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Generation of clinical grade recombinant adeno-associated virus

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BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Thesis

**GENERATION OF CLINICAL GRADE RECOMBINANT ADENO-
ASSOCIATED VIRUS**

by

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B.S., University of Vermont, 2004

Submitted in partial fulfillment of the
requirements for the degree of
Master of Science

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ASSOCIATED VIRUS**

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ABSTRACT

Recombinant adeno-associated viruses (rAAV) remain one of the most encouraging gene therapy vectors for treating patients with genetic abnormalities. rAAV can safely deliver long-lasting expression of a therapeutic transgene to a wide range of cell types. One challenge with therapeutic rAAV is the ability to generate enough virus for clinical trials and commercial supply. Doses administered systemically for neuromuscular or blood disorders can exceed 1×10^{14} viral genomes per patient. Approximate yields from a rAAV production are around 1×10^4 viral genomes per cell, meaning batch cell numbers would need to exceed 1×10^{10} for a single dose. This amount of therapeutic virus will require a production platform that can reliably generate sufficient quantities of therapeutic rAAV to meet patient demand. Common expression platforms used in academia and industry are insufficient for generating this amount of virus, let alone in a controllable or reproducible setting. More advanced systems based on stable cell lines paired with alternate viruses offer avenues for achieving more efficient production of virus, however there is no clear agreement on which system is most amenable to regulatory approval while also delivering safe and cost efficacious therapies to patients. This paper will outline the basic biology of AAV and rAAV vectors. The

common methods used to produce rAAV will be evaluated and a roadmap for producing clinical grade rAAV at scale will be provided.

PREFACE

Working with living systems requires that biological understanding be paired with advanced technology and an ability to execute production in a robust manner. Protein production manufacturing plants, based on mammalian expression systems, are multi-billion dollar facilities and individual production campaigns can run into the millions of dollars. The complexity of harnessing these living systems increases development, characterization and production costs to the point where promising therapies may be abandoned as commercially non-viable. The reemergence of gene therapy builds upon existing biologics production capabilities while creating a host of additional considerations. The pricing model pertaining to protein biologics for chronic diseases often defers the revenue stream over years of repeat administration. Gene therapies, in promising a cure, must recuperate the cost of development and production into what is likely to be a single administration or small number of doses (Brennan & Wilson, 2014). To realize the promise of gene therapy, the production method must be highly productive to constrain costs while maintaining consistent product quality; for the sake of regulatory approval and patient safety (Wright, 2008). Given a standard per cell productivity, Table 1 lists the approximate cell culture volume or surface area required to achieve a given viral yield in suspension and adherent based systems. The numbers are staggering when doses for systemically administered treatments may exceed 1×10^{14} viral genomes (Vg) per patient.

Table 1. Volume and surface areas to achieve a given viral genome (Vg) yield.

Assuming 1×10^4 vg/cell productivity, the volume of suspension and adherent dependent processes in order to achieve a given viral genome (VG) requirement is listed (Cecchini, Negrete, & Kotin, 2008).

<i>Vg</i>	<i>Cells</i>	<i>Volume (L)</i>	<i>Surface (cm²)</i>
1×10^{10}	1×10^6	0.0005	10
1×10^{11}	1×10^7	0.005	100
1×10^{12}	1×10^8	0.05	1000
1×10^{13}	1×10^9	0.5	10 000
1×10^{14}	1×10^{10}	5	100 000
1×10^{15}	1×10^{11}	50	1 000 000
1×10^{16}	1×10^{12}	500	10 000 000
1×10^{17}	1×10^{13}	5000	100 000 000
1×10^{18}	1×10^{14}	50 000	1 000 000 000
1×10^{19}	1×10^{15}	500 000	10 000 000 000

These figures suggest that the potential of the gene therapy field does not solely rest on the efficacy of the treatment; but is heavily contingent upon an ability to generate sufficient virus at a tractable cost. The aim of this thesis is to provide a review of the available methods for producing rAAV as it specifically pertains to generating clinical grade, and quantities, of virus. Academic and small industry groups can readily generate rAAV for research purposes and to power small clinical trials, however it will be shown that these methods are not suitable for powering large trials let alone approval and commercialization of a product. Furthermore, small scale methods are neither robust nor regulatory compliant. The literature surrounding more advanced stable cell line systems is sparse, and there is no consistent roadmap for groups looking to transition from

promising pre-clinical candidates to the clinic and beyond. This document will detail and evaluate the available methods, then provide a recommended workflow for how a group with a promising candidate therapy ought to build a commercial ready platform that would be suitable for meeting patient and market demands.

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LIST OF ABBREVIATIONS

AAV	Adeno-Associated Virus
AAVS1	adeno-associated virus integration site 1
AcNPV	<i>Autographa californica</i> Polyhedrosis Virus
Ad5	Adenovirus serotype-5 virus
BHK	Baby Hamster Kidney
CHO	Chinese Hamster Ovary
CMV	Cytomegalovirus
CSTR	Continuous Stirred-Tank Reactor
GFP	Green Fluorescent Protein
GMP	Good Manufacturing Practice
GOI	Gene of Interest
HEK	Human Embryonic Kidney
HPV	Human Papilloma Virus
HSV	Herpes Simplex Virus
IEX	ion-exchange chromatographic
ITR	Inverted Terminal Repeat
mRNA	Messenger Ribonucleic Acid
NGNA	N-glycolylneuraminic Acid
PCL	Producer Cell Line
PEI	Polyethylenimine
PTM	Post Translational Modifications

rAAV.....	Recombinant Adeno-Associated Virus
Rf.....	Replicative form
ssDNA.....	Single Stranded Deoxyribonucleic Acid
UF/DF	Ultrafiltration/Diafiltration
UL	Unique long
US	Unique Short
Vg.....	Viral genomes
VP	Viral Protein
α -gal	terminal- α -1,3-galactose

Introduction

AAV

Adeno-Associated Virus is a small non-enveloped single stranded DNA (ssDNA) virus belonging to the parvovirus family. The virus was given its name as it was discovered as a contaminant alongside adenovirus infected monkey cells in the 1960's (Atchison et al., 1965). AAV is non-pathogenic, as it is not known to cause disease in humans, but still maintains the ability to infect a wide variety of cell types as is seen in Table 2. The lack of a pronounced immune response in humans (Mingozi & High, 2013), and diverse tissue tropism has made AAV a strong candidate as a gene therapy vector (Srivastava, 2016). Further AAV has been known to lead to long term expression of transgene payload.

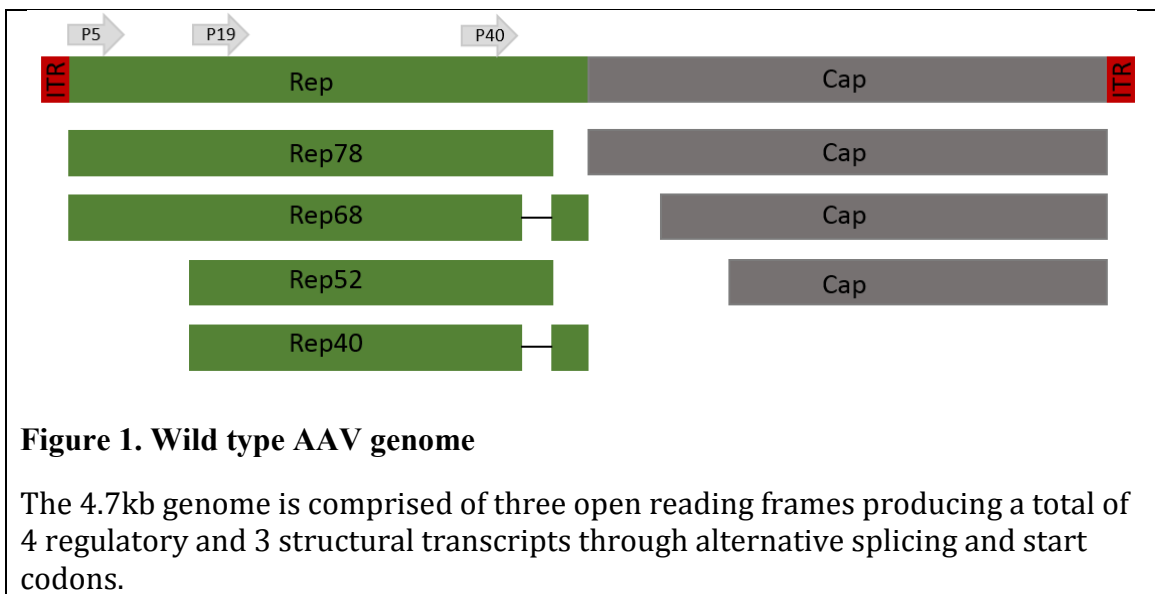
Devoid of helper functions, the wild-type virus readily integrates into the adeno-associated virus integration site 1 (AAVS1) locus on chromosome 19q13.42 and lead to long-term latency (Linden et al., 1996). The targeting of this locus is directed by the Rep protein, encoded within AAV, and a consensus binding site in the human genome at the AAVS1 locus (Luo et al., 2017). The virus is classified as a *dependovirus* and thus remains dormant until viral helper functions are presented by a second virus, often Adenovirus or Herpes Simplex Virus (HSV) (Geoffroy & Salvetti, 2005), which will transition the virus into a lytic phase (Hüser et al., 2010). The proviral genome is excised from the genome, a function of Rep (Pereira et al., 1997), replicated as a double stranded replicative form (Rf), followed by single stranded viral gene expression which is

subsequently loaded into the capsid encoded by the Cap gene products (Lochrie et al., 2006).

The small single stranded genome of 4.7kb encodes two genes with three open reading frames flanked by inverted terminal repeats (ITRs) as can be seen in Figure 1. Like many viruses that have evolved over millennia with a high mutation rate, the genome is highly efficient and packages several overlapping reading frames. The genome gains further efficiency by making use of splice variants to generate multiple mRNA transcripts and thus functional proteins with related but independent roles.

The Rep gene contains two open reading frames driven by the native P5 and P19 promoters (Weitzman et al, 1996). The promoters generate the Rep78 and Rep 68 transcripts and the Rep 52 and Rep40 transcripts, respectively. The Rep68 and Rep40 are generated as splice variants as can be seen in Figure 1. The genes themselves are named according to their protein molecular weight. Rep genes are trans-activated by the presence of helper functions, chiefly E1A from Adenovirus. The two larger transcripts are involved in regulatory and life cycle functions such as insertion and excision from the AAVS1 locus as well as DNA replication and modulation of host cell gene expression (Balakrishnan & Jayandharan, 2014). The virus has evolved with human hosts, and thus viral gene functions additionally create a microenvironment suitable for viral purposes by creating a permissive environment for the viral life cycle. Rep78 will specifically lock the host cell into the S-phase through hypophosphorylation of retinoblastoma and through DNA nicking at naturally occurring Rep binding sites ensuring that the appropriate genomic replication machinery is in abundant supply to support viral needs (Young et al.,

2000). The smaller two Rep proteins are known to have helicase functionality that is imperative for capsid loading during viral life cycle maturity. Interestingly these two transcripts suppress P5 promoter activity in the absence of helper viral proteins (Pereira et al., 1997).



The Cap Gene encodes the VP1, VP2, and VP3 genes that comprise the structural proteins of the Capsid (Rose et al., 1971). The three transcripts are under control of the P40 promoter with two splice variants arising from downstream alternative ACG and canonical ATG start codons (Balakrishnan & Jayandharan, 2014) as is displayed in Figure 1. The three transcripts, and their respective proteins comprise the icosahedral capsid that protects the viral genome and confers infectivity toward target cells. The tropism of each serotype, shown in Table 2, is a product of the three-dimensional interaction of the capsid proteins and the respective composition of target cell receptors, some of which are not fully defined. The capsid also plays a critical role in intracellular

trafficking. Following endocytosis, the nuclear signaling domains of VP1 and VP2 are become exposed due to the acidic environment of the endosome (Nonnenmacher & Weber, 2015). Further, endosomal escape is catalyzed by the exposure of a phospholipase A2 domain contained within VP1. The ratio of proteins can vary based on serotype, but generally fall into a 1:1:10 ratio.

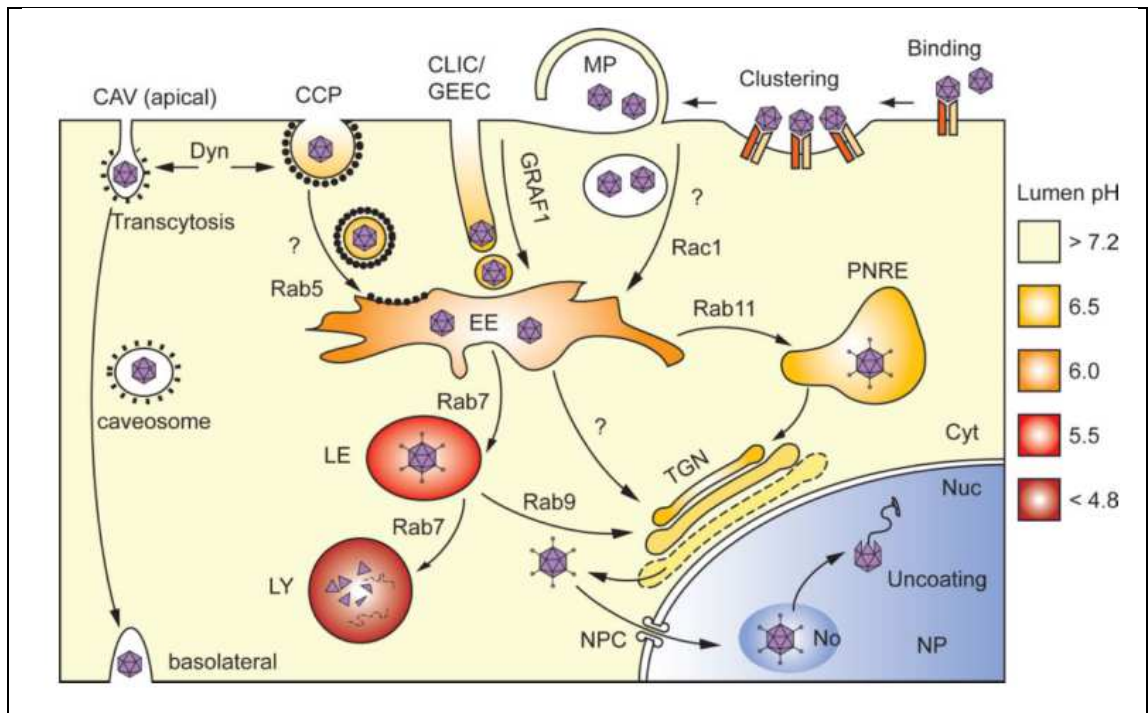


Figure 2. Trafficking of AAV

AAV gains cellular entry through binding of receptor and co-receptors based on the serotype specific capsid. Entry is through endocytosis, endocytosis in clathrin coated pits, or caveolar endocytosis. Viruses are traffic through early endosomes to late endosomes. Acidification of endosomes exposes PLA2 and subsequent release from endosomal compartments and trafficking through nuclear pores. Uncoating takes place in the nucleus (Nonnenmacher & Weber, 2015).

The Rep and Cap genes are flanked by two 145 base pair inverted terminal repeat (ITR) sequences. Each ITR contains a 125bp sequence that is self-complementary to itself and thus forms a hairpin structure with an unpaired 20 base pair D-sequence (Yan et al., 2005). The free 3' hydroxyl of the ITR forms the basis for second strand synthesis, which comprises the stable, double stranded expression competent complex. A strand displacement model allows for single stranded genome replication as the viral life cycle commences in the presence of helper functions.

Table 2. AAV serotype tropism

Multiple naturally found capsids are readily able to target a host of tissue types in humans (Srivastava, 2016).

<u>Serotype</u>	<u>Tissue Target</u>
AAV1	Muscle, Heart, Eye, CNS
AAV2	CNS, Kidney, Muscle
AAV4	Lung
AAV5	Lung, CNS, Eye, Pancreas
AAV6	Lung, Heart
AAV7	Muscle, Liver
AAV9	Liver, Muscle, Eye, CNS, Heart

rAAV

Recombinant adeno-associated virus differs from wild-type AAV in several key ways that make it a versatile tool for gene delivery. AAV is termed a gutless vector as the only element required to generate an infectious particle is the flanking ITRs *in cis* and the core of the wild-type viral coding regions, Rep and Cap, can be delivered *in trans* to actuate a system capable of replication. The genome coded between the ITRs dictates the

sequence to be packaged into the capsid in the presence of helper functions. The packaging capacity of the plasmid is generally considered to be under 5kb (Wu et al., 2010). The various workable configurations of these elements and the respective biological systems will be discussed in subsequent sections. The removal of Rep from the genome also alters the way the vector behaves in target cells. Without Rep positioned within the ITRs, the vector will no longer target integration at AAVS1, and will instead be maintained as extrachromosomal units (Schultz & Chamberlain, 2008). There are two competing models on how AAV creates stable double stranded conformationally stable units upon entering the nucleus (Nakai et al., 2000). Capsid un-coating takes place immediately prior to, or simultaneous with transport into the nucleus (Lux et al., 2005). The first model is the second strand synthesis model suggests that the self-priming nature of the free 3' hydroxyl of the ITR hairpin initiates complementary strand synthesis using host polymerases (Weitzman et al., 1996). The competing view is that sense and antisense genomes, which are present in equimolar ratios, anneal together to form stable double stranded units (Nakai et al., 2000). Unpaired single stranded genomes will be degraded over time, while double stranded units will circularize into monomeric or multimeric episomes. The conformationally stable, double stranded, episomal structures are what confer long-term expression of genes transferred by rAAV in quiescent cell types (Duan et al., 1998). One of the primary upsides of rAAV is the avoidance of disruptive genomic integration events that could lead to activation of oncogenes, or silencing of tumor suppressors. To the contrary, rAAV is only effective in quiescent cells as the episomes would not replicate with cell division, and thus be present in

proportionally fewer cells with time, and finally be lost as the infected cells die or are eliminated.

rAAV Generation

Generation of rAAV requires three core inputs. First, Rep and Cap are to be delivered *in trans*, meaning they will be removed from the AAV genome and provided externally to the two ITR sequences. In the absence of helper functions, the three promoters driving the component transcripts will be silenced. Second, the gene of interest is provided *in cis*, and thus replaces the wild type orientation of Rep and Cap between the ITR elements. The *in cis* elements will be packaged into the capsid and thus compose the gene to be transferred by the vector. The gene of interest must be driven by a promoter known to be active in the tissue type to be targeted by the therapeutic vector. Constitutive promoters, such as the human cytomegalovirus (CMV), are often the most powerful drivers of transcription. However, AAV serotypes while somewhat specific, will often target multiple disparate tissues as is can be seen in Table 2. It may be wise to sacrifice the potency of a CMV promoter for one that is specifically active in the disease tissue of interest, thereby abrogating untoward effects of the transgene in unintentionally targeted tissues (De Leeuw et al., 2016). Third, secondary viral helper functions must be present to transactivate the three promoters and thus trigger the AAV life cycle and generation of packaging capsids (McLaughlin et al., 1988).

As has been noted, the generation of rAAV requires a gene of interest *in cis* with the transgene, the viral Rep and Cap genes *in trans*, and additional viral helper functions.

There are four commonly used platforms that use varying configurations and thus confer different advantages and disadvantages. Each group must take stock of their needs for speed and productivity and how those weigh against their abilities and laboratory technology. Furthermore, some methods will be shown as more regulatory compliant and thus better choices for progressing therapeutics toward the clinic despite the additional complexity. The next section will delve heavily into the four main configurations for the generation of rAAV and how they are suited for addressing the generation of clinical grade rAAV therapeutics.

Transient Transfection

Many groups working with AAV in early development are most interested in generating small amounts of virus to work with and are less concerned with robustness or product quality. The most direct method for generating rAAV is a transient transfection method often referred to as triple transfection. A general view of this method is seen in Figure 3.

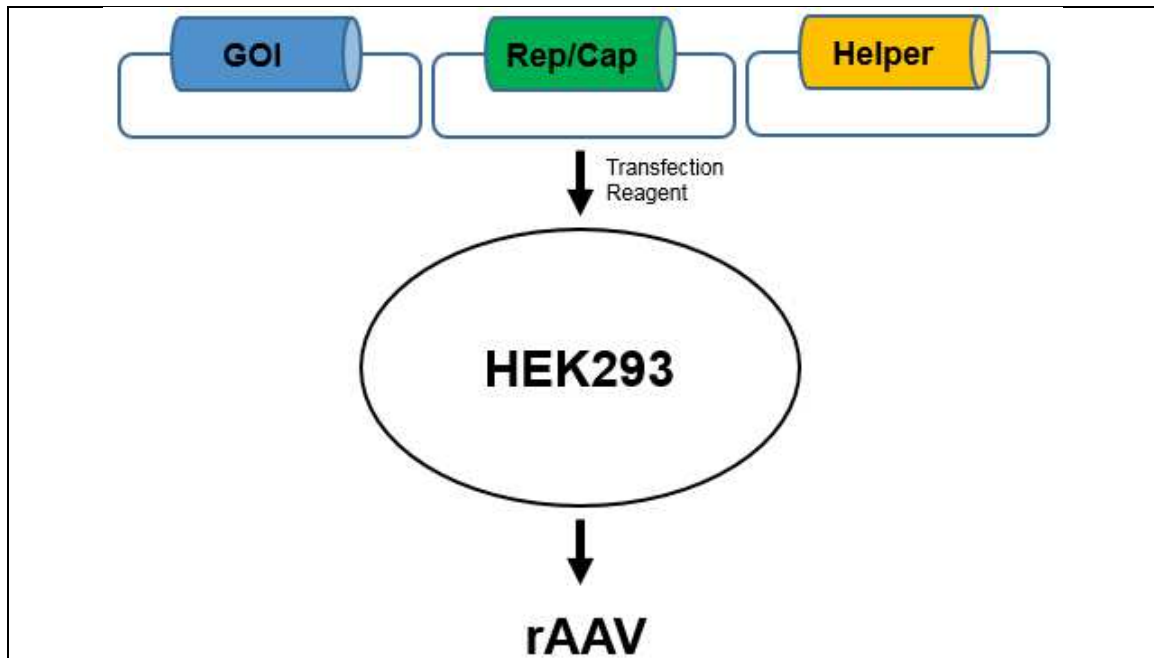


Figure 3. Transient transfection

Three plasmids encoding the ITR flanked GOI, Rep and Cap, and helper functions are transfected into HEK293 cells. AAV is generated from transient (non-integrated) DNA transcription in the nucleus.

This method involves mammalian cells, most commonly Human Embryonic Kidney (HEK) 293 cells. HEK-293 cells were originally transformed by infection with adenovirus type 5, and thus contain the E1A and E1B sequences. The consequence of E1A and E1B being integrated into the HEK-293 genome is that helper functions can be delivered with E1 deleted (E1 Δ). This can take two forms. The first, and less commonly used would be using E1 Δ Ad5, which is a replication incompetent form of the virus. Ad5 is pathogenic, and thus an incompetent form of the virus is a preferable option from a safety standpoint, but also due to the burden placed on downstream purification and analytical assays to remove and validate clearance of the virus. The more common

approach is to encode the helper functions onto a plasmid, without E1, and deliver it simultaneously with a plasmid encoding Rep and Cap, and another plasmid encoding the gene of interest flanked by ITRs (hence the term “triple transfection”). Helper functions used in the transient system are most readily those cloned from Adenovirus, E1a, E1b, E2a, E4orf6 and VA RNA genes (Matsushita et al., 2004).

The independent coding on separate plasmids allows transgenes, ITR elements and capsids to be interchanged more readily than with cell lines generated with these components integrated stably into the genomic backbone. The transient nature means that researchers can rapidly generate material, albeit in small quantities. The plasmids are commonly delivered using cationic lipids to HEK-293 cells. Cationic lipids form a micelle surrounding the negatively charged plasmid DNA and the lipid bilayer which allows the complex to pass through the cell membrane (Safinya, 2001). The complex traffics through lysosomal and endosomal compartments before transporting to the nucleus where transcription can take place.

HEK-293 cells are most readily transfected as an adherent cell line grown in commercially available media supplemented with fetal bovine serum. Adherent growth limits volumetric productivity by restricting biomass to the culture surface. HEK cells can grow in suspension, serum-free conditions; but they are not readily transfected as a suspension cell line due to poorly understand changes in the physiology, and thus permissiveness of the cells upon suspension adaptation. The adherent nature of this system greatly reduces the scalability of the system. A promising method for exploiting the permissive nature of adherent cell lines while maximizing cell densities and thus

productivities is the use of microcarrier technology (Rafiq et al., 2017). Microcarriers are small polystyrene beads with a cationic amine surface modification that make them amenable as surface substrates for anchorage dependent cell lines. Cells adhered to microcarriers can be grown to high cell densities in continuous stirred-tank reactors (CSTR) all the while putatively maintaining their permissiveness to uptake of cationic lipids containing plasmid DNA.

Currently there is not enough evidence for the microcarrier technology which renders triple transfection as an adherent system the predominant method. The major drawback remains the difficulty in achieving scale and reproducibility, two elements critical for generating enough material of similar product quality to meet the rigorous standards for clinical applications. An additional burden is the raw material requirements. Transfection reagents, such as polyethylenimine (PEI), and plasmid DNA made in good manufacturing practice (GMP) environment are expensive when required for the 100's or 1000's of liter scale. A standard method for scaling adherent cultures is to gravitate away from dishes and T flasks to hyper-stacks, where multiple layers of tissue culture plastic are stacked upon one another in a single vessel. The extreme of this technology is the HS-36 which provides 18-square meters of tissue culture surface in a single vessel at a cost of several thousand dollars each. These vessels also are burdened by multiple ports, air vents, and required manipulations for passaging and feeding. Each of these steps, when multiplied across many replicate vessels, creates a high risk for contamination and thus costly production failures.

Herpes Simplex Virus

The necessity for large amounts of bio-comparable rAAV to power clinical trials has been addressed through the development and refinement of more scalable or stable cell line production platforms. The replication deficiency for AAV, and rAAV, necessitates the presence of helper functions, primarily from Adenovirus. However, adenovirus is not the only virus capable of providing the regulatory and microenvironment altering helper functions required for AAV generation. The Human Papilloma Virus (HPV), Vaccinia virus and herpes simplex virus type-1 (HSV) can also confer the requisite helper functions (Wold & Toth, 2012). The first two have not been explored as viable beyond their innate ability to confer helper functions (Schlehofer et al., 1986). HSV has a growing track record as a suitable hybrid method for generating AAV. HSV has a large doubled stranded linear 152kb genome comprising fewer than 100 genes (Blits & Bunge, 2006). The viral icosahedral capsid is surrounded by a proteinaceous layer termed the tegument that itself is shielded by a lipid bilayer studded with glycoproteins (Akhtar & Shukla, 2010). The genome itself is segmented into Unique Long (UL) and Unique Short (US) segments, and many of the HSV genes are non-essential which enables transgenes to be readily inserted into the genome without hampering the critical effectiveness of the virus. HSV-1 contains a set of genes, differing from those found in adenovirus, capable of regulating and stimulating the production of rAAV. Within HSV, Weindler conducted mutation studies to identify the minimal set of genes required as UL5, UL8, UL52 and UL29 (Weindler & Heilbronn, 1991). UL5, UL8 and UL52 form an enzyme complex that has helicase and primase functionality, while

UL29 is a DNA binding protein. It's worth noting the differences between the regulatory nature of adenovirus helper functions and the DNA replication nature of HSV helper functions.

rAAV generation from the HSV platform can take many forms. The earliest system developed is an amplicon system where the core of the AAV genome, with the capsid of interest are cloned into a plasmid also containing the HSV origin and packaging signal as can be seen in Figure 4 (Conway et al., 1997). The plasmid can be transfected along with wild-type HSV DNA or replication defective HSV itself to provide the *trans* helper functions required for generation of rAAV genome and packaging. In the same paper Conway's group showed that in the place of plasmid DNA containing the rAAV genome, rAAV virions and even rAAV genomes integrated into the host cell line are viable methods when complemented with the HSV helper functions.

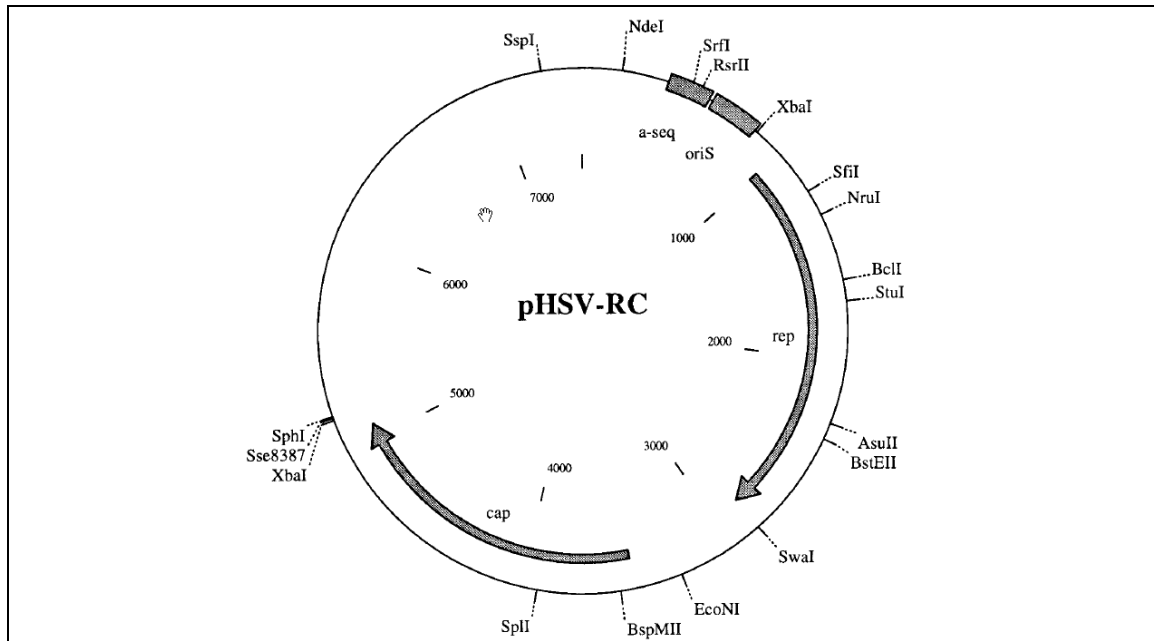


Figure 4. Plasmid delivery of Rep and Cap in HSV system

Plasmid delivery of HSV origin of replication, Rep2 and Cap2, and alpha sequence that confers the packaging signal (Conway et al., 1997).

From a raw materials perspective, the HSV amplicon system has several drawbacks. First, HSV is highly pathogenic and thus creates a safety concern within the work environment, but also demands an active approach to viral management. Robust purification methods must be instituted which will lead to concomitant reductions in rAAV yield. Additionally, state of the art analytic techniques must be developed and applied to prove full removal of contaminating HSV virus. The second complicating factor is the complexity of managing three precursors; HSV-RC, mutated HSV, and the source of the rAAV genome.

The next iteration of the HSV/AAV system relies upon the generation of a recombinant HSV virus containing the requisite AAV genes. Both generated rHSV-

RC by way of a plasmid containing homologous sequences to the UL43 region of the HSV genome. The homologous regions flanked the Rep/Cap genome sequence, respectively, allowing for homologous recombination (Booth et al., 2004). rAAV is generated by infecting Vero, BHK or HEK cells with the rHSV-RC and a plasmid containing the *in cis* gene of interest. Booth found this system to be 100-times more productive than the straight amplicon system.

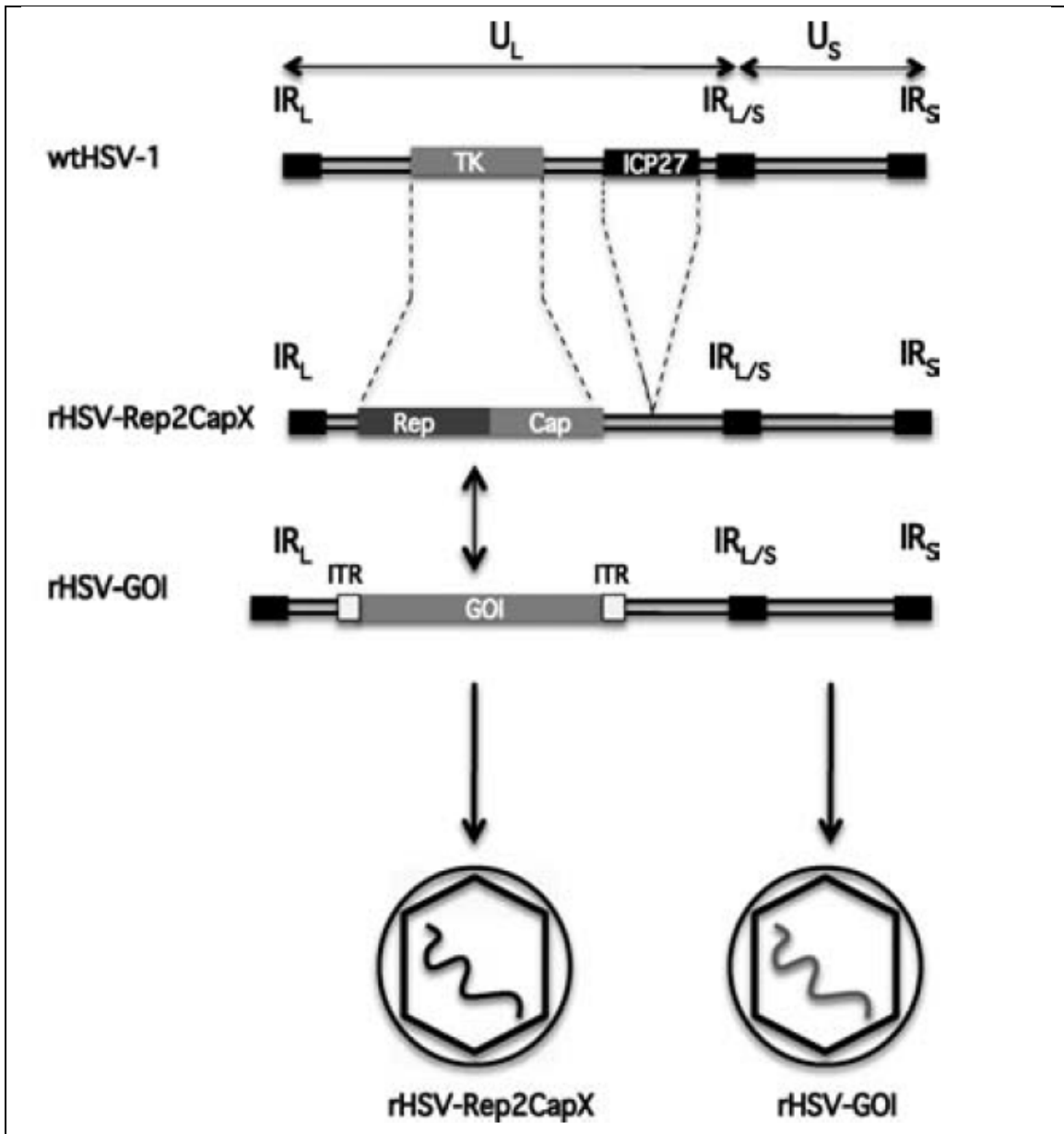


Figure 5. Herpes simplex virus and vector.

Top: Wild type genomic locus for targeting showing the thymidine kinase gene and ICP27 gene. Middle: the rHSV-RC and rHSV-GOI genomes with disrupted TK and deleted ICP27 genes. Bottom: Packaged rHSV virions (Clément et al., 2009).

One of the current leading methods for rAAV production, by Conway's group, furthers the hybrid method. Rather than rely upon transfection to deliver any components, they generated two independent seed stocks, one rHSV-RC and one rHSV-rAAV. Both were inserted via homologous recombination into the thymidine kinase (TK) gene and thus provided a basis for selection using the toxicity associated with ganciclovir addition (Conway et al., 1999). Simultaneously, the key HSV replication gene ICP27 was deleted rendering HSV replication incompetent. The two viral seed stocks were propagated in Vero or V27 cells expressing HSV ICP27 to complement the viruses upon infection. It should be noted that the sequestration of the two seed stocks conveys a beneficial level of flexibility for mixing and matching capsids and genes of interest that isn't available in systems fully engineered with both components. Subsequently the two rHSV viruses were used to infect a final BHK cell line that was amenable to growth in suspension as is seen in Figure 6. This method rarely produced replication competent, wild type, AAV, likely due to the partitioning of the *cis* and *trans* components during seed stock generation.

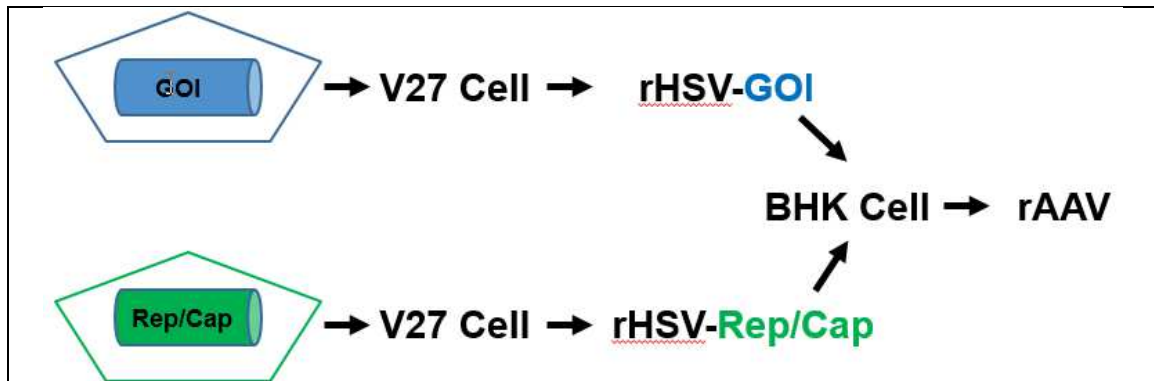


Figure 6. RAAV production system from HSV hybrid system.

Two HSV viral seed stocks are generated in Vero cells. The viral seed stocks infect BHK cells to generate rAAV using the HSV helper functions.

Baculovirus

The necessity for helper functions is both essential for rAAV production, but also a burden for purification and yield considerations. Another key element is the ability to grow and transfect, or infect, the cells used as a viral factory in suspension to concentrate enough cell mass into manageable vessels. Researchers have been exploring viruses that infect insect cells, baculoviruses, as another approach for generating stable, highly productive clinical grade rAAV. Insect cells, mainly Sf9 cells, readily grow to high densities in suspension, serum-free culture. Despite their invertebrate phylogeny it has been shown that baculoviruses not only infect and propagate within insect cells but that the key AAV viral proteins are readily generated and functional within these cells (Madore et al., 1999; Christensen et al., 1994). Figure 7 shows a key study by Urabe proving that baculovirus are readily able to propagate AAV structural proteins (Urabe et al., 2002). Thus, baculoviral systems based within the Sf9 cell type have been developed using similar strategies as the HSV system to generate rAAV.

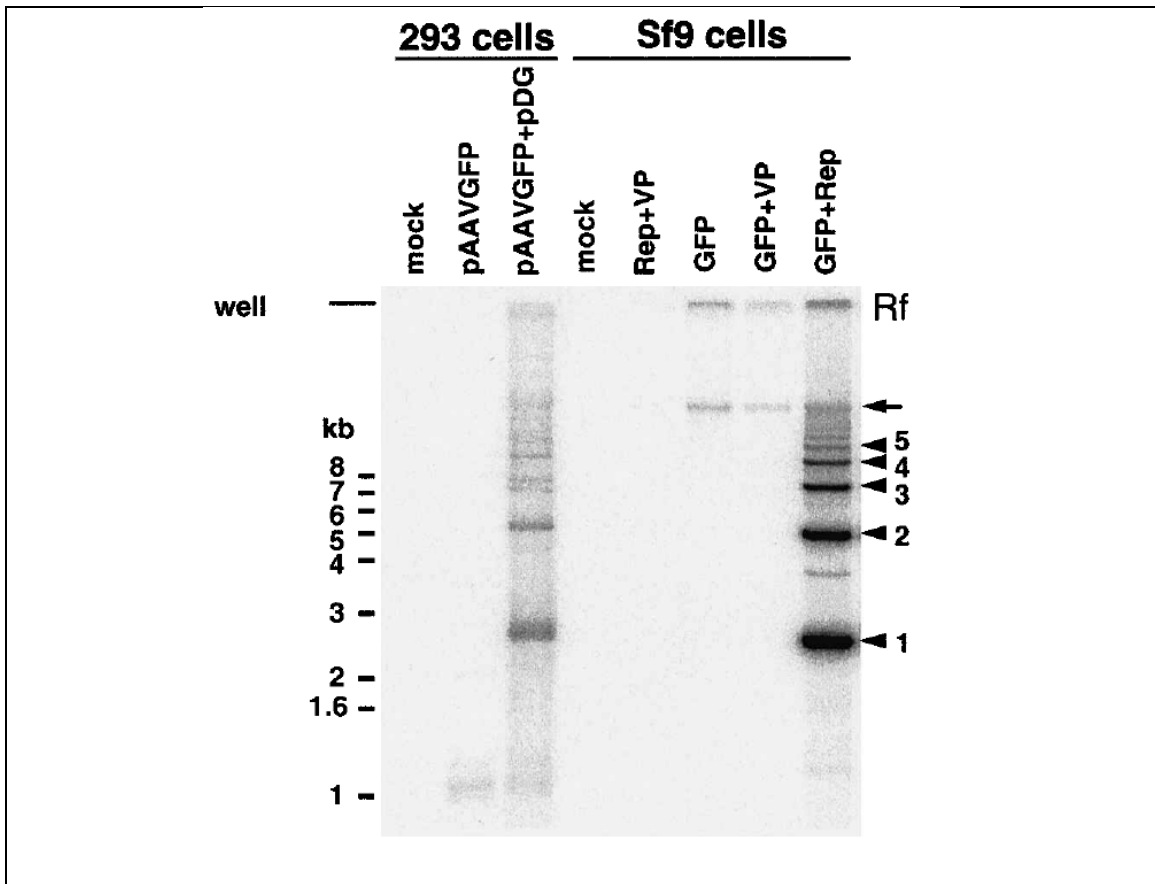


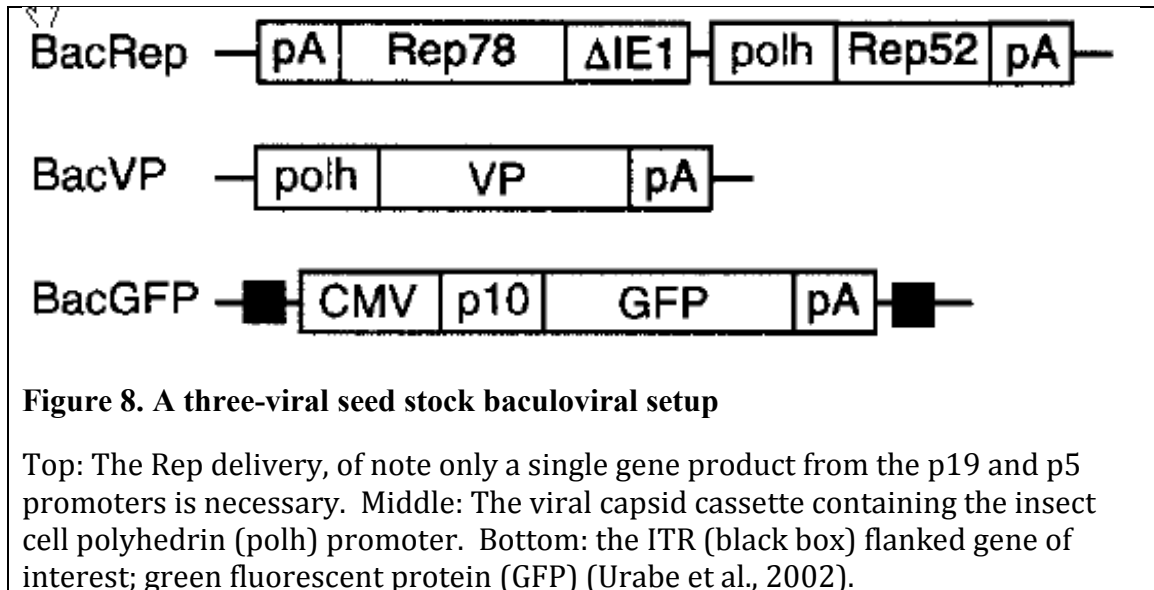
Figure 7. AAV genome replicates in Sf9 insect cells.

Southern blot showing that GFP-AAV genome can successfully be generated in the presence of Rep in SF9 cells. The monomer is denoted as 1, with dimers and higher order structures denoted with 3-5. Arrow denotes the input baculoviral genome (Urabe et al., 2002).

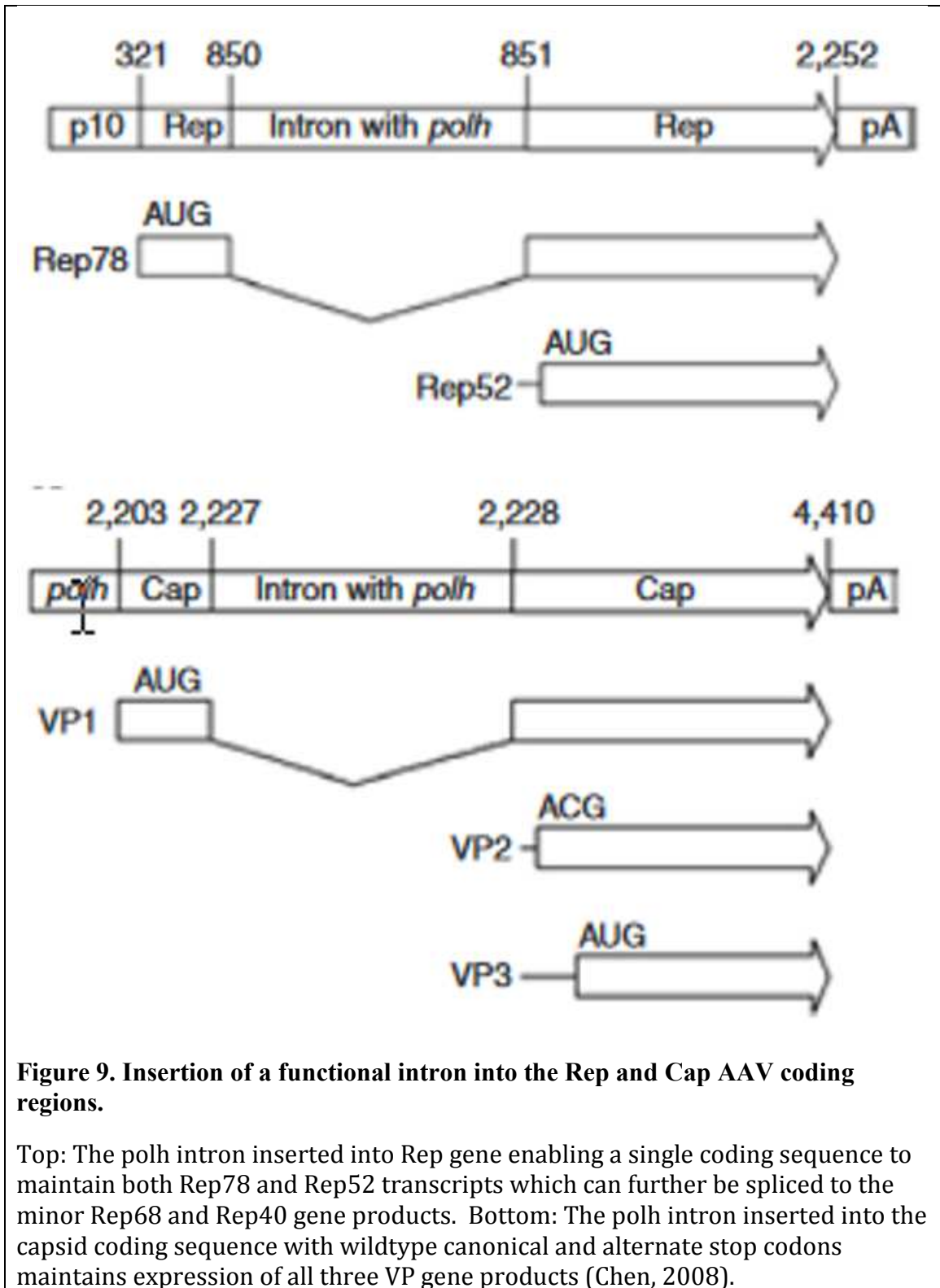
Like HSV, these systems require an *in cis* transgene cassette and *trans* AAV factors to catalyze the rAAV lifecycle. Here, though, initial efforts focused on three individual viral seed stocks to be generated as can be seen in Figure 8, with the Rep, Cap, and Gene of interest residing on individual precursors. Insect cells are not known to properly transcribe and process variants from overlapping open reading frames, so the Urabe group found it necessary to partition the p5 (Rep78 and Rep68) and p19 (Rep52 and

Rep40) coding sequences. As was noted in the introduction, each of the two open reading frames produces two transcripts through one splice variant; hence single coding of the two full length transcripts is sufficient for generation of all four gene products and thus full activity of the regulatory machinery. Many groups employ the *Autographa californica* polyhedrosis virus (AcNPV) in conjunction with Sf9 cells to generate rAAV. The system functions based on a strong p10 promoter which performs the analogous role to the CMV promoter in the producer cell line. The p10 has strong activity and can drive transgene sequences.

A paramount concern when using an invertebrate cell line for generation of biotherapeutics is the effect of using non-mammalian cells on post translational modifications (PTMs), such as *O*-linked and *N*-linked glycosylation, gamma-carboxylation, and phosphorylation. For standard protein therapeutics, these attributes are intricately involved in bioactivity, stability, clearance, and interaction with the immune system. PTMs are the primary reasons why complex mammalian expression systems are warranted in lieu of fast growing, inexpensive bacterial systems. While the critical quality attributes for rAAV are not well defined for rAAV therapies; concern regarding the potential immunogenicity of viral particles is a central focus of researchers and regulators (Thacker, 2009). Biotherapeutics produced in human cells are the least likely to confer an immune-reactive PTM. It would reason that swapping to an invertebrate system, despite the advantages, would warrant additional purity and PTM analytical package validation and scrutiny from regulatory agencies.



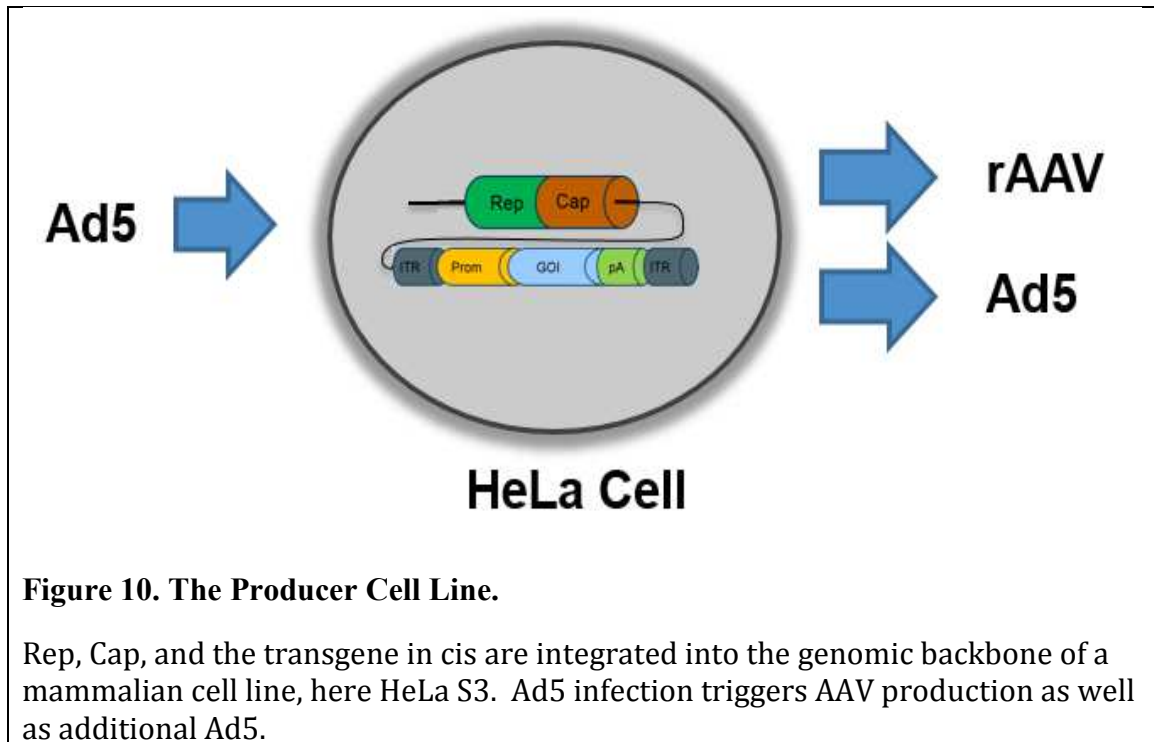
As previously mentioned, insect cells are not known to express from overlapping open reading frames. As Figure 1 shows, the p19 promoter is embedded in the coding region of the p5 Rep78 and Rep68 major gene products. The Urabe group initially overcame this limitation by partitioning the p5 and p19 gene products, as a continuous intact coding sequence. It was found in passaging studies of the individual seed stocks that the titer of Bac-Rep declined steadily with serial culturing suggesting that the head-to-tail Rep78 and Rep52 orientation was not stable and this led to a direct reduction in overall rAAV productivity (Kohlbrener et al., 2005). A single intron was identified in the IE-1 gene of AcNPV that appeared to be functionally processed in Sf9 cells. Figure 9 shows an elegant setup where the Chen group inserted this intron with part of the polyhedron promoter into the Rep and Cap genes allowing for a single Baculoviral sequence for each component and realized normalized stability with high level expression system.



Producer and Packaging Cell Lines

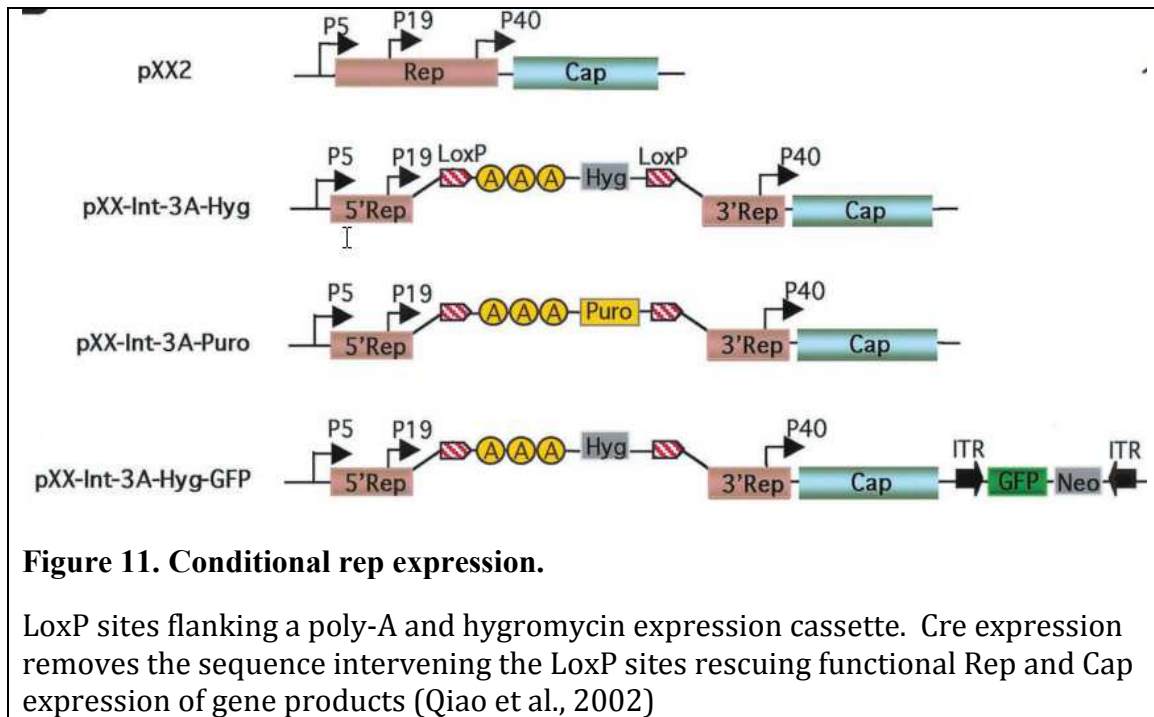
Production of biotherapeutics is most commonly performed in mammalian cell lines. Mammalian cell lines can be grown in suspension to high cell densities and will confer, depending on the cell line chosen, less immunogenic post-translational modifications. However, even within accepted practices, Chinese Hamster Ovary (CHO) cells are the mainstay of protein therapeutic expression systems, the various lineages (dg44, CHOK1, CHO-S) confer non-human N-glycolylneuraminic Acid (NGNA) and terminal- α -1,3-galactose (α -gal) immunogenic epitopes. These epitopes must be rigorously characterized and screened out during cell line development activities (Berkowitz et al., 2012). The gene therapy field has been restricted to a small number of viable cell line options, requiring a human cell line capable of high levels of transgene expression, an ability to grow in suspension serum-free conditions, all without imposing tumorigenic risk.

The producer and packaging cell lines are a modified extension of stable antibody, and protein, therapeutic cell lines. Packaging cell lines integrate the Rep and Cap sequences into a production host cell line's genomic DNA. The host cell then relies upon the delivery of the rAAV genome through transfection with the simultaneous infection of a wildtype human Adenovirus serotype-5 virus (Ad5), or an infection of recombinant Ad5 containing the rAAV genome of interest as a payload. Producer cell lines (PCL) integrate the Rep, Cap, and rAAV genomes into the genomic backbone as can be seen in Figure 10.



In the absence of helper functions, the PCL will not generate rAAV and thus mimics the latent phase of an AAV infection in nature. The p5 and p19 promoters will be silent and thus the productive lifecycle of the virus cannot begin. This is advantageous, as AAV generation and Rep activity are known to be toxic to host cells. Interestingly, HEK cells are not a good substrate for the generic PCL setup. As previously mentioned, HEK are transformed with adenoviral E1a and E1b which transactivate the p5 Rep gene leading to toxicity (Pereira et al., 1997). The level of toxicity prohibits the accumulation of sufficient healthy cell mass using the HEK background to make it a viable system without instituting some level of conditional repression of the Rep protein. One novel example of conditional rep expression is shown in Figure 11 (Qiao et al., 2002). Placing conditional repression on the p5 promoter does not silence the p19 activity and

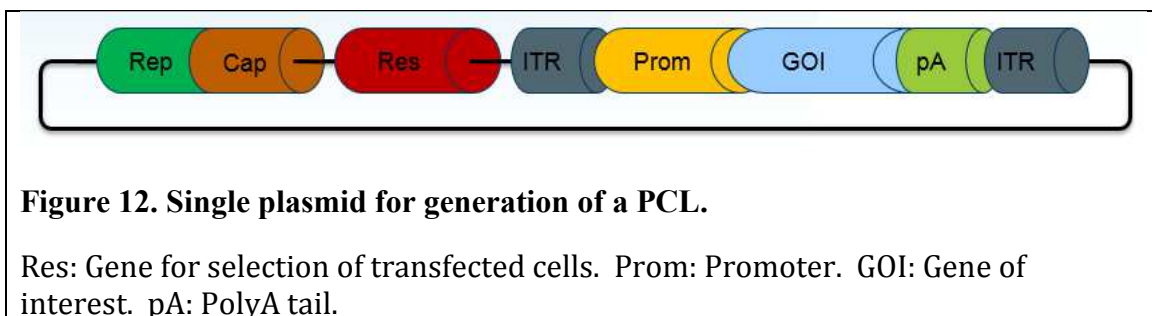
subsequent toxicity due to the presence of a binding site for the E1a protein (Chang et al., 1989). The Qiao group navigated this pitfall by interrupting p19 expression with potent disruption of gene expression comprised of multiple poly-A termination signals and a hygromycin cassette all flanked by LoxP sites. *Cre* recombinase expression delivered as a recombinant E1 deleted Ad5 loops out the cassette allowing for normal expression of the p5 and p19 Rep gene products and thus initiation of the rAAV viral life cycle



The two cell types most commonly used in PCL and packaging cell lines are the HeLa and A549 transformed cell lines. HeLa is an immortalized human cervical carcinoma cell line and A549 is a human adenocarcinoma cell line. HeLa is one of the most common cell lines used in academic and industrial settings. HeLa has portions of the HPV-16 genome integrated; which may be one reason that no approved

biotherapeutics, aside from vaccines, have been generated in this cell line. One advantage to these two cell lines is that, unlike in insect cells, normal splicing is functional in mammalian cell lines; allowing for integration of the intact AAV Rep and Cap genes. CHO cell lines, despite their coveted status as a protein platform workhorse, are not thought to be useful as an AAV production platform. The PCL system relies upon delivery of helper functions by human Ad5 virus which enters the cell using the coxsackievirus receptor (CAR) (Tomko et al., 1997). The CAR receptor is not meaningfully present on CHO cells meaning this key step in production will be highly attenuated. The inability of Ad5 to infect CHO as efficiently as these human lines has further bolstered the use of HeLa and A549 as PCL substrates.

The recombinant PCL is generated by transfecting either two plasmids, one containing the Rep and Cap native sequences and another with the gene of interest cassette in *cis* with the ITRs, or commonly a single plasmid containing these elements as is seen in Figure 12 (Martin et al., 2013). Integration will be a random event with no known preference for the AASV1 locus.



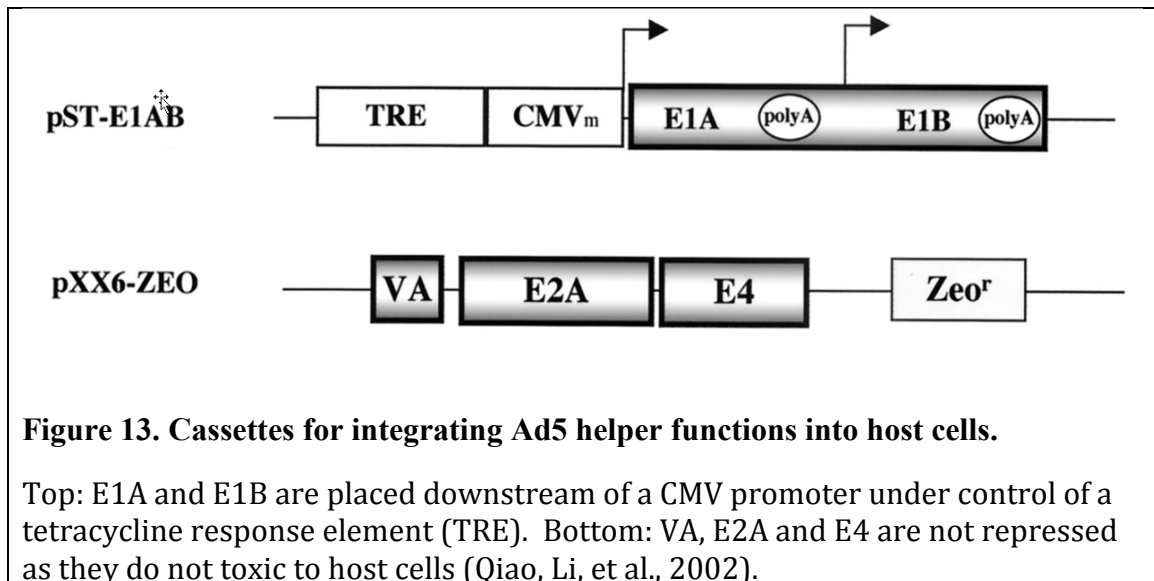
Caution must be taken to ensure overlap between the sequences do not provide an opportunity for recombination which can lead to the generation of wild type AAV *in*

vitro. Stable cell lines are established by the application of an antibiotic agent in conjunction with the presence of a selective gene on the plasmid.

rAAV production is catalyzed by the delivery of helper functions, which does not in principal exclude strains of virus other than Ad5. Ad5 is the primary choice for several reasons. Ad5 readily infects human cells; key for AAV production in human cell lines, but also for generation of Ad5 as a raw material. Ad5 is a precursor in the PCL process and it can be readily made at high titers in the same A549 or HeLa background used to generate the PCL. Ad5 is also more heat labile than AAV providing the basis for selectively inactivating Ad5, as opposed to rAAV, in a post-production processing step.

Ad5 E1a transactivates the silent p5 promoter initiating AAV viral gene production to pair with the constitutive expression of the gene of interest (Weitzman et al., 1996). Ad5 also has a dramatic effect on the host cell, and prepares the microenvironment for viral production at the expense of cellular functions beneficial to the host cell (Defer et al., 1990). Ad5 will force the host cell into S-phase and therefore ensure replication machinery is abundant for genome replication. E2a plays a role in the replication centers, E4 is involved with AAV replication and second strand synthesis, while the viral associated RNA induces expression of AAV regulatory and structural proteins (Balakrishnan & Jayandharan, 2014). The presence of Ad5 creates a burden as a precursor and a byproduct. The production of Ad5 as a byproduct during rAAV generation consumes cellular resources that would be better focused toward generation of the therapeutic. The industry is turning toward integrating the key Ad5 helper functions into host cells which are used to develop PCL manufacturing lines (Qiao et al., 2002).

In the system developed by Qiao, VA RNA, E2A and E4 are allowed free expression as they are not known to be toxic to host cells and do not activate AAV viral genes. E1A and E1B are placed under the control of a tetracycline response element, maintaining tight repression until tetracycline or an analogue, commonly doxycycline, is added to the cell culture media and activates expression. The cassettes used to generate these cell lines are shown in Figure 13.



Discussion

rAAV approaches to gene therapy are reemerging as relevant vectors for gene transfer to patients suffering from diseases caused by genetic abnormalities. The low immunogenicity of AAV, ability to target a wide variety of cell types and capacity to confer long term high levels of gene expression in target cells are the primary reasons rAAV is one of the most promising gene therapy vectors. Unlike with recombinant proteins, generation of sufficient quantities of the therapeutic to treat diseases with

systemic administration is a formidable challenge. Neuromuscular disorders may require greater than 1×10^{14} Vg per patient. The complexity, and thus cost, of generating virus goes well beyond standard protein based therapeutic platforms. The more advanced systems require complex management of raw materials and precursors beyond cell culture media and a single GMP cell bank. Viral risk management of precursor, or byproduct, viruses further add expensive downstream costs. Gene therapy production costs will exceed that of small molecule and protein therapeutics, which already pose as a contentious political and public health issue.

The most realistic method for containing cost will be choosing the most productive method of rAAV generation possible. The four methods discussed differ greatly in their productivity profiles and scalability. The two most productive platforms appear to be the baculoviral and the PCL. Reports for both suggest that 1×10^{13} - 1×10^{14} vg/L are obtainable, though the evidence cited are sparse and the gene of interest in each study is likely to play a profound role on productivity (Clément et al., 2009; Martin et al., 2013). Beyond simple per cell productivity, is the ability to scale the cell line into large single use disposable vessels, primarily stirred tank bioreactors to maximize rAAV volumetric productivity during costly production runs. Many of the transient systems, short of expensive and unproven microcarrier technology, are adherent based as is the viral precursor generation for rHSV; despite the production run taking place in a BHK suspension cell line. The baculovirus and PCL systems are realistically the only two systems that can operate end-to-end in suspension bioreactors.

The bioreactor environment enables high cell growth, but also robust control over the cell culture environment through a mixture of online and offline monitoring of cell growth (overall cell mass, viable cell density, cell viability), dissolved gas and acidity of culture medium (dissolved oxygen, CO₂, pH), and metabolic inputs and waste products (glucose, glutamine, glutamate, ammonia, lactate). Key nutrients can be fed back in using concentrated feeds or isolated inputs such as glucose, glutamine or base to maintain key parameters within optimized ranges. Monitoring and control allows optimal growth conditions to be identified and banded for production runs enabling reproducible batches, but also providing a basis for rejection criteria for batches that deviate from normal and expected conditions. The precise product quality attributes that will be scrutinized by regulatory authorities are not well-defined, as approved therapies administered directly to humans are lacking. Presumably the ratio of empty to full particles, the ratio of the capsid proteins, and the amount of non-intended DNA packaged (host cell or plasmid derived) will be important. Post translational modifications as assessed by mass-spectroscopy will assuredly build greater understanding around which quality attributes are important, and how consistent production platforms and processes apply them. The baculovirus system may be a risky choice should the insect cells confer immunogenic epitopes to the viral capsid. Even BHK, mammalian cells, will need to provide “absence of” evidence for PTMs should regulators gain comfort with human cell line production systems. Historical perspective for biotechnology therapeutics would suggest that reproducibility will be an additional highly scrutinized aspect during regulatory vetting. The production system, and the use of highly controlled production vessels will be the

best mitigation strategies. Transient and HSV systems in full, or in-part, will again fall short as adherent cultures, regardless of their scale are largely uncontrolled.

Another consideration of high importance to regulatory bodies is the presence of animal derived products in the derivation or production process. Adherent cells are generally grown in the presence of fetal bovine serum (FBS) on tissue culture-treated plastic. Adherent growth vastly reduces the number of cells that can be grown in a vessel, which becomes further restricted by incubator space. But most importantly, serum is looked upon with great skepticism by regulatory bodies due to the potential for contaminating adventitious viruses to be transferred from the animal derived raw material to the therapeutic. Mammalian based protein therapeutics which have a much broader track record with regulatory approvals, are often generated in Chinese Hamster Ovary (CHO) cells adapted to grow in animal component free media. Industry groups with cell culture focused groups can generate fully chemically defined cell culture media that allow for reproducible formulations with consistent control over raw materials.

Complexity of precursor management will have an impact on the likelihood of success as well as the cost of the generation of material. The cost of plasmid and transfection reagents for the transient method makes this method unrealistic for any scale beyond the laboratory bench. rHSV and baculovirus systems too have biological precursors that must be generated in large quantities to initiate large scale production runs. Baculoviral systems have further been plagued by instability of Rep containing recombinant viruses. Only recently have genomic engineering solutions begun to show that this may be a tractable issue.

This leaves the PCL as the most realistic platform for meeting the needs of a clinical grade rAAV production system. The PCL is easily scaled as a suspension cell line in animal component free media. As a human derived cell line, the likelihood of immunogenic epitopes being conferred onto the viral capsid are unlikely. HeLa and A549 are tumorigenic cell lines. Therapeutics generated in these cell lines will have to be thoroughly vetted to ensure that the unwanted tumorigenic sequences do not end up packaged into the capsid. Considering the size of the genome, this seems like an unlikely scenario, however analytical methods such as Next Generation Sequencing should be able to readily identify and quantify contaminating DNA. The other major drawback to PCL is the use of pathogenic Ad5 virus to initiate production. This is problematic, but as will be detailed there are methods to clear Ad5 from the drug product stream. As was discussed there are methods to engineer the key helper functions in an inducible system into the host cells. The major drawback to the PCL system is the time required to generate the cell lines and the lack of flexibility a single cell line with the viral AAV genes and gene of interest has. Disease indications requiring small amounts of virus, such as those directly administered to the ocular compartment, may not require the scalability, and thus upfront time and resource commitment, intrinsic to this system.

In practice, the PCL is a logical extension of existing protein therapeutic workflows for generation of the stable cell line and execution of the production runs. What follows will be guidance on the practical attributes of how the execution and downstream steps may unfold to generate clinical material.

Production runs would begin with the thaw of the PCL cell line in a viral free environment and grown up in iteratively larger combinations of shake flasks, wave bags or CSTR bioreactors. As the scale increases, centrifugation of cell culture becomes impractical (and is generally not used at all in production facilities at any stage). The bioreactor prior to the production run, termed an N-1 reactor, may employ a modified fed batch or a perfusion reactor to achieve sufficient cell densities to seed the production reactor at the highest cell density that will support rAAV production. These N-1 steps are critical to keeping the volume of cell culture transferred to the production reactor as minimal as possible. This will reduce transfer of inhibitory waste products and maximize the proportion of fresh media in the production vessel. The N-1 inoculum will be transferred to a designated viral suite where the Ad5 viral bank will be thawed and inoculated into the production reactor to a specific multiplicity of infection (MOI) containing acclimated PCL cells at the intended density. The N-1 and production, but realistically all, vessels will be single use disposables. The current maximum scale of single use bioreactors caps this process at 2000L, though various commercial suppliers may push this scale larger as the industry demands higher scale. The production run generally unfolds over several days with minimal feed and nutrient additions, or pH adjustments as required.

Harvest conditions will be contingent on the viability of cells and how the respective capsid dictates the virus will be partitioned as some serotypes are retained in the cell while some are secreted. A lysis step may be necessary in conjunction with an enzymatic digestion of free DNA from lysed host cells or unpackaged AAV or Ad5. Cell

debris is most easily removed through depth filtration, which is followed by a buffer exchange and concentration step using ultrafiltration and diafiltration (UF/DF) to prepare the production stream for purification.

Purification steps can be sequenced interchangeably, and in principal are dictated by the binding properties of the various loading and elution buffers to maximize yield off each step. Prior to, or after, UF/DF an ion-exchange chromatographic (IEX) step to exploit the charge difference between the two viruses present can provide upfront meaningful removal of Ad5. The two critical purification steps are a subsequent Anion Exchange (AEX) and affinity based chromatography. The AEX step will exploit the charge difference conferred upon full rAAV capsids by the presence of a negatively charged genome. This difference allows for resolution of empty and full particle. Full particles can represent equal or greater quantities of the virus produced depending on the serotype and system chosen, and thus would represent an undue challenge to a patient's immune system (Gao et al., 2014). While there is no clear regulatory guidance for the level of full capsids warranted, a robust removal step must be developed. Affinity chromatography exploits serotype specific binding to the capsid and thus removal of extraneous proteins and debris. As previously mentioned a heat inactivation step also must be implemented to further inactivate Ad5 and undetected adventitious virus, and thus provide an orthogonal method for Ad5 removal. The final step will employ UF/DF to formulate the affinity eluate to a buffer compatible for stability and administration to the compartment of the body the drug is intended for.

Gene therapy using rAAV is reemerging as one of the most promising therapeutic vectors for addressing a host of devastating genetic diseases. rAAV is not known to cause pathology in humans, can infect a host of disparate cell types and has been shown to lead to long lasting expression of a transgene. Large quantities of virus will be needed to dose patients intravenously or into central nervous system compartments. The methods widely used by groups working in this field, primarily the transient transfection system, are insufficient in terms of productivity or robustness. The transient and HSV systems rely on poorly scalable adherent systems that require problematic animal derived products. The HSV and baculovirus systems require complex management of precursor viruses and plasmids. The baculovirus system is marred by precursor instability and questions regarding post translational modifications. The PCL system requires a long development time line; however, it avoids the pitfalls that afflict the other systems. The PCL is scalable as a suspension line, can be grown in chemically defined serum-free media and requires only two precursors; Ad5 and the PCL itself. The PCL is likely to be looked upon well by FDA regulators do to its foundation in protein production system technology that has been embraced by regulatory bodies for decades. It's hopeful that companies moving gene therapy candidates toward the clinic will embrace highly productive and physiologically reproducible systems. This will be critical to contain costs for an already strained healthcare industry and thus ensure the promise of gene therapy becomes a reality for patients suffering from debilitating diseases.

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