greater than 2000 bp are not able to diffuse in the cytoplasm due to the viscous cytoplasmic environment (Lukacs et al., 2000). Considering the distance between the cytoplasm and nucleus, free DNA will complex with host cellular proteins and move across the cytoplasm by active transport via the microtubule network. During this process, a large amount of host cell factors, such as transcription factors and DNA-binding proteins, play a role in the formation of the host protein-DNA complex and in the microtubule-mediated movement (Badding et al., 2013; Miller et al., 2009). The plasmids-host cell factors complex will interact with the molecular motor, such as dynein, and nuclear localization signal (NLS) peptides to reach the nucleus (Salman et al., 2005; Vaughan and Dean, 2006).

Nucleus entry is another significant bottleneck. It has been shown that only 10% of plasmids delivered to cytoplasm were detected in the nucleus, indicating the nuclear envelope as a hurdle for the transfection (Coonrod et al., 1997; Tseng et al., 1997). During cell division, the nuclear membrane breaks down, allowing the plasmids in the cytoplasm access to the nuclear area (Brunner et al., 2000; Tait et al., 2004). In the slow dividing or non-divided cells, the protein-DNA complex or DNA binding with the NLS peptides enters the nucleus through the nuclear pore complex via using the importin machinery. Generally, actively dividing cells have ten times higher chance to achieve gene expression compared to non-divided cells (Fasbender et al., 1997). DNA nuclear targeting sequences (DTSs), specific sequences promoting nuclear import, have been investigated for improved nuclear import of plasmids for non-dividing cells (Dames et al., 2007; Le Guen et al., 2021; Sacramento et al., 2010). Several general DNA nuclear targeting sequences (DTSs), short consensus motifs recognized by specific transcription factors, were identified as host cell factors binding sites for efficient plasmid trafficking. After the binding of transcription factors to DTSs, the plasmid-protein complex containing enhanced nuclear localization signals (NLSs) will utilize the NLS-mediated import machinery for nuclear entry. The general DTSs, such as SV40 enhancer (Dean, 1997), glucocorticoid receptor binding sites (Dames et al., 2007), NF-κB-binding sites (Le Guen et al., 2021; Mesika et al., 2001), and hypoxia response elements (Sacramento et al., 2010) have shown the improvement of plasmid delivery in mammalian cells. It is worthwhile to note that Guen et al. recently added 3NFs, an optimized short nucleotide sequence that can bind with transcription factor nuclear factor kB (NF-kB), into the plasmids for more efficient plasmid delivery in mammalian cells in vitro and in vivo (Le Guen et al., 2021). In addition, the corresponding host cell factors interacting with the reported DTSs also play an important role in plasmid delivery. The ubiquitously expressed transcription factors (Bai et al., 2017; Dynan and Chervitz, 1989), such as AP1, AP2, AP3, NF-κB, Oct-1, TEF—I, Sp1, and other nuclear import machinery factors (Wilson et al., 1999), such as importinα/β and RAN, interact with SV40 DTS and promote plasmid trafficking in mammalian

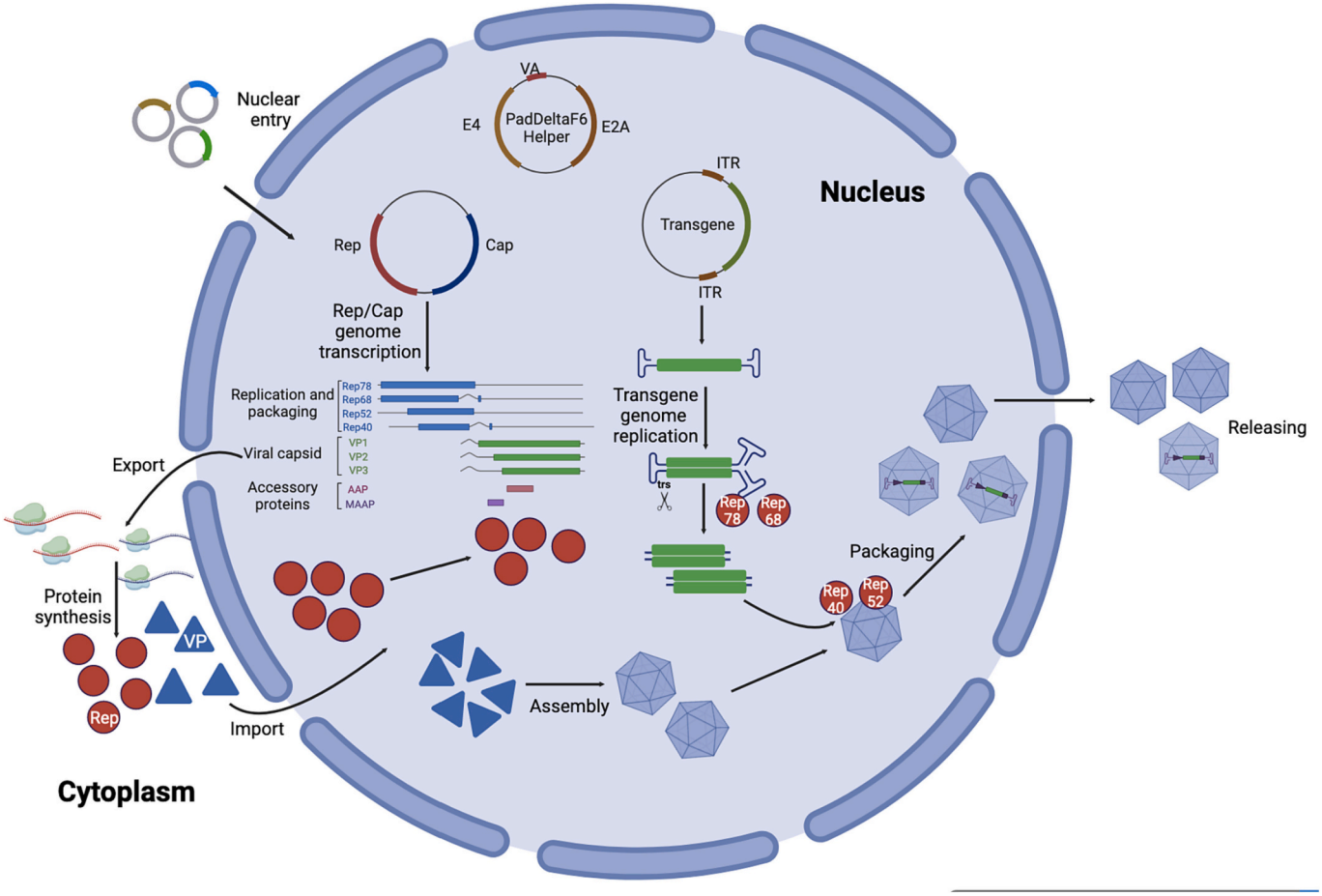

Fig. 2. Major steps involved in rAAV production: heterologous gene transcription and viral protein synthesis, capsid assembly, genome replication, and genome packaging. Rep gene transcription will be activated by E1A inside the nucleus. Once Rep proteins are translated in the cytoplasm, they will enter the nucleus and activate the transcription of cap gene encoding viral structural proteins. Viral structural proteins will assemble and form capsids. Plasmids carrying GOI will undergo genome replication with the assistance of helper proteins, large Rep proteins, and host cell factors. With the help of small Rep proteins, the synthesized genome will be packaged into preformed capsids. Some capsids will remain as empty ones. Both empty and filled capsids will be secreted to the cytoplasm.

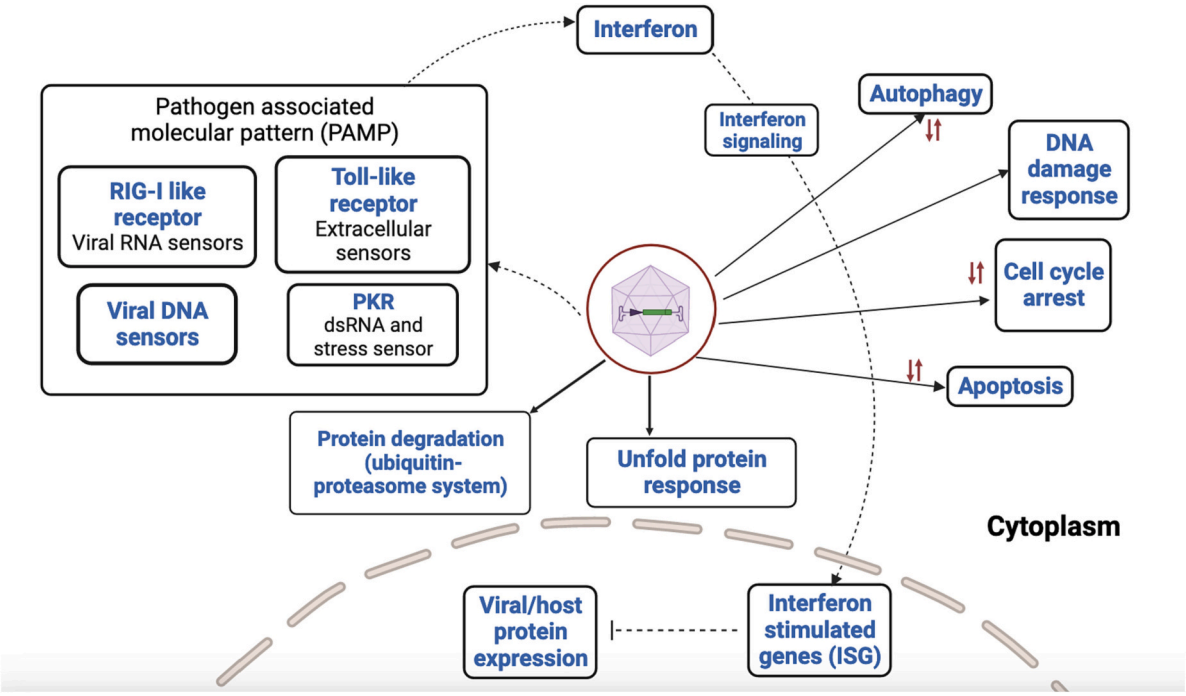

Fig. 3. The overview of the host-virus interactions and host cell responses triggered during the rAAV vector production. Host cell responses, such as antiviral immune responses, ER stress, DNA damage responses, cell cycle arrests, etc., together regulate the viral protein expression, the genome replication and finally affect the rAAV production.

cells. Other than reported DTSs, the CMV promoter has been engineered to eliminate the binding site of host trans-repression factor YY1 and RBP-Jκ, and it was also shown to enhance the transcription efficiency up to 50% (Johari et al., 2022). Transcription factors including ARNT, CREB, E4F, Sp1, ZBED1, JunB, c-Rel, and NF-κB were identified as key elements for the enhanced CMV activity (Johari et al., 2022). Apart from the key transcription factors used in the active shuttle, many other host cell proteins such as DNA/RNA binding proteins, NLS-receptor proteins, chaperones, etc. can be identified via the proteomic study. The characterized process can facilitate our understanding of the host factors involved in cytoplasm trafficking and nuclear entry. The corresponding host cell factors are summarized in Table 1.

Based on the mechanistic understanding shown above, several potential strategies can be applied to enhance transfection efficiency and therefore improve viral vector production: (1) enhance the nucleus entry of plasmids for gene expression by improving the cell growth performance, such as an increase in the cell growth rate. With increased cell growth rate and more active cell division, the nuclear entry of plasmids can be improved; (2) identify host cell factors involved in the formation of DNA-protein complex and overexpress those specific host cell factors to increase trafficking efficiency; (3) integrate enhanced DTSs in the plasmids to facilitate more efficient nuclear import; and/or (4) engineer the host factors binding sites in the plasmids to allow enhanced binding of transcription factors or eliminate the binding of trans-repression factors.

3.2. Heterologous gene transcription and protein synthesis

After the nuclear entry of three plasmids, those heterologous genes in the plasmids, including *E2A, E4orf6, Rep,* and *cap* genes, will be transcribed to mRNAs, exported to the cytoplasm and used for protein translation in the ribosome as shown in Fig. 2. Synthesized proteins in the cytoplasm will be transported back to the nucleus for later virion biosynthesis. This section describes the host cell responses involved in this process and the related host cell factors. The host cell responses are summarized in Fig. 4 and the corresponding host cell factors are summarized in Table 1.

ER stress and Unfold protein response (UPR): During vector production, viral protein synthesis relies on host cellular translational machinery, leading to an inevitably large amount of unfolded or misfolded proteins (Kim et al., 2008). Inherent cellular stress response and UPR will then be triggered by the accumulation of unfold/misfold proteins and Endoplasmic Reticulum (ER) stress, resulting in translational arrest, mRNA degradation, and protein degradation (ER-associated degradation and autophagy-lysosome degradation) (Hetz, 2012). To promote cell survival, the transcription of ER chaperones will be activated as an antiapoptotic signal to mediate translation attenuation, facilitate protein folding, and release cellular stress. However, heightened exposure to UPR and ER stress can escalate the expression of genes related to inflammation and potentially trigger cell apoptosis (Smith, 2014). UPR utilizes adaptive mechanisms to maintain the optimal rate of protein production and keep the balance between survival and death. Detailed summary of virus-induced ER stress and UPR can be found in this review (Zhang and Wang, 2012). It has been shown that AAV activated three major UPR pathways during its infection that include endoribonuclease inositol-requiring enzyme-1 (*IRE1α*), activating transcription factor 6 (*ATF6*) and PKR-like ER kinase (*PERK*), and different viral capsids determined the strength and type of UPR activation (Balakrishnan et al., 2013; Sen et al., 2014). Additionally, tunicamycin, a potent UPR and ER stress inducer, significantly reduced the rAAV2 vector yields (Mary et al., 2019b). More importantly, pharmacological UPR inhibitor inhibits the expression of inflammatory genes (Balakrishnan et al., 2013). Moreover, the attenuation of UPR with these small molecule additives leads to a 2.8-fold increase in the expression of the transgene in the AAV transduction (Balakrishnan et al., 2013). Also, by overexpressing multiple ER protein processing genes, including *PDIA2, HSPA5, and XBP1*, Formas-Oliveira et al. achieved up to 97% increase in r-RV volumetric productivity (Formas-Oliveira et al., 2020). The improvement achieved in AAV transduction and lentiviral vector production indicates that the modulation of these stress pathways is beneficial for protein synthesis, and potentially can be applied for rAAV production. Thus, it is worthwhile to regulate the UPR activated by viral protein synthesis (e.g.,

Table 1
Host cell responses and factors involved in rAAV early production process, specifically plasmid trafficking and gene transcription and protein synthesis.

rAAV production process	Host cell responses /Host cell factors	The details of host cell factors	Description (function, strategies, and improvement)	Relevance	Reference
Cytoplasmic trafficking and nuclear entering	Host cell factors	Importin α/β	Importin machinery factors are required to carry the DNA-protein complex through the NPC into the nucleus.	Others	(Bai et al., 2017)
		AP1/2/3, NF-kB, Oct-1, TEF1, Sp1	Transcription factors interact with SV40 DTS and facilitate nuclear import.	Others	(Dynan and Chervitz, 1989)
	ER stress and unfold protein response	ATF6, IRE1α, PERK	AAV infection upregulated three major signaling proteins, including ATF6, PERK, and IRE1α, involved in the UPR pathways. UPR pathway inhibition improved transgene expression by 2.8-fold and attenuated the subsequent inflammatory responses in AAV transduction.	AAV infection	(Balakrishnan et al., 2013; Sen et al., 2014)
		PDIA2, HSPA5 and XBP1	The pathways of ER-related protein processing influenced vector productivity in HEK293 cells. e.g., improved retroviral vector productivity up to 97%.	Others, Vector production in HEK293	(Formas-Oliveira et al., 2020)
Heterologous gene transcription and protein synthesis	Autophagy	SMURF1 and FANCC	The SMURF1 and FANCC are identified as the mediators of viral autophagy to target HSV-1. Inhibition of autophagy lead to decreased synthesis of adenovirus structural protein and poor replication; whereas the promotion of autophagy result in increased adenovirus titer.	Others, HSV-1, Adenovirus infection	(Orvedahl et al., 2011; Rodriguez-Rocha et al., 2011; Sumpter Jr et al., 2016)
	Ubiquitin-proteasome system	SPOP	SPOP as the E3-ubiquitin ligase interacts with AAV capsid protein VP1. Knockout of SPOP resulted in up to 3-fold increase in the total amount of capsids, and enhanced transduction through proteosome inhibition.	rAAV transduction	(Njenga, 2020)
		Ubc9	Ubc9 was identified as the host factor involved in restricting AAV. Transiently targeting SUMOlation can effectively enhance transduction.	rAAV transduction	(Hölscher et al., 2015)
		RNF	RNF121 as an E3 ubiquitin ligase is important in the regulation of AAV genome replication. Knockout of RNF121 significantly decreased AAV transduction. mRNA syntheses were found markedly decreased in KO cells.	AAV infection and rAAV transduction	(Madigan et al., 2019)

overexpression of ER protein processing genes, the addition of small molecule UPR inhibitors or attenuators) and further understand its detailed role in AAV vector production.

ER-associated degradation (ERAD) via *ubiquitin-proteasome system (UPS)*: ER-associated degradation, or ERAD, is a critical eukaryotic process that involves the ubiquitination of misfolded proteins followed by their degradation via UPS. Not only synthesized viral proteins are subject to degradation, but assembled capsids are also vulnerable to the host cell's UPS. Douar et al. found that a large amount of AAV vectors were degraded by the ubiquitination/proteasomal machinery when they performed AAV transduction experiment (Douar et al., 2001). Several ubiquitin ligases, such as SPOP (Njenga, 2020), RNF121 (Madigan et al., 2019), and Ubc9 (Hölscher et al., 2015), were found to restrict the AAV life cycle and transduction by different mechanisms. SPOP regulated the turnover of both unassembled and assembled capsids; SPOP knockout decreased the proteasome inhibition, leading to an enhanced transduction (Njenga, 2020). RNF121 regulated AAV genome transcription as a decrease in transduction was observed with RNF121 knockout (Madigan et al., 2019). In another study, a decrease of viral vector degradation was observed when specific amino acids on the viral capsids are modified via phosphorylation or ubiquitination (Gabriel et al., 2013). Based on these observations, we believe that the proteasomal system also plays a role in rAAV production through undetermined mechanisms, and additional investigation will be necessary to clear the gap between the proteasomal system and viral production. No literature has been reported about the connection between host cell ubiquitination and AAV vector productivity. It is worthwhile investigating if proteasome inhibitors or the regulation of UPS protect AAV capsids or other viral-related proteins from degradation, thus increasing the nuclear accumulation of viral capsids and potentially vector productivity.

ER stress and autophagy: The presence and accumulation of misfolded/unfolded protein will be harmful to cells, thus it has to be eliminated by specialized processes, such as ERAD and autophagy (Lin et al., 2008). If the ERAD mechanism is insufficient to recover ER and restore homeostasis due to the presence of excessive stress, other response pathways like autophagy are promoted to eliminate misfold/unfold proteins and damaged ER (Chipurupalli et al., 2021). Autophagy acts as a restriction response to viral replication and pathogen spread, delivering the cytoplasmic material to the lysosome for degradation (Dreux and Chisari, 2010). The process of autophagy can be utilized selectively to degrade viral components, viral particles, or host factors that are necessary for viral replication in the viral infection (Choi et al., 2018). The SMURF1 and FANCC are identified as the mediators of viral autophagy to target HSV-1 (Orvedahl et al., 2011; Sumpter Jr et al., 2016). However, some viruses can hijack the autophagic pathways to promote their own replication (Jackson et al., 2005). In adenovirus replication, autophagy inhibition leads to a decrease in structural protein synthesis, while the promotion of autophagy increased viral titer yield, indicating the positive correlation of adenovirus-induced autophagy with viral replication (Rodriguez-Rocha et al., 2011). Detailed discussion about the autophagy and viral life cycle can be found in another review paper (Choi et al., 2018). So far, no publication has shown autophagy in rAAV production. Considering the dual roles of autophagy, the correlation between autophagy and rAAV vector production deserves further investigation.

Apoptosis: In addition, Rep protein synthesis and helper gene

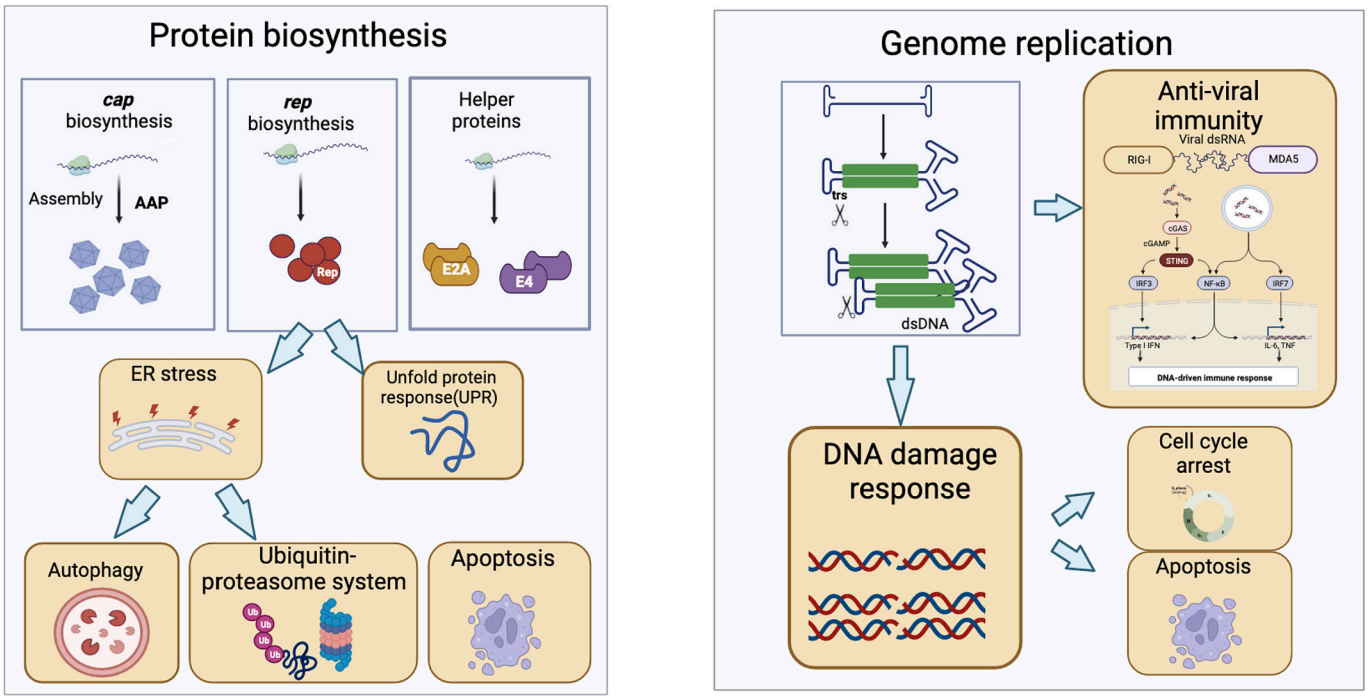

Fig. 4. Host cell responses are triggered during protein biosynthesis and genome replication in AAV infection or vector production. In the phase of protein biosynthesis, ER stress and UPR will be activated by a large accumulation of unfolded/misfolded protein. The presence of those proteins will be detrimental to cells; thus it has to be eliminated by UPS and autophagy. In the phase of genome replication, DDR can be activated and coupled with a series of other cellular responses, such as cell cycle arrest, and apoptosis. Additionally, the antiviral immune response will be initiated once host cells detect foreign genes and proteins.

expression are often associated with viral infection and vector production. Viral replication in host cells can lead to the production of a large number of viral proteins, including the Rep protein, which can trigger apoptosis in host cells. Rep proteins can induce DNA damage, which can activate cellular pathways leading to apoptosis. In addition, they can interfere with the normal functioning of the host cell by disrupting the cell cycle, inhibiting DNA repair mechanisms, and altering gene expression. The Rep78 protein was reported to activate the caspase-3 and induce cell apoptosis (Schmidt et al., 2000). Also, the Rep78 protein would decrease Cdc25A activity and block the cell cycle in the S phase (Berthet et al., 2005). E4orf4 protein has multiple functions in regulating viral infection progression and inhibiting the cellular DNA damage response (Rosen et al., 2019). E4orf4 can induce a mode of cell death independent of the p53 and caspase activation, leading to caspase-dependent apoptosis in some mammalian cell lines (Marcellus et al., 1998).

To reduce the cytotoxic effects caused by the expression of Rep proteins to the host cells during rAAV production, controlling the ratio of Rep proteins with overlapping functions, such as Rep78 and Rep68, can be employed to improve rAAV productivity (Emmerling et al., 2016). Since Rep78 and Rep68 have similar functions, downregulating Rep78 can reduce the negative effects caused by this protein and alleviate the burden of heterologous protein expression on cells. Similarly, this strategy can be applied to other types of Rep proteins such as Rep52/40 (Urabe et al., 2002). In addition, the cytotoxicity associated with viral components similarly remains as the main limitation for establishing constitutive lentiviral vector (LV) production. T26S point mutation in viral protease of plasmids (Tomás et al., 2018) and codon-optimized viral components by recombinase mediated cassette exchange (RMCE) (Sanber et al., 2015) were both reported to lower cytotoxicity without affecting virus maturation and infectivity and used to establish new improved and safer high titer LV producer cell lines. Similar codon optimization and point mutation approach can be applied to rAAV production: Seyffert et al. found the novel mutant AAV2 rep proteins support efficient AAV2 replication but have reduced impact on apoptosis and Rep-specific DNA damage response (Seyffert et al., 2017); the systematic mutagenesis can reveal the comprehensive AAV capsid fitness that leads to the machine guided rational design and engineering (Ogden et al., 2019). Deep mutational scanning, an advanced and commonly utilized technique (Ogden et al., 2019), to Rep genes and helper genes, allows screening for the additional mutant candidates that can facilitate rAAV production while demonstrating reduced cytotoxicity toward host cells. Furthermore, a mechanistic study of the cytotoxicity associated with Rep genes and helper genes from the host cell perspective will be useful for rational modifications to enhance the tolerance of the host cells and enhance the manufacturing capacity. To allow the constitutive expression of Rep and helper genes but not strong enough to cause cell death, the inducible promoters can be used to regulate the gene expression level of viral components. Along with screening libraries that target apoptosis, cell cycle, and DNA damage pathways, CRISPR-Cas9 screening can be employed to identify essential biomarkers involved in cytotoxicity. Once the particular pathway is identified, rational modifications can be implemented to mitigate and address the issue of cytotoxicity.

3.3. *Important host cell responses and factors for AAV genome replication*

AAV genome replication is accompanied with the transcription of non-structural viral genes and the synthesis of Rep protein (Pereira et al., 1997). With viral ITR structure as the origin of replication, strand displacement replication, and DNA synthesis take place using host replication machinery (Earley et al., 2020) (Fig. 2). A series of defense and stress responses are activated in the host cells to target the viral life cycle steps and eliminate the viral spread in both wild-type viral replication and the viral vector production process. Host cellular proteins involved in these response mechanisms, such as antiviral immune responses, DNA damage responses, cell cycle arrest, and cell apoptosis, will be highly regulated to maintain the survival of cells. The triggered host cell responses are described in Fig. 4, and the corresponding host cell factors are summarized in Table 2.

Table 2
Host cell responses and factors involved in rAAV later production process, specifically genome replication, capsid assembly, genome encapsidation and secretion.

rAAV production process	Host cell responses /Host cell factors	The details of host cell factors	Description (function, strategies, and improvement)	Relevance	Reference
		IFNB1, IRF7, IFIT2, OASL	A robust inflammatory and antiviral responses were triggered during rAAV production. Multiple pathogen patterns sensor, signal transducer and downstream interferon stimulated cytokines have been shown upregulation based on RNA seq data.	rAAV production	(Chung et al., 2023)
		IFITMs	AAV infection was shown to be resistant to all IFITMs antiviral effects.	AAV infection	(Tartour et al., 2017)
		TRIM19/PML	PML is a viral restriction factor inhibiting uncoating to transcription during AAV infection. Overexpression of PML inhibit AAV second strand synthesis, genome replication and vector production. Knockout of PML was shown to significantly enhance rAAV transduction.	AAV infection, rAAV transduction, and rAAV production	(Mitchell et al., 2014)
		RSAD2	RSAD2 enhances type I interferon response after dsDNA detection. It couples innate immune signaling with the production of antiviral nucleotides.	Others, Viral production in HEK293T cells	(Crosse et al., 2021; Dumbrepatil et al., 2019)
	Inflammatory and anti-viral immune response	OAS1, PKR, LDLR	OAS1 and LDLR were two host restriction factors identified in HEK293T cells impeding lentiviral vector production. Knockout of these antiviral related genes led up to 7-fold increase in viral titer.	Others, Vector production in HEK293T cells	(Han et al., 2021)
		DNA-PKcs, ATM, Ku70, Ku86	AAV/Ad coinfection induced a robust DNA damage response mediated by DNA-PKcs and ATM. Recruited repair proteins, such as Ku70 and Ku86, and other phosphorylated host factors RPA32 were found interact with Rep protein and accumulated in the replication center. The inhibition of DNA-Pkcs significantly reduced the AAV replication.	AAV/Ad coinfection	(Y.-K. Choi et al., 2010; Collaco et al., 2009; Schwartz et al., 2009)
	DNA damage response	ATR, DNA repair polymerase η, κ	During AAV/Ad coinfection, ATR plays an important role in replication. Genome replication-initiated DDR, which initiated the repair process that contributed to the genome amplification. The knockout of DNA repair DNA polymerase κ, η significantly reduced the wt AAV replication and rAAV production in HEK293T cells.	AAV/Ad coinfection and rAAV production	(Ning et al., 2023)
		SKA2, ITPRIP	SKA2 is involved in cell cycle progression, regulated by p53. It is related to cellular processes, such as DNA damage response, cell cycle arrest and apoptosis. ITPRIP regulates intracellular calcium signaling. The overexpression of SKA2 and ITPRIP increased genome replication and packaging, thereby improving the final titer by 3.8 fold.		(Barnes et al., 2021)
		S phase arrest	Thymidine mediated S phase arrest increased rAAV production by 1.6–2.4 fold depending on the treatment period.	rAAV production	(Barnes et al., 2021)
	Cell cycle arrest	G2/M phase arrest	Addition of nocodazole (anti-mitotic agent) and M344 arrests cells in G2/M phase, resulting in 3-fold increase in genome titer for rAAV production in HEK293 cells.	rAAV production	(Scarrott et al., 2023)
	Apoptosis	BCL2	The overexpression of anti-apoptotic gene, BCL2, increased 53% volumetric productivity for gamma-retroviral vector in HEK293 cells.	Others, Vector production in HEK293 cells	(Formas-Oliveira et al., 2020)
		NPM1	DNA binding protein NPM1 forms steric load block to DNA transcription, leading to the repression of viral DNA replication. Knockdown of NPM1 resulted in significant improvement in AAV vector production.	rAAV production	(Satkunanathan et al., 2017)
		ZF5	ZF5, a ubiquitously expression transcription factor, represses p5 promoter, thus restricting both AAV replication and rAAV production.	rAAV production	(Cathomen et al., 2001)
AAV genome replication	Other host cell factors	RPA, PCNA, polymerase δ	These DNA binding proteins are identified as required host factors for AAV DNA replication and important for AAV production as well.	AAV/Ad coinfection and rAAV production	(Ni et al., 1998)
Capsid assembly and genome packaging	Host cell factors	YB1	DNA binding protein YB1 competes with AAV capsids for binding to ITR sequence. Downregulation of DNA binding protein YB1 lead to 45 fold increase in AAV vector genome titer and reduced the percentage of empty particles in the product.	rAAV production	(Satkunanathan et al., 2014)
		VSVG, rabiesG	Intracellular synthesized VSVG stimulated the formation of transport vehicle, facilitating rAAV trafficking to the plasma membrane.		(Kotterman et al., 2015)
		Tetraspanin CD9	Tetraspanin CD9 is one of the component of exosomes. The overexpression of tetraspanin CD9 increased the exosome release of AAV producer cells and thus increased the amount of *exo*-AAV.		(Schiller et al., 2018)
rAAV releasing	Host cell factors			rAAV production	

Innate immune responses: Host cell innate immunity often acts as the first line of defense against the spread of viruses. Once a pathogen-associated molecular pattern (PAMP) (e.g., viral nucleic acids) is detected by the host's pathogen recognition receptors, such as RIG-I like receptor, Toll-like receptor, Viral DNA sensor, and PKR receptor, a series of signaling pathways is triggered, leading to the production of type I interferon (IFN) and the transcription of interferon-stimulated genes (ISGs) (Raftery and Stevenson, 2017) (Fig. 3). The expression of these genes results in the negative regulation of viral replication and the restriction of the viral production (Raftery and Stevenson, 2017).

Several recent RNA-seq studies identified a strong cellular defense response to rAAV production: inflammatory and antiviral responses with upregulated interferon-stimulated cytokines and chemokines (Chung et al., 2023; Wang and Fu et al., 2024). A detailed summary of restriction factors that affect the viral life cycle has been provided in one recent review paper (Chemudupati et al., 2019). Several reported antiviral factors are closely related to the AAV life cycle: IFITMs (Tartour et al., 2017), TIRM19/PML (Mitchell et al., 2014), RSAD2 (Crosse et al., 2021; Dumbrepatil et al., 2019), etc. Only a small proportion of antiviral factors have been identified as specific restriction factors for inhibiting viral replication. This is because viruses have the ability to hijack various host proteins to secure their effective replication, even in the presence of host defense mechanisms (Kluge et al., 2015). Therefore, additional verifications, such as siRNA-mediated screening/gene silence (Xiao et al., 2016), and Crispr-mediated screening/ gene knock-out (Han et al., 2021; Shin et al., 2022), will help to illustrate the roles of these antiviral factors and enable the effective rational design to boost manufacturing capacity (Collaco et al., 2009). To illustrate, the successful improvement (7–11 fold increase) in lentiviral vector productivity in HEK293T cells by knockout of antiviral-related genes further demonstrates the importance of the regulation in antiviral responses (Han et al., 2021). Similar antiviral gene silence/knockout strategies or the supplementation of small molecule inhibitors targeting antiviral factors can be applied in the production of rAAV based on transcriptomic studies (Wang and Fu et al., 2024).

The immune response induced by the viral nucleic acid in rAAV production can also be modulated through sequence design based on codon optimization. The methylation by CpG motif of mammalian cells and viruses is distinct, thus leading to an immune response (Krieg, 2000). Hence, the CpG of the plasmid to the host cell should be one of the critical parameters, and optimizing the CpG of the plasmid can decrease the immune response and thus improve rAAV productivity (Faust et al., 2013; Pan et al., 2022). Several codon optimization tools are available to aid in sequence design and CpG control (Chin et al., 2014). Furthermore, mutation of ITRs, codon optimization of transgenes, and use of synthetic/reverse polyA tails have been shown to enable AAV to escape innate immune response during the AAV transduction (Li and Samulski, 2020; Pan et al., 2022). Similar strategies can be applied to minimize the host cell immune responses to AAV for rAAV production.

DNA Damage Response (DDR): Other than innate immune responses, AAV genome replication can also trigger a robust DDR (Schwartz et al., 2009). The activation of DDR is triggered by DNA double-strand break (DSB) or the accumulation of single-strand DNA (Weitzman and Fradet-Turcotte, 2018). Sensor proteins, such as MRE11-RAD50-NBS1 (MRN) complex and RPA, recognize damaged DNA and activate the downstream host signal transducer proteins, including ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3 related (ATR), or DNA-dependent protein kinase catalytic subunit (DNA-PKcs) (Weitzman and Fradet-Turcotte, 2018). The signal cascade will lead to the recruitment of cellular factors to repair the lesion and the activation of the cell cycle checkpoint to prevent the replication of damaged DNA. During the productive AAV replication with adenovirus coinfection, DNA-PKcs (Collaco et al., 2009; Schwartz et al., 2009) and ATR (Ning et al., 2023) have been reported as the primary mediators of damage signaling. With HSV coinfection, the DDR signaling is mainly through ATM (Millet et al., 2015; Vogel et al., 2012). Helper virus mentioned above, Adenovirus and HSV, facilitates the formation of viral replication center, where recruited repair proteins accumulate, colocalize with Rep protein and mediate the viral replication. In the case of Adenoviral mediated AAV coinfection, DNA-PKcs and recruited cellular protein Ku70/86, as Rep interacting proteins, are reported as required for AAV replication (Schwartz et al., 2009). Additional downstream host cell factors, such as Chk1, H2AX, XRCC4, and RPA32, have also been phosphorylated and featured during AAV replication induced DDR signaling (Collaco et al., 2009; Schwartz et al., 2009). The regulation of sensor proteins was shown to exhibit significant impacts on AAV replication: Inhibition of DNA-PKcs by a DNA-PKcs inhibitor, wortmannin, or siRNA targeting DNA-PKcs significantly decreased the AAV replication (Choi et al., 2010); Knockout of polymerase (Pol) η and Pol k involved in ATR-mediated repair process significantly decreased wild-type AAV2 replication and recombinant AAV2 production in HEK293T cells (Ning et al., 2023); DDR senor protein MRN complex negatively regulates AAV replication during coinfection with adenovirus, whereas positively regulates AAV replication during coinfection with HSV (Millet et al., 2015). All suggest the important role of DNA damage response and recruited repair proteins in AAV replication. Investigation should be conducted on crucial host repair proteins that are enlisted during the replication process. Additionally, cell line engineering techniques, such as the overexpression of these host factors, can be employed to delve deeper into their associations with DNA damage response (DDR) pathways and AAV productivity.

DDR and cell cycle arrest: DDR triggers a series of phosphorylation events that synchronize with cellular processes, such as DNA repair, cell cycle arrest and apoptosis. Host cell cycle arrest has been constantly observed in the life cycle of viruses (Davy and Doorbar, 2007). With induced cell cycle arrest, the cellular condition can achieve the level beneficial for viral replication by increasing the deoxynucleotide pool, preventing early cell death, and promoting the virus assembly (Bagga and Bouchard, 2014). Increasing evidence has shown that AAV also modulated host cell cycles to facilitate viral genome replication: Rep78 arrest cells in the S phase (Saudan et al., 2000); AAV genome replication mainly occurs in the S/G2 phase (Winocour et al., 1988). One genome-wide screening study identified SKA2 and ITPRIP, involved in cell-cycle progression, as important genes whose modulation can enhance the cell capacity to produce rAAV vector (Barnes et al., 2021). By modulating the progression of the cell cycle, the upregulation of these two genes in the producer cell line resulted in a significant increase of vector genome titer up to 3.8-fold, successfully boosting the genome replication and promoting the genome packaging (Barnes et al., 2021). In the same study, it was found that an increased S phase population with the addition of thymidine improved AAV productivity by 2 fold depending on treatment time (Barnes et al., 2021). Induction of G2/M phase arrest has been found favorable for viral replication by establishing a pseudo-S phase state, an active environment with available substrates and machinery (Davy and Doorbar, 2007). Scarrott et al. proposed the addition of small molecule bioactive chemical additives, Nocodazole (an anti-mitotic agent) and M344 (a selective histone deacetylase inhibitor) via modulating cell cycle in the G2/M phase, that resulted in the 3-fold of the rAAV productivity (Scarrott et al., 2022). Results from both genome-wide screening and supplementation screening with small molecule additives indicate that cell cycle modulation plays an essential role in viral replication and thereby affects productivity. Additional investigation might be worthwhile in identifying more key host cell factors involved in the cell cycle arrest response and further elucidating their fundamental mechanisms.

DDR and induced apoptosis: Major sensor proteins DNA-PK (Lees-Miller et al., 1992; S. Wang et al., 2000), ATR (Lakin et al., 1999) and ATM (Banin et al., 1998) involved in DNA damage responses have been reported to induce p53 phosphorylation, the signal that causes cell apoptosis. AAV replication can be maintained without significant cell death in p53-proficient cells via H2AX-p53-p21 mediated G2/M arrest.

However, the lack of this pathway in p53-deficient cells results in apoptosis (Chen and Qiu, 2010). The overexpression of the anti-apoptotic gene *BCl2* improved r-RV productivity by up to 97% (Formas-Oliveira et al., 2020). A similar control strategy, such as the overexpression of antiapoptotic genes, and the silencing of proapoptotic genes, can be applied to alleviate apoptotic stress for rAAV production.

Additional host cell factors: Furthermore, many other host cellular factors have been reported to have impacts on genome replication by directly or indirectly interacting with the AAV genome. DNA binding protein nucleophosmin (NPM1) interacts with G-quadruplex DNA sequence (GQRS), forms steric roadblocks to DNA transcription and replication, and leads to the repression of viral DNA replication (Scognamiglio et al., 2014). Knockdown of NPM1 achieved a significant increase in AAV vector production (Satkunanathan et al., 2017). Additionally, a genetic screen found that ZF5, a ubiquitously expressed transcription factor, led to the repression of p5 promoter, thus reducing AAV2 replication and lowering the vector productivity (Cathomen et al., 2001). Other than these cellular factors that have demonstrated effects on vector production, some host factors directly involved in wild-type AAV genome replication, such as replication protein A (RPA), proliferating cell nuclear antigen (PCNA), and polymerase δ, have been extensively studied and should also play essential roles in the production process (Nash et al., 2007; Ni et al., 1998).

Overall, host cell responses and factors play critical roles in the AAV replication process. In addition to the host cell factors mentioned earlier, high throughput screening tools, such as multi-omics or genome-wide screening, can contribute to the identification of additional novel and essential host factors. Rational modifications that manipulate these host cell factors can be later proposed and implemented to enhance productivity. Furthermore, optimizing the input materials, including AAV plasmids that contribute to the host cell responses, can be pursued to facilitate the vector production process.

3.4. Host cell factors in capsid assembly and genome packaging

Viral structural proteins will form preassembled capsids with the help of AAP (Maurer et al., 2018). The preformed capsids will be transported to the nucleus, either staying inside the nucleus for later genome packaging or secreting out as empty capsids from the nucleus into the cytosol (Fig. 2). Virion encapsidation remains as a challenging and imperfect step potentially due to (1) the poorly coordinated timeline of capsid synthesis and genome replication and (2) the hinder of capsid production due to the repressive function of the Rep protein at the later phase (Nguyen et al., 2021). This leads to the fact that only a small fraction of rAAV crude harvest contains therapeutic transgene, and a large number of empty particles pose extra difficulties in downstream purification steps.

The reported mutation of structural proteins, such as the N or C terminus of VP3 (Wu et al., 2000) and the five-fold (5F) axis of the assembled capsid (Bleker et al., 2005), decreased the capsid assembly and packaging efficiency, revealing their important roles in capsid assembly and stability. The mutation of 5F disrupts the shape and prevents the ability for the rep protein to dock and mediate genome encapsidation. Other than viral components themselves, understanding the host responses to the intrusion of virion assembly and the host factors' role in genome encapsidation will shed light on innovative strategies for enhanced vector manufacturing.

Host cells are required for AAV assembly; capsid proteins failed to assemble to proper capsids without the addition of cell lysates (Steinbach et al., 1997). Additional evidence also has shown the presence of host cell factors that restrict capsid assembly and thus block viral production: with more than 90% transfection efficiency, only less than 10% of cells had assembled capsids in their nucleus (Dash et al., 2022; Maurer, 2019). Based upon that observation, Maurer and her colleagues conducted an unbiased genome-wide screening and proteomics to identify potential cellular assembly and restriction factors. Proteins such as Hsp70 (folding viral protein) and NPM1 (folding and nuclear transport chaperone), were confirmed indirectly to affect the assembly and production machinery (Maurer, 2019). Further investigation is required to gain a comprehensive understanding of the assembly process and the specific host cellular machinery involved in order to improve rAAV production.

Several factors, such as Rep52/40 proteins (Yoon-Robarts et al., 2004), the specific sequence of ITR (Wang et al., 1996), and host cellular proteins(Zhou and Muzyczka, 1998), were considered to play important roles in the genome packaging step; however, the detailed mechanism of packaging is not clearly defined yet. Reconstitution of genome packaging in vitro also requires the addition of cell lysate, confirming the role of host proteins (Zhou and Muzyczka, 1998). One study identified several important AAV-associated cellular proteins like DNA binding protein YB1 with proteomics study (Satkunanathan et al., 2014). Downregulation of YB1 resulted in a 45-fold increase in vector genome titer for AAV2 and reduced the percentage of empty particles in the final product. It has been suggested that YB1 plays an important role in AAV packaging by competing with AAV capsid proteins for binding to the ITR sequence (Satkunanathan et al., 2014). This indicated the essential role of cellular proteins in vector genome packaging. Further investigations on host factors involved in genome packaging will be necessary to understand the limited packaging capacity of host cells (Bennett et al., 2017). For example, up to 95% full packaging ratio has been reported in wtAAV replication (Zeltner et al., 2010). The comparison experiments between wtAAV replication and rAAV production can be assessed to identify the direct/indirect packaging host cell factors via high-throughput screening approaches, such as genome-wide CRISPR screening, transcriptomics, and proteomics.

Additionally, the assembly and nuclear entry of AAV capsids are regulated by AAP protein, one essential component encoded in the Cap gene(Grosse et al., 2017). The overexpression of AAP might promote capsid assembly and improve capsid yields, but simultaneously lead to insufficient post-translational modification (PTM) of VP1, 2, 3 in the cytoplasm, important in capsid protein modification. The literature reported 52 PTMs, such as glycosylation, phosphorylation, ubiquitination, SUMOylation, and acetylation cross different serotypes in AAV capsids (Mary et al., 2019a). Disrupting the glycosylation site in AAV2 significantly decreased the packaging efficiency (Mary et al., 2019a). PTMs of AAV capsid proteins were also able to regulate viral infectivity and cell-type transduction preferences (Mary et al., 2019a; Nonnenmacher and Weber, 2012; Xie and Butler, 2023). Delaying or decreasing the expression of AAP could potentially prolong the presence of VP1/2/3 in the cytoplasm, thereby increasing the occurrence of PTM in these proteins (Grosse et al., 2017). The balance between capsid assembly/yield and the PTM by the modulation of AAP can be achieved after further investigation. Furthermore, a mismatch in the expression timing between Rep and Cap proteins results in substantial production of empty capsids (Nguyen et al., 2021). A recent study has demonstrated that regulating the timing of capsid expression using an inducible promoter can effectively reduce empty capsids, improving both viral quantity and quality (Ohba et al., 2023). Therefore, the regulation on timing and expression level of Rep and Cap can help to resolve the mismatch between capsid formation and genome replication, and further enhance the packaging efficiency.

3.5. rAAV release/secretion

In the late stage of rAAV production, due to the unfavorable intracellular environment, only a small portion of the host cells lyse through lysosome and apoptosis, releasing rAAVs to the media/supernatant. Most assembled virus particles stay inside the host cells, which requires extra steps to lyse the cells and harvest rAAV. This further complicates the downstream purification process when compared to the secreting vectors. Technically, being the non-enveloped viruses, AAV can't be secreted like other enveloped viruses (e.g., Retrovirus). However,

research reported that serotype-dependent rAAV associated with extracellular vesicles (EVs) were found in the supernatant without cell lysis (Maguire et al., 2012; Vandenberghe et al., 2010). It is named as exosome associated-AAV (*exo*-AAV) (Schiller et al., 2018). The process of rAAV release/secretion is illustrated in Fig. 1, and the corresponding host factors are summarized in Table 2.

The continuous and efficient secretion of rAAV particles into the media will offer significant benefits for the manufacturing process. Ongoing secretion of rAAV simplifies the purification process, and exosome AAVs have been shown to improve the effectiveness of drug delivery and offer protection against the anti-AAV neutralizing antibodies (Meliani et al., 2017; Schiller et al., 2018). However, only a few literatures reported potential approaches to enhance viral secretion in rAAV production. The research identified the membrane associated accessory protein (MAAP) as a novel factor for AAV secretion (Elmore et al., 2021). The expression of viral envelop proteins, such as VSVG, and rabiesG, in the packaging cell triggered the active secretion of rAAV particles (Kotterman et al., 2015). Intracellular synthesized VSVG stimulated the formation of transport vehicle, facilitating rAAV trafficking to the plasma membrane (Müsch et al., 1996). Additional validation experiments indicated that VSVG-mediated secretion relied on the cellular exosome pathway (Kotterman et al., 2015). Furthermore, the overexpression of tetraspanin CD9 increased the exosome release of AAV producer cells and thus increased the amount of exo-AAV, with enhanced transduction efficiency and effective immune escape (Schiller et al., 2018). However, the mechanism(s) by which AAV exits the cell or associates with EVs and the involved cellular machinery remains to be determined.

In light of the concept that AAV can utilize exosomes as a means of secretion, forthcoming research can focus on exploring the modification of capsid proteins that facilitate efficient entry of rAAV particles to exosomes and subsequent secretion, thereby enhancing the manufacturing process. The identification of biomarkers that enhance exosome production will be followed by a cell line engineering approach, specifically the overexpression of genes associated with exosome biosynthesis and secretion (Gurung et al., 2021; Hessvik and Llorente, 2018; Jafari et al., 2020). This strategy aims to improve the production and secretion of rAAV particles.

4. Cell-line engineering strategies

Host cells play an essential role in providing raw materials and cell machinery for rAAV production as cell factory. This review addresses every step occurring throughout the rAAV production and summarizes the host cell responses and related host cell factors involved. The cell line engineering strategies from host cells and input materials (e.g., rAAV plasmids) perspectives are generally listed below. Specific control strategies elaborated in this review paper are summarized as the following:

(1) To overcome the trafficking barrier and enable successful gene expression, approaches that promote cytoplasmic trafficking and enhance nuclear entry will be beneficial for viral production.
(2) During the productive expression of viral proteins, the strategies, such as overexpression of genes encoding chaperone or activating chaperone transcription to alleviate the UPR can release the cell stress thereby potentially enhancing rAAV productivity.
(3) During viral vector production, detection of viral DNA/RNA triggers the antiviral immune response, which can negatively regulate genome replication. Therefore, inhibition of the interferon signaling pathway and codon optimization of transfected plasmids can minimize the host cell immune response, thus positively regulating genome replication and viral productivity.
(4) The regulation of DDR would also benefit genome replication. The recruited damage repair proteins interact with the rep protein and accumulate in the replication center. They are important and required for genome replication. Overexpression of essential damage repair proteins might be useful to enhance productivity.
(5) Reducing the cytotoxicity effects caused by *Rep* and helper gene expression can be employed to improve rAAV productivity: overexpression of antiapoptotic related genes; point mutation in *Rep* and helper proteins that can support viral replication but trigger less cytotoxicity; CRISPR-Cas9 screening to identify biomarkers involved in cytotoxicity induction.
(6) Strategies that can regulate the cell cycle to match the viral life cycle would facilitate vector production.
(7) The regulation of expression level and expression timing of *Rep* and *Cap*, such as inducible promoters, can help to resolve the timing mismatch between capsid formation and genome replication, thereby enhancing the packaging efficiency.
(8) Strategies to improve the rAAV secretion will also simplify the downstream purification process and facilitate the manufacturing process.

5. Concluding remarks

Identifying key molecular features and understanding host cell physiology changes during viral production remain as major challenges hindering the viral vector productivity improvement in the current rAAV production process. Here, we elucidated the biological processes during rAAV production and summarized the important host cell responses and factors that have been discovered during viral replication and vector production. The fully characterized cellular processes and mechanisms will promote the rational modification of the input materials and host cell factory, thereby improving rAAV production. Given that similar biological processes take place within the host cells, it would be valuable to assess any insights gained from the transient transfection production system in the stable production system. Recent advances in omics approaches and high throughput genome-wide screening technologies will unveil the biological drivers of vector production. With genome editing tools, host cells can be engineered to be more robust for viral vector production while reducing their negative impact on the production process.

Declaration of competing interest

All the authors declare no conflict of interest.

Data availability

No data was used for the research described in the article.

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