Biotechnology and Bioengineering

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BIOTECHNOLOGY AND BIOENGINEERING

REVIEW OPEN ACCESS

What Makes an "Ideal" Cell Line for Recombinant Adeno-Associated Virus Production?

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Received: 30 January 2026 | **Revised:** 6 May 2026 | **Accepted:** 27 May 2026

Funding: Enterprise Ireland under its Research, Development and Innovation Fund, Grant/Award Number: 180753/RR; Irish Research Council (Taighde Éireann), Grant/Award Number: EBPPG/2022/38; Joint Appointment Research Professorship Award, Grant/Award Number: 23/RP/11975

Keywords: cell lines | chemical additives | genetic modifications | phenotypic traits | production systems | recombinant adeno-associated virus (rAAV)

ABSTRACT

Recombinant adeno-associated viruses (rAAVs) have been utilized extensively as the vector-of-choice for *in vivo* delivery of gene therapy products. Nonetheless, there are several disadvantages associated with current rAAV production systems, as they struggle to meet ever-growing clinical demands. To date, eight different host cell systems have been used to produce rAAVs, with particular cell lines possessing characteristics that make them more suitable for rAAV manufacturing than others. These traits, along with other modifications that have been shown to improve rAAV productivity, are reviewed here to develop a consensus on what an "ideal" cell line for rAAV production might look like. In doing so, we describe traits that will help to identify and deliver novel host cell systems that can support the widespread adoption of rAAV-based gene therapy products.

Abbreviations: AAP, assembly-activating protein; AAV, adeno-associated virus; Ad5, adenovirus 5; ATMP, advanced therapeutic medicinal product; ATP, adenosine triphosphate; BAK1, Bcl2 homologous antagonist/killer 1; BAX, Bcl2 associated X-protein; BEV, baculovirus expression vector; BHK, baby hamster kidney cells; CAP, CEVEC's amniocyte production cells; CASP8, caspase-8; CCL5, C–C motif chemokine ligand 5; CCNB1, cyclin B1; CCND1, cyclin D1; CD, chemically defined; CHO, Chinese hamster ovary cells; CREB, cyclic AMP response element binding protein; CRISPR, clustered regularly interspaced short palindromic repeats; CSP, cell-specific productivity; DNA, deoxyribonucleic acid; DTS, DNA nuclear targeting sequences; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; FDA, Food and Drug Administration; GMP, Good Manufacturing Practice; HAT, human amniotic epithelial cell line for gene and cell therapy; HBoV1, human bocavirus 1; hcDNA, host cell DNA; HCP, host cell protein; HDAC, histone deacetylase; HEK293, human embryonic kidney 293 cells; HER, human embryonic retinoblasts; HIF-1α, hypoxia-inducible factor 1-α; HPV, human papillomavirus; HSV, herpes simplex virus; HVEM, herpesvirus entry mediator; IFIT2, interferon-induced protein with tetratricopeptide repeats 2; IFIT5, interferon-induced protein with tetratricopeptide repeats 5; IFNAR1, interferon alpha/beta receptor subunit 1; IFNAR2, interferon alpha/beta receptor subunit 2; IFNGR2, interferon gamma receptor 2; IL-8, interleukin-8; IRF7, interferon regulatory factor 7; IRF9, interferon regulatory factor 9; ITR, inverted terminal repeat; JAK1, Janus kinase 1; JAK2, Janus kinase 2; kb, kilobases; kg, kilogram; L, liter; M, mitosis; MAAP, membrane-associated accessory protein; mL, milliliter; MLP, major late promoter; MVB, multivesicular body; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PEI, polyethyleneimine; PLK1, polo-like kinase isoform 1; PSG4, pregnancy-specific β-1-glycoprotein 4; rAAV, recombinant adeno-associated virus; RNA, ribonucleic acid; SFRP5, secreted frizzled-related protein 5; SNAP23, Synaptosome associated protein 23; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptors; STAT1, signal transducer and activator of transcription 1; STAT2, signal transducer and activator of transcription 2; STX7, syntaxin-7; SV40, simian virus 40; TESSA, Tetracycline-Enabled Self-Silencing Adenovirus; TGFB1, transforming growth factor beta 1; TRIF, TIR-domain-containing adapter inducing interferon-β; USD, US Dollars; VA RNA, virus-associated ribonucleic acid; vg, vector genomes; VP, viral protein; ZFP91, zinc finger protein 91; μM, micromolar; Z-VAD-FMK, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

1 | Introduction

1.1 | Adeno-Associated Viruses

The adeno-associated virus (AAV) was first identified in 1965 as a contaminant of adenovirus preparations (Atchison et al. 1965), and belongs to the Parvoviridae family of viruses. Structurally, AAV is a nonenveloped virus that consists of an icosahedral capsid and a single stranded, 4.7 kb viral genome (Fu et al. 2023). The viral genome contains two genes (*rep* and *cap*) which are flanked by hairpin-like structures known as inverted terminal repeats (ITRs); these are essential for genome replication, packaging, and gene expression. Three promoter sequences are located within the viral genome. The p5 promoter drives expression of the *Rep78* and *Rep68* genes, while *Rep52* and *Rep40* are expressed from the p19 promoter (Sha et al. 2021). Two alternatively spliced mRNAs are generated from the p40 promoter, which are translated to yield three different capsid proteins (VP1, VP2, VP3), whilst alternative start codons give rise to the membrane-associated accessory protein (MAAP) and assembly-activating protein (AAP) (Meier et al. 2020). The *cap* gene determines the AAV serotype as well as the tissue tropism. Naturally occurring AAV serotypes have been discovered that can target liver, neural, retinal, and lung tissue among others (Issa et al. 2023). This range has been expanded by the generation of novel engineered capsid variants (Zolotukhin and Vandenberghe 2022).

Recombinant adeno-associated virus (rAAV) vectors have been utilized extensively in the field of gene therapy due to their non-pathogenicity, broad tropism, and low immunogenicity. To compensate for a genetic defect, the virus's *rep* and *cap* coding sequences are replaced by the gene of interest, along with promoter and regulatory sequences required for *in vivo* expression. Of the 47 cell and gene therapy products currently approved by the FDA, eight are rAAV-based therapies (Bogdanovic et al. 2025; U.S. Food & Drug Administration 2025).

Despite the growing number of rAAV-based gene therapy products, there are several limitations associated with some rAAV manufacturing systems, including poor scalability and the generation of large amounts of empty capsids (not containing the target genome), which may cause an immune response without providing a therapeutic effect (Escandell et al. 2022). Current rAAV production methods also struggle to meet clinical demand, with the recommended dose of certain systemically delivered gene therapies being upwards of 1×10^{14} vg/kg of a patient's bodyweight (Novartis 2023). These issues, along with the high cost associated with a number of production materials (such as GMP-grade plasmids and transfection reagents), contribute to the expensive price-tag associated with these therapies; for instance, a single dose of onasemnogene abeparvovec (Zolgensma) is priced at $2.1 million USD (Wong et al. 2023).

Given the challenges and costs associated with rAAV production, there is great interest in identifying opportunities to improve production efficiency. The host cell used for rAAV production is a key element, as factors such as culture viability, growth rate, transfection ability, the cellular response to the production of viral Rep and Cap proteins and the presence of helper genes have a strong influence on product yields and quality. To date, eight host cell production systems have been used to produce rAAVs, and these will be explored further below (also summarized in Table 1).

1.2 | Current Hosts Used for rAAV Production

1.2.1 | HEK293 Cells

The HEK293 cell line is the most commonly used host for rAAV production (Sha et al. 2021). This cell line was originally established in 1977, by transforming human embryonic kidney cells with sheared DNA derived from the adenovirus 5 (Ad5) genome (Graham et al. 1977). The 293rd attempt proved successful: a 4.3 kb sequence containing Ad5's *E1A* and *E1B* genes (nucleotides 1–4344 of the Ad5 reference genome) were stably integrated into the coding sequence for the pregnancy-specific β-1-glycoprotein 4 (*PSG4*) gene on Chromosome 19 (Louis et al. 1997). The E1A protein functions as a master transcriptional regulator that is required for the expression of other factors during wild-type adenoviral infections. In contrast, *E1B* expression produces the E1B-19K and E1B-55K proteins, which inhibit apoptosis induced by Bcl-2 homologs (Bax and Bak) and p53, respectively (Meier et al. 2020). Several HEK293 variants were subsequently developed, including HEK293T (transformed with the large T-Antigen from SV40), HEK293F (adapted to suspension culture and chemically defined medium), and HEK293E (stably transfected with Epstein–Barr virus nuclear antigen-1 to enable replication of plasmids containing oriP) (Lin et al. 2014; Yuan et al. 2018).

Triple transfection of the HEK293 cell line is the most commonly used rAAV production platform for manufacturing clinical material (Clément and Grieger 2016). Using a cationic transfection reagent, HEK293 cells are transfected with three plasmids that encode elements required for rAAV production. The viral genome plasmid contains the therapeutic transgene flanked by ITRs derived from AAV serotype 2 (AAV2). The adenoviral helper genes (*E2A*, *E4orf6*, *VA RNA*) required to support rAAV production are provided on the helper plasmid. Finally, AAV *rep* and *cap* genes are provided on a third plasmid known as pRep/Cap. The *rep* coding sequence is usually derived from AAV2 to match the ITRs on the viral genome plasmid. On the other hand, any *cap* gene can be used, depending on the desired tropism of the rAAV product. The triple-transfection method can also be used with suspension-adapted HEK293 cells to improve process scalability, with rAAV yields greater than 10^{10} vg/mL being reported (Grieger et al. 2016).

A number of modified transfection-based HEK293 production systems have also been developed in recent years. These include the AAVone system, which incorporates the AAV *rep* and *cap* genes, adenoviral helper genes, and ITR-transgene cassette into a single 13 kb plasmid. A 2–4-fold increase in rAAV yield relative to triple transfection was reported. Differences in the profile of contaminant DNA were observed compared to the triple transfection system, with an increase in the mispackaging of *cap* and VA RNA sequences and reduced levels of plasmid backbone elements being observed with the AAVone system.

TABLE 1 | Different host cell systems used to produce rAAVs.

Cell line	Technology	Yield (vg/mL)	CSP (vg/cell)	Advantages of cell line	Disadvantages of cell line	References
HEK293	Triple transfection	~10^{10}	1×10^5	• Flexibility of transfection-based systems • Improved yields observed with AAVone and TESSA systems • Human origin - native post-translational modifications	• Traditional triple transfection approach struggles to deliver sufficient yields for systemic therapies • Difficult to scale-up transfection-based processes • Immortalization with oncogenic DNA from Ad5 may raise safety concerns	Grieger et al. (2016)
	AAVone	10^{11}–10^{12}	1×10^6			Yang et al. (2025)
	Triple transfection including genes from HBoV1	2-fold increase relative to control				Z. Wang et al. (2018)
	Producer cell lines	~10^{10}	$3–4 \times 10^3$			Lu et al. (2024)
	TESSA system	~10^{12}	10^5–10^6			Su et al. (2022), Roach et al. (2024)
Sf9	Three rBEVs	9×10^{10}	5×10^4	• Mono-Bac system shown to deliver improved yields • Reduced risk of contamination with adventitious agents that can infect human cells	• Nonmammalian origin – potential differences in post-translational modification, full/empty ratio, in-vivo transduction rates	Urabe et al. (2002)
	Two-Bac	8×10^{10}	7×10^4			Smith et al. (2009)
	Mono-Bac	7×10^{12}	4×10^6			Galibert et al. (2021)
	One-Bac	2×10^{11}	1×10^5			Aslanidi et al. (2009)
HeLa	Triple Transfection	~10^9	5×10^3	• Moderate yields • Human origin – native post-translational modifications	• Presence of oncogenic DNA from HPV-18 may raise safety concerns • Ethical/legal concerns around use without informed consent	Reed et al. (2006), Beskow (2016)
	E1-transformed line with two modified vaccinia virus vectors	1×10^{12}	2×10^5			Q. Wang et al. (2017)
	Producer cell line	2×10^{11}	10^4–10^5			Escandell et al. (2023)
CHO	Transfection-based	3×10^8 vg per 2×10^9 capsids	N.A.	• Widespread acceptance of CHO-derived products by regulatory authorities	• Very poor yields from transfection-based system – potential incompatability between nonhuman cell line and adenoviral helper system	Cao et al. (2024)
	Recombinant Herpes Simplex Virus vectors	10^9–10^{10}	4×10^3			Nagy et al. (2023)
BHK	Recombinant Herpes Simplex Virus vectors	2×10^{11}	~10^4	• Process has been adapted to be free from animal-derived byproducts	• Concerns around stability of rHSVs	Thomas et al. (2009), Sha et al. (2021)
A549	Packaging/producer lines	~10^{10}	~10^4	• Nontransformed human cell line	• Use of cell line has not progressed since early 2000s	Gao et al. (2002), Farson et al. (2004)

(Continues)

TABLE 1 | (Continued)

Cell line	Technology	Yield (vg/mL)	CSP (vg/cell)	Advantages of cell line	Disadvantages of cell line	References
HAT	Triple transfection	~10^{11}	7×10^4	• Moderate yields with comparable quality to HEK293-derived material	• Recently developed – not yet used for manufacture of clinical material	Hirai et al. (2025)
Yeast	Transformed *S. cerevisiae* strains	~10^8	N.A.	• Easy and inexpensive culture methods	• Poor yield has limited further use of this cell line	Barajas et al. (2017)

However, the AAVone system did not generate significantly higher amounts of replication-competent AAVs compared to the triple transfection system (Yang et al. 2025). In another study it was shown that adding the *NP1* and *NS2* genes from human bocavirus 1 (HBoV1) to the helper plasmid resulted in a 2-fold increase in rAAV yield compared to triple transfection controls, highlighting how targeted genetic modifications can improve transfection-based rAAV production (Z. Wang et al. 2018).

In contrast, numerous attempts have been made to generate HEK293-based packaging and producer cell lines. The main distinction here is that only the *rep* and *cap* genes are integrated into the host genome in packaging cell lines, whereas in producer cell lines both the transgene and *rep*/*cap* genes are integrated (Fernandes et al. 2023). Initial attempts were hindered by the cytotoxicity of constitutive *rep* expression (Berthet et al. 2005; Fernandes et al. 2023). In 2022, inducible producer cell lines were generated by Lee et al. (2022) although the observed rAAV yields were relatively low (approx. 10^8–10^9 vg/mL). However, yields were subsequently improved by reducing transgene expression during production, changing the doxycycline induction conditions, and inhibiting capsid protein degradation through treatment with the proteasome inhibitor MG132 (Lu et al. 2024). Given the expense of GMP-grade plasmids used in transfection systems, producer cell lines offer an alternative to produce rAAV cost-effectively.

rAAVs can also be produced in HEK293 cells using a wild-type or modified adenovirus to provide helper functions, although the potential for helper virus contamination of resulting products has limited industrial application of this approach. To address this issue, a novel E1/E3-deleted adenovirus was developed. Here, the viral major late promoter (MLP) was modified to include the tetracycline operator sequence, and the coding sequence for the tetracycline repressor was also placed under control of the MLP (Su et al. 2022). This generated a self-attenuating helper adenovirus known as the Tetracycline-Enabled Self-Silencing Adenovirus (TESSA), which can only replicate in the presence of doxycycline as structural proteins under MLP control will not be expressed while the tetracycline repressor protein is active (Roach et al. 2024). The authors demonstrated that a dual-TESSA vector system could deliver 10–30-fold increases in rAAV yield compared to the triple transfection. Furthermore, rAAVs produced with this system exhibited higher transduction efficiency *in vitro*. Significantly reduced levels (approx. 7-log fold lower) of contaminating adenovirus were also present, demonstrating the improved suitability of this system for rAAV manufacturing.

1.2.2 | Sf9 Cells

The Sf9 cell line was developed from ovarian tissue of the insect *Spodoptera frugiperda* (commonly known as the fall armyworm) (Kwiatkowska et al. 2025). This cell line was first used to produce rAAVs at the beginning of the 21st century, when Urabe et al. (2002) demonstrated that coinfection of Sf9 cells with three recombinant baculovirus expression vectors (BEVs) (modified with the coding sequence for *rep*, *cap*, and the ITR-transgene cassette, respectively) could produce rAAVs with

yields around 9×10^{10} vg/mL. However, this system was originally only used to produce rAAV2, and the palindromic orientation of the expression cassettes for Rep78 and Rep52 in the *rep*-containing BEV was also shown to be genetically unstable (Joshi et al. 2021; Kohlbrenner et al. 2005). In an attempt to address these limitations, vector systems were subsequently developed using two BEVs (known as "Two-Bac") (Chen 2008; Smith et al. 2009), and then a single BEV ("Mono-Bac" and "One-Bac") (Aslanidi et al. 2009; Galibert et al. 2021; Joshi et al. 2019, 2021).

Although volumetric yields of around 7×10^{12} vg/mL have been reported in a study that employed the Mono-Bac system (Galibert et al. 2021), significant differences in the biological properties of Sf9-derived and HEK293-derived rAAV preparations have been observed. For instance, rAAV vectors generated with the Sf9/BEV system have shown altered post-translational modifications of the capsid, reduced VP1 and VP2 levels, and poor full/empty ratios relative to HEK293-derived material. These issues resulted in an overall reduction in vector potency and infectivity (Giles et al. 2023; Rumachik et al. 2020). However, other studies reported that rAAVs produced in Sf9 cells demonstrate improved full/empty capsid ratios, elevated *in vitro* transgene expression and higher *in vivo* transduction rates compared to material produced in HEK293 cells (Kondratov et al. 2017; Liu et al. 2024). These conflicting reports highlight the difficulties that can arise when comparing rAAV preparations from different cell lines and laboratories, adding further layers of complexity to the selection of rAAV production cell lines.

1.2.3 | *HeLa Cells*

The HeLa cell line was the first immortalized human cell line to be cultured indefinitely in a laboratory setting and has contributed to countless developments in biomedical research (National Institutes of Health (Office of Science Policy) 2025). rAAVs have been generated for research purposes in HeLa using several different technologies. Although HeLa-derived rAAVs have been used for several clinical trials and shown a favorable safety profile (Clément and Grieger 2016; Tatalick et al. 2005), possible encapsidation of the oncogenic *E6* and *E7* genes from HPV18 remains a key barrier to the widespread adoption of this cell line for rAAV manufacturing (Escandell et al. 2023). Transfection-based rAAV production in HeLa cells has been shown to generate yields in the 10^9 vg/mL range, although it is noted that the adenoviral *E1A* and *E1B* genes were not included in the plasmid system for these transfections (Reed et al. 2006). In another study, suspension HeLa cells (HeLa-S3) were stably transfected with the *E1A* and *E1B* genes, generating an E1-transformed HeLa clone which the authors named QW158-7. Using two recombinant vectors (modified vaccinia virus carrying AAV *rep* and *cap* genes, and an E1/E3-deleted adenovirus carrying the rAAV genome), the QW158-7 cell line was shown to produce rAAV yields of approximately 1×10^{12} vg/mL. The rAAV vectors produced with this system contained significantly higher amounts of the VP1 protein in their capsid, which may improve the transduction capacity of these vectors compared to rAAVs generated with the triple transfection method (Q. Wang et al. 2017). Additionally, several HeLa-based rAAV packaging and producer cell lines have been developed (Figueroa et al. 2017). In a recent publication, stable producer cell lines were developed using the HeLaS3 cell line; using rAAV wtAd5 to induce production and supply helper functions, yields above 1×10^{11} vg/mL were reported (Escandell et al. 2023).

1.2.4 | *CHO Cells*

Chinese hamster ovary (CHO) cells are a well-established host system to produce biotherapeutics, with approximately 80% of approved antibody therapies being produced in this cell line (G. Walsh and E. Walsh 2022; Xu et al. 2023). The widespread regulatory acceptance of CHO-derived products may also be beneficial for developing rAAV-based gene therapies. In 2024, Cao et al. presented a transfection-based system that produced rAAVs in suspension CHO cells. However, very low viral genome and capsid yields were reported (3×10^8 vg per 2×10^9 capsids) (Cao et al. 2024), which may be due to incompatibilities between nonhuman cell lines and the adenoviral helper system employed (Buller et al. 1979). In a further study, suspension-adapted CHO cells were modified to express the HSV-entry factor HVEM (herpesvirus entry mediator). Two recombinant Herpes Simplex Virus vectors (rHSV) (carrying the *rep/cap* genes and an ITR-transgene cassette, respectively) were then used to produce rAAVs in the HVEM-expressing cell line, achieving viral genome yields in the 10^9–10^{10} vg/mL range (Nagy et al. 2023).

1.2.5 | *BHK Cells*

The baby hamster kidney (BHK) cell line was first established in the 1960s (Hernandez and Brown 2010; Macpherson and Stoker 1962; Stoker and Macpherson 1964) and has been previously used to produce foot-and-mouth disease virus for animal vaccines (Park et al. 2021). Suspension-adapted BHK cells have also been used to produce rAAV products for clinical trials (Knop et al. 2010). rAAVs are usually produced in BHK cells through coinfection with two recombinant herpes simplex viruses, with one vector containing an ITR-transgene cassette and the other containing the AAV *rep* and *cap* genes (Thomas et al. 2009). This system delivered rAAV yields as high as 2.4×10^{11} vg/mL, although both the rHSV and rAAV manufacturing steps of this production system were serum-dependent. Subsequently, the authors developed an animal-derived-byproduct-free production process that could deliver rAAV yields comparable to the original process (Knop et al. 2011).

1.2.6 | *A549 Cells*

Stable cell lines for rAAV production have also been generated using A549 cells, which were originally derived from primary lung carcinoma tissue in 1973 (Giard et al. 1973). An adherent, A549-based packaging cell line was developed by Gao et al. in 2002 (K209), while a suspension-adapted producer cell line was later created which generated rAAV yields above 10^4 vg/cell (approx. 10^{10} vg/mL) (Farson et al. 2004; Fernandes et al. 2023; Gao et al. 2002).

1.2.7 | *HAT Cells*

The HAT (human amniotic epithelial cell line for gene and cell therapy) cell line was developed specifically for ATMP manufacturing through the immortalization of primary human amniotic epithelial cells with the catalytic subunit of human telomerase (*hTERT*) (Shitova et al. 2024) and stable transfection of the Ad5 *E1* genes. A suspension-adapted HAT clone was then obtained, which produced viral genome yields in the 10^{10}–10^{11} vg/mL range across six rAAV serotypes. rAAVs derived from the HAT cell line also displayed improved *in vitro* transduction rates and full/empty ratios compared to HEK293-derived material, indicating a further potential benefit of this cell line (Hirai et al. 2025). Although to our knowledge HAT cells have not yet been used for GMP-grade rAAV manufacturing, the use of an amniocyte-derived cell line for rAAV production has been explored industrially, with one of Cytiva's ELEVECTA producer lines being derived from CAP cells (CEVEC's amniocyte production cells) (Cytiva 2024).

1.2.8 | *Yeast*

rAAVs have also been produced in *Saccharomyces cerevisiae*. Here, the coding sequences for AAV Rep (Rep78 & Rep52), Cap (VP1, VP2 and VP3) and AAP proteins were transformed into the *S. cerevisiae* strain YPH501, generating a stable cell line that could produce rAAVs. However, low yields (approx. 10^8 vg/mL) have limited the further application of this technology (Barajas et al. 2017; Donohue et al. 2021).

2 | Desirable Traits for rAAV Production

So far, this review has examined the eight cell types that have been used to produce rAAVs to date. The remainder of this review focusses upon identifying the desirable traits that a cell line might possess to form a consensus view from our investigations on what an "ideal" rAAV production cell line might look like (visualized in Figure 1). All the traits described here apply to both transfection-based and stable rAAV production systems, with the exception of the subheading titled "Transfection Efficiency." Chemical and genetic modifications that have been shown to impact rAAV production in the HEK293 cell line are discussed throughout (and summarized in Table 2), as these alterations may prove to be useful in the development of more effective production systems in the future. As highlighted in Table 2, each of these modifications may have only been tested in a single serotype, or a small number of rAAV serotypes. Further research would be required to verify whether such modifications (e.g., resistance to apoptosis) are transferable to other rAAV serotypes. However, it is likely that certain characteristics (e.g., stable integration of *E1* genes, growth in chemically defined medium and suspension culture) would be beneficial for all serotypes.

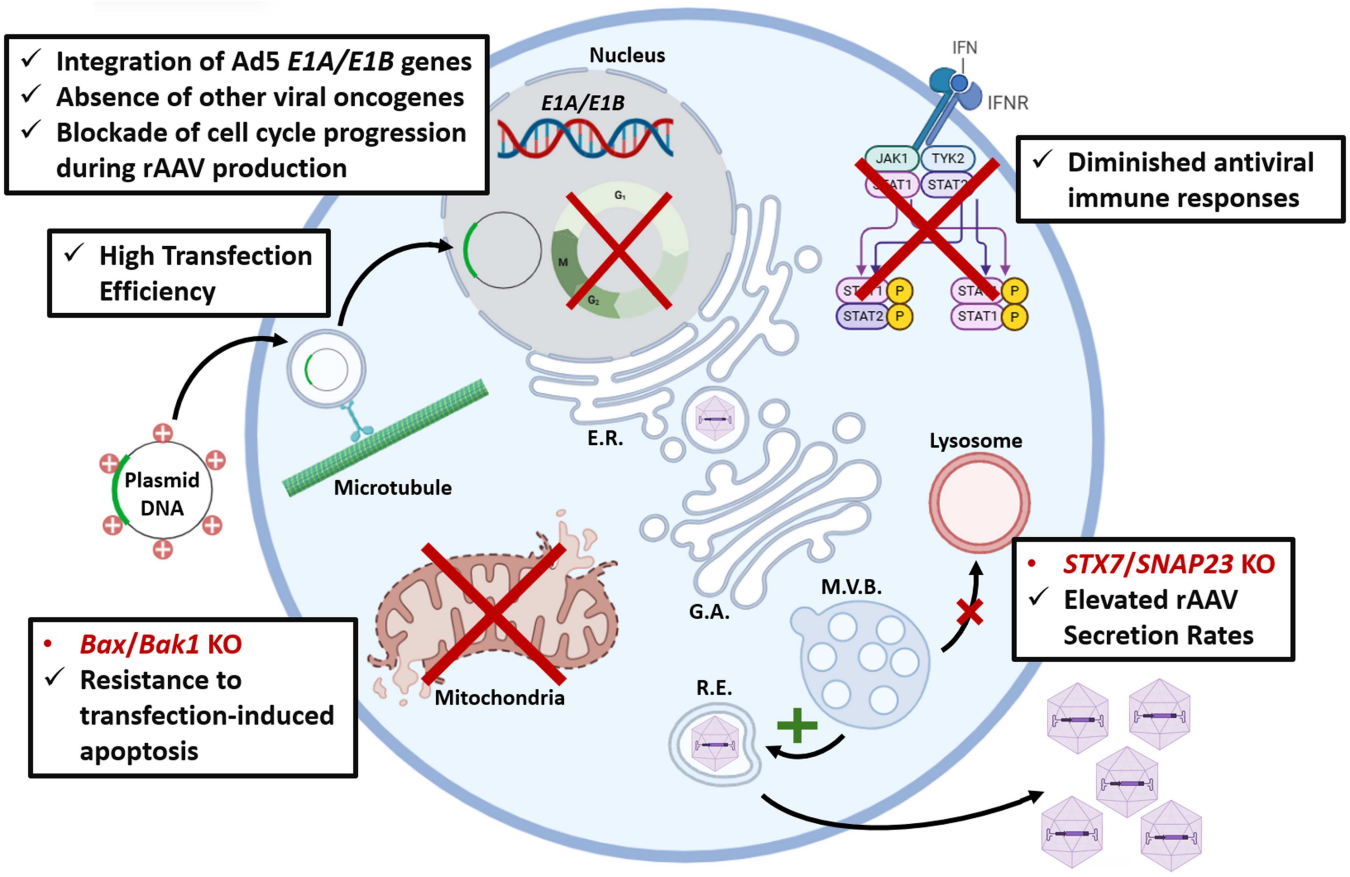

FIGURE 1 | Desirable traits in a host cell system for rAAV production. Note that positive charges represent the cationic transfection reagent (e.g., PEI) used to deliver plasmid DNA in transfection-based rAAV production systems. E.R., endoplasmic reticulum; G.A., Golgi apparatus; IFN, interferon; IFNR, interferon receptor; M.V.B., multivesicular body; R.E., recycling endosome (created with Biorender.com).

TABLE 2 | Genetic modifications and chemical inhibitors that impact rAAV production in HEK293 cells.

	Link to cell line characteristics	Modification	Capsid serotype/s tested	Mechanism of action	Effect on rAAV yield	References
Genetic modifications	Secretory capacity	*STX7* KO, *SNAP23* KO	AAV1, AAV2, AAV5, AAV9	Knockout prevents formation of lysosome/endosome hybrids and degradation of rAAVs present in endosomal compartments	3-fold increase in supernatant yield and 2-fold in total yield	Kuz et al. (2025)
	Resistance to apoptosis	*BAX/BAK1* KO	AAV2, AAV9	Reduces apoptosis activation following PEI-based transfection	1.6-fold increase in total genome titer, no effect on full/empty ratio	Park et al. (2025)
Chemical inhibitors	Cell cycle activity	Thymidine	AAV2	DNA synthesis inhibitor, causes cell cycle arrest at G1/S boundary	2-fold increase in total yield	Barnes et al. (2021)
		Nocodazole	AAV2	Inhibits microtubule dynamics, leads to cell cycle arrest at G2/M	1.7-fold increase in total yield	Sun et al. (2025)
		HMN-214	AAV1-9, AAVrh10	PLK1 inhibitor, arrests cell cycle at G2/M	2-fold increase in total yield	Fisher et al. (2025)
	Secretory capacity	Chloroquine	AAV5	Inhibits endocytosis and endosomal acidification	1.3-fold increase in total yield	Strasser et al. (2021)
	Resistance to apoptosis	Z-VAD-FMK	AAV2	Pan-caspase inhibitor, prevents caspase-mediated apoptosis	25% decrease in capsid titer without having significant effect on viral genome yield	Ladiwala et al. (2025)
	Diminished activity of immune and inflammatory pathways	Ruxolitinib	AAV8	JAK1/JAK2 inhibitor, diminished activity of antiviral interferon pathways	2-fold increase in total yield	Kahlig et al. (2024)
	Cell metabolism	PX-478	AAV8	HIF-1α inhibitor. Target identified through multi-omic profiling	2.5-fold increase in capsid yield and reduced viral genome yield	Zehetner et al. (2025)
	Cell cycle activity	Givinostat, Ricolinostat, Belinostat	AAV9	HDAC inhibitors, identified as target through transcriptomics and pathway analysis	Up to 1.8-fold increase in vg titer compared to DMSO control	Tworig et al. (2024)
		Vindesine, Colchicine, Vinblastine		Microtubule inhibitors, identified as target through transcriptomics and pathway analysis	Up to 3.2-fold increase in vg titer compared to DMSO control	

2.1 | Stable Integration of *E1* Genes

As mentioned above, one reason for the selection of the HEK293 cell line for rAAV production was the stable expression of three adenoviral proteins (E1A, E1B-19K, and E1B-55K) that facilitate rAAV replication. In cell lines that do not stably express these genes, their absence leads to significant reductions in vector yields when using adenoviral helper systems (Matsushita et al. 2004). Findings from a recent study suggest that duplication of the adenoviral integration site in the HEK293 genome may be linked to elevated rAAV yields, which could prove to be a potential avenue for genetic manipulation in the future (Herzog et al. 2025). Expression levels of these adenoviral genes may also influence rAAV production, as downregulated *E1A* and *E1B-55K* expression has been observed in high rAAV-producing HEK293 cells compared to a low-producing subpopulation (Mullick et al. 2025). In addition to HEK293, several other E1-complementing cell lines have been developed for adenoviral vector production, including cell lines developed from A549, HeLa, and HER (human embryonic retinoblasts) cells (Kovesdi and Hedley 2010). Some of these cell lines could also prove to be useful in the context of rAAV production which, to our knowledge, has not been explored yet.

2.2 | Transfection Efficiency

Consistently high transfection efficiency is desirable for transfection-based rAAV production, with transfection efficiencies above 70% being reported in suspension HEK293 cells across several studies (de Los Milagros Bassani Molinas et al. 2014; Grieger et al. 2016; Zhang et al. 2025). It is well established that different cell lines exhibit varying degrees of transfectability (Horibe et al. 2014; Maurisse et al. 2010), while particular transfection reagents may also be more effective than others for a given cell line (Yamano et al. 2010). Interestingly, suboptimal complex uptake and/or trafficking of cargoes to the nucleus have been identified as key factors that can cause a given cell line to be difficult to transfect, providing an insight into potential biological underpinnings of this phenomenon (Figueroa et al. 2017). Improvements in transfection efficiency might be achieved in difficult-to-transfect cell lines through the addition of DNA nuclear targeting sequences (DTS) to plasmids. The SV40 enhancer, CREB (cyclic-AMP response element binding protein) binding sites, and NF-κB binding sites have been identified as putative transfection enhancers, aiding in the transport of plasmid DNA to the nucleus of nondividing cells through their interaction with transcription factors and importin-β that promote trafficking along microtubules (Bai et al. 2017). This strategy may not be required or effective in actively dividing cells though, where breakdown of the nuclear envelope already facilitates the transfer of plasmid DNA from the cytoplasm to the nucleus. Additionally, DTS were not shown to effectively improve nuclear uptake of plasmids in other studies (Prasad and Rao 2005; van Gaal et al. 2011). To our knowledge, the effectiveness of this approach has not yet been investigated in the context of plasmid transfection for rAAV production.

2.3 | Ability to Grow in Chemically Defined Medium and Suspension Culture

Compared to serum-supplemented medium, the use of chemically defined (CD) medium offers several advantages for the production of biotherapeutics, such as lot-to-lot consistency and the absence of components that could introduce adventitious agents (Gorfien et al. 2000; Ling et al. 2015). In recent years, several different types of CD media have been made commercially available for use with the HEK293 line. This could prove to be a roadblock in the development of new GMP-compliant rAAV production processes with other cell lines, however, as only a limited number of human cell lines have been cultured in serum-free media to date (Yao and Asayama 2017).

If a cell line can be cultured in serum-free media, the absence of cell attachment factors such as fibronectin provided by FBS may also enable the adaptation of the cell line to suspension culture (Gstraunthaler et al. 2013; Hayman et al. 1985; Koblinski et al. 2005; Piazza et al. 2024). This can provide several advantages over adherent-based culture methods including growth at higher cell densities and improved scalability of production processes. Protocols have been published on the adaptation of cell lines to growth in serum-free media and suspension culture (Mather 1998; Sinacore et al. 2000), although this process can be time-consuming and success may not be guaranteed (Bellani et al. 2020).

Interestingly, the adaptation of an adherent, producer cell line for rAAV production to serum-free, suspension culture led to a 50-fold reduction in cell-specific productivity (Kuo et al. 2025), highlighting the impact that these adaptations can have on rAAV productivity. rAAV production using adherent cells on microcarriers is another alternative and has been accomplished with the HEK293T cell line previously (Ladd et al. 2022). The use of microcarriers may also prove useful for rAAV production with other cell lines, allowing production processes to harness the scalability of suspension culture while still achieving the high cell-specific productivities associated with adherent culture methods.

2.4 | Cell Cycle Activity

It is well established that different cell lines display different growth rates/population doubling times (Bonfim-Silva et al. 2019; Grob et al. 2024). To our knowledge, the effect of a given cell line's growth rate on rAAV production has not yet been directly investigated. In theory, a cell line with a high growth rate would be desirable for the production of most biopharmaceuticals to enable rapid scale-up from vial thaw to production. However, this property of a cell line may be diametrically opposed to that which best supports rAAV production.

A recent study found that attenuation of cell cycle progression is a key characteristic that allows a cell to produce high quantities of rAAVs (Mullick et al. 2025). Previous work had identified that only around 10% of transfected cells in the bulk HEK293 population produced significant amounts of rAAVs

(Dash et al. 2022). To further investigate this, producer and nonproducer populations were separated by fluorescence-activated cell sorting (FACS) and analyzed via RNA-seq to identify differentially expressed genes. Upregulated expression of genes such as *TP73*, *CASP8*, and *TGFB1* and downregulation of other genes including *MYC*, *CCND1*, and *CCNB1* in the producer population were taken to reflect a block on normal cell cycle progression, illustrating the effect that the cell cycle can have on rAAV production.

Cell cycle disruption has previously been shown to improve the productivity of other bioprocesses, specifically for the production of biotherapeutics in CHO cells (Donaldson et al. 2021; Fussenegger et al. 1997). Numerous studies have also investigated the impact of treatment with cell cycle inhibitors on rAAV production. For instance, approximate 2-fold increases in rAAV yield have been achieved through treatment with the DNA synthesis inhibitor thymidine, which causes cell-cycle arrest at the G1/S boundary (Barnes et al. 2021). Additionally, treatment with the chemotherapeutic and antimitotic agent nocodazole at a concentration of 6 μM has been shown to generate 1.7-fold increases in viral genome titers in HEK293 cells compared to untreated controls (Sun et al. 2025). Nocodazole inhibits microtubule formation and depolymerization through binding to β-tubulin (Blajeski et al. 2002), leading to cell cycle arrest at the G2/M phase. Altered expression of the *SFRP5* and *ZFP91* genes was observed following nocodazole treatment, and the authors also found that similar increases in rAAV yield could be delivered through the exogenous/plasmid-based overexpression of *ZFP91* and silencing of *SFRP5*. These results also align with findings from other research, where the PLK1 (Polo-like kinase isoform 1) inhibitor HMN-214 was shown to increase rAAV yields across several serotypes, therapeutic transgenes, and HEK293 cell lines through blockage of cell cycle progression at the G2/M phase (Fisher et al. 2025).

2.5 | Secretory Capacity

rAAV particles can be secreted from producer cells while attached to extracellular vesicles, through a process that likely involves the AAV's MAAP (Elmore et al. 2021; Kuz et al. 2024). rAAV secretion has previously been shown to be dependent on the rAAV serotype, with higher fractions of rAAV2 and rAAV6 particles being retained intracellularly compared to other serotypes due to the affinity of these capsids for heparin (Vandenberghe et al. 2010). Potentially, improved secretion rates could allow the harvesting of rAAVs directly from the culture medium, which in turn could simplify downstream purification and reduce overall manufacturing costs (Kotterman et al. 2015).

Results from a recent study suggest that certain genetic modifications can be introduced to improve vector secretion from producer cells. Kuz et al. (2025) identified two SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors) proteins, known as syntaxin-7 (STX7) and Synaptosome associated protein 23 (SNAP23), which colocalized with MAAP in both wtAAV2-infected and rAAV2-producing HEK293 cells. Knockout of the *SNAP23* and *STX7* genes was subsequently shown to generate more favorable rAAV production profiles in HEK293 cells across several rAAV serotypes, with roughly 3-fold increases in rAAV supernatant yields and 2-fold increases in total viral genome yield being observed. Mechanistically, the authors hypothesized that knockout of these SNARE proteins prevents the fusion of rAAV-containing late endosomes with lysosomes, directing these vectors toward recycling endosomes where they are ultimately released in association with microvesicles (Kuz et al. 2025). Interestingly, these findings align with results from previous research, where treatment with an inhibitor of endosomal acidification (chloroquine, at 5 μM) was shown to increase secreted and intracellular rAAV5 yields from HEK293 cells (Strasser et al. 2021). Overall, these findings demonstrate that elevated rAAV secretion rates can be achieved in producer cells through targeted genetic and/or chemical modifications.

2.6 | Resistance to Apoptosis

rAAV production has been shown to induce apoptosis in HEK293 cells; for PEI-based transfection systems, this process is mediated by the caspase pathway (Ladiwala et al. 2025; Park et al. 2025). Various reports have been published regarding the effects of apoptosis modulation on rAAV production. For instance, Park et al. (2025) found that knockout of the pro-apoptotic *BAX* and *BAK1* genes improved yields of rAAV2 and rAAV9 in HEK293T cells without significantly changing full/empty capsid ratios. Meanwhile, apoptosis inhibition through treatment with the pan-caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (also known as Z-VAD-FMK) at a concentration of 50 μM was shown to yield improved full/empty capsid ratios without affecting total rAAV yield in Expi293F cells (Ladiwala et al. 2025). Although the findings from these studies may not align completely, it has been well illustrated that apoptosis modulation can significantly affect rAAV production, which may be a key factor to consider as part of developing more efficient rAAV production systems.

2.7 | Diminished Activity of Immune and Inflammatory Pathways

Several antiviral response pathways are activated during the host response to viral infections (Sengupta and Chattopadhyay 2024). Interestingly, numerous transcriptomics studies have demonstrated that transfection-based rAAV production systems upregulate the expression of genes involved in innate immune responses, including *CCL5*, *IL-8*, *IFIT2, IFIT5, IRF7*, and *TRIF* (Chung et al. 2023; Y. Wang et al. 2023). In addition, several genes involved in interferon-signaling pathways (*STAT1*, *STAT2*, *IRF9*, *IFNAR1*, *IFNAR2*, *IFNGR2*) were upregulated in a low-producing HEK293 clone, compared to a high-producing clone. Subsequently, the authors showed that treatment with the JAK1/JAK2 inhibitor ruxolitinib could lead to significant increases in rAAV yield in the low-producing HEK293 clone, illustrating that the manipulation of antiviral response pathways can affect rAAV production (Kahlig et al. 2024). Similar insights could be used to identify cell lines for rAAV production that intrinsically possess diminished

antiviral responses, or could be introduced genetically into producer cell lines to improve productivity.

2.8 | Cell Metabolism

Metabolic engineering has been shown to improve the specific productivity of several bioproduction processes, involving hosts such as *Escherichia coli* (Mahalik et al. 2014) and CHO cells (Gupta et al. 2017; Richelle and Lewis 2017). rAAV production is a highly energy-dependent process, with ATP being consumed to express viral proteins, replicate the rAAV genome, and package genomes into rAAV capsids (Sha et al. 2021). Therefore, differences in metabolism between cell lines could affect the availability of ATP for rAAV production and consequently the overall productivity.

A recent study found that over 99.8% of cellular resources remained directed toward cellular growth during rAAV production in HEK293 cells. In an attempt to redirect metabolic resources away from cellular growth, a chemical inhibitor known as PX-478 was used to inhibit the transcriptional regulator hypoxia-inducible factor 1-α (HIF-1α) in suspension HEK293 cells during rAAV production. Whilst increased capsid levels were observed following HIF-1α inhibition, there was a concomitant reduction in viral genome yields, resulting in lower full/empty capsid ratios. Interestingly, the authors attributed the reduced viral genome production to altered nucleotide synthesis through the pentose phosphate pathway, highlighting the effect that cellular metabolism can have on the quality of the resulting rAAV product (Zehetner et al. 2025).

2.9 | Absence of Viral Oncogenes

The genome of certain cell lines contains viral oncogenes, including HeLa (Cantalupo et al. 2015) and HEK293 (Russell et al. 1977; Tessier et al. 2021). In the context of rAAV production, the presence of viral DNA within a cell line can raise safety concerns due to the potential for viral oncogenes to be packaged into rAAV capsids. The presence of the *E6* and *E7* oncogenes in the HeLa genome has proven to be one of the main factors limiting the commercial application of HeLa-based rAAV production systems (Escandell et al. 2023). The adenoviral *E1A* and *E1B* genes present in the HEK293 genome can also be oncogenic (Singhal et al. 2013; Tessier et al. 2021). That being said, significant reductions in rAAV yield are observed using adenoviral helper systems when these genes are absent (Matsushita et al. 2004), which may explain why possible safety risks associated with their presence are accepted.

2.10 | Origin and Availability

The origin of a cell line used for rAAV production can affect the subsequent selection of helper genes, and vice versa; for instance, relatively low rAAV yields have been observed when adenoviral helper genes are used in CHO cells (Cao et al. 2024) while recombinant herpes simplex viruses have been shown to support more effective rAAV production in CHO cells (Nagy et al. 2023). As mentioned above, differences in the biological and physicochemical properties may also be observed between rAAV preparations produced in different cell lines, which can affect the overall potency and effectiveness of the material and may lead to a given host system being favored over another. The origin of certain human cell lines may also raise concerns around their availability to commercial entities, with many cell line depositors stipulating that certain cell lines will only be made available for research-use only (American Type Culture Collection n.d.; Culture Collections n.d.).

2.11 | Favorable Impurity Profiles

An acceptable impurity profile must be demonstrated during the regulatory approval process for biotherapeutics and was raised as a concern during the EMA's assessment of Glybera in 2012 (European Medicines Agency 2012). Impurity profiles can vary significantly depending on the host cell used during rAAV manufacturing, especially between cell lines of human (e.g., HEK293, HeLa), mammalian (e.g., CHO, BHK), or non-mammalian (e.g., Sf9, Yeast) origin. Impurities can include host cell DNA (hcDNA), partial genomes, plasmid DNA, and host cell proteins (HCP) (Bracewell et al. 2021; Kontogiannis et al. 2024, 2025), and vector preparations from Sf9 cells have been shown to contain reduced levels of mispackaged DNA (Kondratov et al. 2017; Liu et al. 2024) but higher HCP amounts (Giles et al. 2023). Additionally, empty and partially filled capsids are another unwanted byproduct of the upstream rAAV production process. It is possible that the full/empty ratio observed in vector preparations from different cell lines may vary due to differences in vector expression and packaging within the host cell (as described for HEK293 and Sf9 cells above, see Section 1.2.2). Although these impurities from the upstream process should be reduced to acceptable levels by an effective downstream process, the identification or development of a cell line that generates vector preparations with a favorable impurity profile (i.e., high full/empty ratio, low levels of HCP and hcDNA) would be extremely beneficial.

3 | Conclusion and Future Outlook

With the demand for rAAV-based gene therapy products predicted to increase over the coming years, it is paramount that more effective production systems are developed to allow the industry to scale. As discussed above, HEK293 is the current standard cell line for rAAV production, which was originally motivated by the integration of the *E1A* and *E1B* genes in the HEK293 genome. That being said, numerous alternative host systems are available, and the advent of modern gene editing tools such as CRISPR has enabled targeted genome editing. Crucially, the choice of cell line can impact the therapeutic efficacy and price of the resulting product, and will be scrutinized along a new therapy's path to regulatory acceptance. Regulators may be more likely to favor established cell lines with proven safety profiles, genetic stability, and history of GMP compliance (e.g., HEK293 cells) over a novel host (e.g., HAT cells), even when the established host has been genetically modified.

Furthermore, a number of genetic modifications have been shown to improve rAAV productivity in the HEK293 cell line. The identification of chemical additives that affect rAAV production has also illuminated several cellular targets and processes that can be modulated to improve productivity. These developments may enable the identification of cell lines that possess the desirable features or "cellular fingerprint" described, including resistance to apoptosis, diminished antiviral immune responses, and a favorable rAAV secretion profile. Moreover, the cellular proteins targeted by these chemical additives may also be suitable for genetic modification in the host cell genome, which may be preferred as a process is scaled toward GMP production to maximize the active culture volume. The genetic stability of these modifications over an extended number of passages would need to be proven using molecular techniques like qPCR and next-generation sequencing (e.g., Illumina sequencing) (Ng et al. 2017). However, alterations that could affect core cellular processes (e.g., altered cell cycle activity, Section 2.4) may not be suitable for permanent modification in the host genome. An alternative to constitutive expression of such targets is to use an inducible expression technology, however, this approach in itself brings issues for large-scale production such as when and how to induce the required expression, the "leakiness" of expression and the stability over time. The effectiveness of many of these ideal traits at larger scales also requires verification, although scale-down platforms such as Sartorius' Ambr systems have been used for rAAV process development (Andaluz et al. 2025; Mangrati et al. 2025) and could help to investigate whether beneficial modifications identified in small-scale studies using cell culture plates and shake flasks can transfer to large-scale bioreactors (Jiang and Dalby 2023).

Future developments could investigate possible synergies and connections between the ideal traits highlighted here, either in HEK293 or another cell line. For instance, apoptosis resistance (Section 2.6) may prove to synergize with an altered cell cycle (Section 2.4), as attenuation of cell cycle activity can be associated with p53 activation and apoptosis. E1A activity (Section 2.1) is known to drive cells to S phase of the cell cycle (Grand et al. 1998; Meier et al. 2020) leading to a blockade in cell cycle progression which can be beneficial during rAAV production (Section 2.5). Enhanced rAAV secretion (Section 2.5) could also lead to more favorable impurity profiles (Section 2.11), as significant vector quantities could be purified solely from the harvest's supernatant fraction. Additionally, a modification to the cell cycle that is constitutively active may impact transfection efficiency, as actively dividing cells are transfected most effectively (Brunner et al. 2000; Thermo Fisher Scientific 2026). Ultimately, combinations of modifications aligned with process developments could be further investigated with the goal of creating a cell line specifically designed to produce significantly enhanced amounts of functional rAAVs.

Author Contributions

Conceptualization: James Conheady, Nicholas Donohue, Niall Barron, and C. Mark Smales. Writing – drafting: James Conheady and Nicholas Donohue. Tables and Figures: James Conheady and Nicholas Donohue. Writing – reviewing/editing: James Conheady, Nicholas Donohue, Alexandra Bogdanovic, Sharon Davin, Brian Glennon, Niall Barron, and C. Mark Smales.

Acknowledgments

The authors thank the following for support: N.D. and S.D.—Enterprise Ireland under its Research, Development and Innovation Fund, Grant Award (180753/RR). A.B.—Irish Research Council (Taighde Éireann) (EBPPG/2022/38). C.M.S.—Joint Appointment Research Professorship Award (23/RP/11975).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

There was no data generated during this research to be made available.

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