CELL & GENE THERAPY INSIGHTS

GENE THERAPY VIRAL VECTORS: ADVANCES AND CHALLENGES
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Clinical translation of viral vectors for gene therapies and beyond

Gerhard Bauer  and Mohamed Abou-El-Enein

Gene therapy has, after years of setbacks, returned as a highly advantageous novel therapy for the treatment of severely debilitating diseases for which there were no treatment options.

Clinical applications of viral vector-mediated gene therapy will soon be approaching their third decade of use. The initial idea of re-engineering naturally arising viruses for efficient integrating and non-integrating gene delivery strategies was groundbreaking [1]. A huge amount of effort went into basic research in the 1980s to make murine oncoretroviruses replication incompetent, adapt them to efficiently transduce human cells and then reliably integrate their payload into the genome. At the same time, adenoviruses were re-engineered to transport genetic information into target cells without gene integration. Soon afterwards, adenoviral vectors were used for human in vivo gene therapy applications, while culture technologies for human T cells and human hematopoietic progenitor cells were developed to allow for retroviral vector transductions ex vivo [2]. At that time, while used under appropriate transduction conditions and transferring a well-designed gene, oncoretroviral vectors based on the murine moloney leukemia virus could be utilized in clinical testing. Unfortunately, the in vivo application of a first-generation adenoviral vector in a clinical trial of gene therapy for ornithine transcarbamylase (OTC) deficiency ended with the death of a young patient in the year 2000, due to a systemic, uncontrollable inflammatory reaction [3]. Similarly, the oncoretroviral mediated clinical trial of hematopoietic stem cell gene therapy for X-linked
severe combined immunodeficiency (SCID) in France caused several patients to develop leukemia, due to insertional toxicity [4]. By carefully evaluating these severe drawbacks, soon a clearer understanding of the safety issues involved in viral-mediated gene therapy was achieved [5]. Safer adenoviral vectors were developed that would not cause systemic inflammatory reactions, and for retroviral vectors, tighter control of transduction conditions and inserted copy numbers, combined with integration site analyses with a better understanding of the integration pattern in transduced cells lowered the inherent risk of insertional toxicities. A major improvement in integrating vector gene therapy was achieved when HIV-based vectors became available, offering a safer integration pattern [6]. The further development and application of adeno-associated viral vectors (AAV) also offered significant improvement in non-integrating in vivo gene therapy, since these vectors showed extremely high safety with almost no toxicities, particularly in gene delivery to the CNS [7].

Soon after, gene therapy clinical trials, particularly using AAV and lentiviral vectors, established clear clinical benefits in many patients [8]. Lentiviral vectors have shown therapeutic efficacy in the treatment of ADA SCID [9], adrenoleukodystrophy [10] and Wiskott–Aldrich syndrome [11]. AAV vectors have been successfully used for treatments of Leber’s congenital amaurosis 2 [12], a rare inherited eye disease with a mutation in the *RPE65* gene, choroideremia [13], an X-linked recessive retinal disease, and hemophilia B [14]. Additionally, an AAV vector, Glybera™ (uniQure), has been approved as the first gene therapy application in Europe, for the treatment of lipoprotein lipase deficiency. Subsequently, other gene therapy products entered the EU market [15]; an oncolytic herpes virus 1 (HSV-1), Imlygic® (Amgen), for the treatment of melanoma, Strimvelis® (GlaxoSmithKline), autologous hematopoietic stem cells (HSCs) transduced with a retroviral vector transferring the ADA gene to treat children with ADA SCID, and most recently, Zalmoxis® (MolMed SpA), allogeneic T cells for the treatment of high-risk hematologic malignancies (in conjunction with stem cell transplantation), transduced with a retroviral vector transferring a herpes simplex virus-1 (HSV-1) thymidine kinase. In the USA, no marketing approval for gene therapy vectors has yet been given; however, several successful gene therapy products, including transduced stem cell products, are in the pipeline for marketing approval.

While Europe has made excellent progress in approving gene therapy vectors and certain cellular gene therapies as marketed products, the USA has made great strides in pioneering a new gene therapy technology in the fight against cancer – chimeric antigen receptor T cells (CAR-T cells) [16]. Autologous patient T cells are genetically engineered using retro- or lentiviral vectors, transferring the genetic information for a new T-cell receptor that can recognize a specific antigen on cancer cells, bind to it and enable the T cells to elicit cytotoxicity on the target cells. Remarkable cancer remissions, particularly in leukemias, could be demonstrated [17], and significant amounts of funding have been directed into the clinical development of this technology. The drawbacks, however, are sometimes
systemic toxicities caused by cytokine storms, which still need to be monitored, controlled and made manageable. To manufacture the gene-modified cellular product, autologous T-cell expansions and transductions need to be performed in a controlled environment, which is currently only possible in specialized centers [18]. However, closed-system culture technologies are under development that will, in the future, allow the generation of CAR-T cells at a much wider scale, and also in areas where Good Manufacturing Practice (GMP) laboratories are not available.

Currently, the transduction of HSCs with integrating vectors, particularly lentiviral vectors for clinical applications is the most complicated procedure, requiring several highly technical steps [19]. To treat ADA SCID, for instance, the patient’s bone marrow or mobilized peripheral blood stem cells are harvested, CD34+ hematopoietic stem and progenitor cells are isolated and cultured in conditions that allow efficient transduction, but at the same time, will not diminish the cells’ long-term engraftment potential. The transduced CD34+ cells are tested extensively prior to the infusion into the recipient. Product safety is imperative, with tests for sterility, endotoxin, mycoplasma and integrated copy number performed. It is vital that as few therapeutic gene copies will be integrated per genome to limit integrational toxicity with any possibility of leukemia generation. The cells will be administered with myeloablation or reduction, to allow for efficient engraftment of the gene corrected cells. It took the field almost 20 years to perfect this protocol. Only a few specialized centers in the world can perform this procedure. Maybe when this application becomes more mainstream, more medical centers will be equipped with specialized facilities and highly trained personnel, able to perform it.

The translation of laboratory research into all these aforementioned clinical applications with therapeutic efficacies, although having progressed significantly, has always been challenging and required the development of unique knowledge and costly manufacturing procedures [20]. Often, both vector manufacturing and cellular manufacturing is required, and both need to be performed under GMP conditions. These controlled manufacturing procedures must be approved by the appropriate regional regulatory agencies and carried out in properly equipped facilities with equally properly trained personnel. Scaling up of the manufacturing process from laboratory scale to clinical scale is not trivial, both in vector and cellular manufacturing. Vector purity, particularly for direct in vivo administration is of utmost importance, and vector titer (with good packaging efficacy and little interference from empty particles) is directly responsible for good clinical efficacy. In the past, retroviral vector could be manufactured using stable producer cells. This production method cannot easily be applied to lentiviral vector or AAV vector manufacturing. Transient plasmid transfection into certified human producer cells is the most common
Transfection efficiency is over 90% in most protocols; however, vector particle yield is largely dependent on the properties of the packaged therapeutic gene, with a high degree of variability. Vector production method, currently. Transfection efficiency is over 90% in most protocols; however, vector particle yield is largely dependent on the properties of the packaged therapeutic gene, with a high degree of variability. Vector purification strategies are also wide ranging, from spin filtration, tangential flow, gradient ultracentrifugation to chromatography methods. Vector certification tests include sterility, endotoxin, mycoplasma, replication competent vector, plasmid DNA, host cell DNA and proteins, other tests may also be necessary; again, the most important aspect of the manufactured vector lot is its safety.

What lies ahead in the future? Most likely, new gene editing technologies with zinc finger nucleases, transcription activator-like effector nucleases and clustered regularly interspaced short palindromic repeat-associated systems will be widely employed in clinical testing for gene therapies. Instead of inserting a new functional gene into cells at random and leaving the old one behind, the non-functional gene will be replaced with the new functional gene, in the correct locus. This will remove the danger of random insertional toxicity with upregulation of oncogenes in the vicinity of the new gene. In vivo gene editing is also being developed; however, any adverse events associated with this novel technology, particularly unwanted genetic editing of germ line cells and off target effects will need to be studied carefully, prior to its implementation in clinical testing.

Gene therapy has, after years of setbacks, returned as a highly advantageous novel therapy for the treatment of severely debilitating diseases for which there were no treatment options. As marketed therapeutic products, gene therapies will add an arsenal of new options to provide life-saving clinical benefits to many patients worldwide.

FINANCIAL & COMPETING INTERESTS DISCLOSURE

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Cell and Gene Therapy Insights - a truly multidisciplinary journal for debate and discussion by all stakeholders committed to progressing this exciting field.

CGTI is an online hub featuring high quality, relevant, engaging and interactive content including live webinars, podcasts, white papers, conference reports and daily news updates on key milestones such as spin-outs, financings, candidates entering the clinic, and approvals.
This issue of *Cell and Gene Therapy Insights* puts adeno-associated vectors (AAV) on the spotlight with two thorough reviews by Dr Srivastava (University of Florida) and Dr Merten (Genethon, France). And this honor is well deserved: over the past 2 years, AAV has been propelled to the front of the line as one of the most efficient, robust, reliable and safe bio-tools to mediate long-term gene expression in human patients with genetic diseases, thanks to key advancements, both at the clinical and technical levels. Both industry and venture capitalists now capitalize on AAV’s most recent clinical successes, creating an unprecedented demand for AAV vector development and production as well as a cash flow toward AAV development and clinical use, on what was not so long ago, a purely academic research interest.

Despite this sudden momentum on AAV vectors, AAV is not the new kid on the block: AAV vectors were in fact ‘born’ more than four decades ago, and the wild-type virus, AAV serotype 2, from which the recombinant AAV are derived from, just celebrated its 50th anniversary [1,2]. In this issue of *Cell & Gene Therapy Insights*, Dr Srivastava provides a very elegant, yet thorough, biography of this viral vector, and we will only emphasize a few key features in this editorial: AAV is a very tiny virus, with a 26-nm
diameter icosahedric protein cap-
sid virus protecting and delivering
its linear DNA genome of less than
5 Kb. AAV’s eight proteins are ex-
pressed from only two major open
reading frames – Rep and Cap –
using a simple yet subtle ‘built-in’
approach by means of splice donor
and acceptor [3] sites; AAV Rep and
Cap proteins play key roles in the
genome replication, gene transla-
tion, packaging, host cell binding
and entry into a wide range of tis-
sues and cell types. AAV has been
described as an opportunist virus:
in ‘healthy’ conditions, or in the ab-
sence of cell trauma or distress, AAV
integrates its genome into a safe and
latent unique site within the human
genome, AAVS1; in the event of su-
per infection by a lytic virus, such
adenovirus or herpes virus, AAV is
rescued from the genome, starts rep-
llicating and packaging, and quickly
evades a dying host cell [4,5].

Two major events changed AAV’s
journey to the human body for ever:
its genome was cloned and fully se-
quenced in the early ’80s [6,7], which
led to the AAV first make-over in Dr
Musyza’s laboratory at the Univer-
sity of Florida, where it got com-
pletely degutted from its core pro-
teins, Rep and Cap, to only retain
its inverted terminal repeats (ITRs)
[7,8]. Since then, AAV vectors car-
rying a plethora of different genet-
ic material, from the jellyfish green
fluorescent protein to the most so-
phisticated human genes, have been
deployed to support research from
basic mechanistic pathways to the
treatment of genetic diseases in hu-
mans. From the original prototype
to the current version of rAAV rela-
tively little has changed in the design
or concept. And it is probably nec-
essary to re-emphasize the fact that,
to date, neither the wild-type virus,
nor its vector counterpart, has been
linked to any human disease, mak-
ing AAV the safest genetic vehicle
currently developed for human gene
therapy [9–11].

The list of advantages that make
AAV one of the most sought after
vectors for gene delivery, exhaus-
tively described, triggered the first
gene therapy trial in the mid-’90s.
However, it is only very recently-
that true clinical successes by
means of therapeutic effect (let’s
not forget that Phase I trials are
to demonstrate safety; and in that
sense, all Phase I trials have been
successful to date) have come to
light, from retinal diseases (Leb-
er congenital amaurosis, [12,13],
Leber hereditary optic neuropathy
[14]) to neurological disorders (ar-
omatic L-amino acid decarboxylase
deficiency) [15], blood disorders
(hemophilia B [16], lipoprotein li-
pase deficiency [17]) and muscular
dystrophies (spinal muscular atro-
phy [NCT02122952] and Pompe
disease [18]). With a current port-
folio of over 80 clinical trials and
an explosion of biotech companies,
AAV is stealing the show in what
most are now calling the ‘renaix-
sance of gene therapy’. From un-
known to famous, from forgotten
to rediscovered, from scarce and
competing government funding to
multi-million dollar venture capi-
tal investment, AAV, and probably
its supporters, seem to be living the
American dream.

But let’s not get ahead of our-
selves, AAV gene therapists. There
are still major challenges on the road
to clinical success, and making AAV
a bio-drug accessible to patients
worldwide. In his review, Dr Srivas-
tava lists the two top challenges for
AAV clinical success to be: pre-ex-
isting antibodies to AAV capsids;
and tissue/cell-targeted expression. In this editorial, we want to emphasize one aspect of AAV development that is too often overlooked and underestimated: manufacturing. Full clinical and commercial success of gene therapy using AAV vectors faces the current difficulties of producing high titers, high quantities and highly pure AAV material. The current lack of well-established and validated processes for commercial scale manufacturing is not only the direct consequence of inherent technical challenges, but also of the relatively sporadic interest and funding availability dedicated to this type of research, both in academia and industry, over the past two decades. The current booming interest from industry has created an unprecedented amount of funding that benefits both industry and academia, often working in partnership, which will lead without a doubt to the manufacturing platform(s) required for the next chapter in AAV’s life: commercialization.

Dr Merten’s review in this issue provides a detailed description of AAV production methods currently in use for clinical manufacturing. The first production method was born when it was shown that the AAV genome bearing only the AAV 2 ITR could be replicated and packaged when Rep and Cap were provided in trans in the host cells, in addition to a helper virus. Since wtAAV is a defective parvovirus, some of the adenovirus or herpesvirus genes are required for its replication [7]. The first protocol relied on simultaneous transfection of two or three plasmids carrying all the required functions to produce AAV in HEK293 cells. 25 years or so later, transfection remains the most utilized method to produce rAAV, both at research and clinical grades, as recently reviewed in [19]. To date, four production methods have been implemented for the manufacture of clinical AAV products (also recently reviewed in [19,20]: transfection in both adherent or suspension cell platform [21–23]; packaging cell lines with adenovirus infection [22–24]; baculovirus expression system (BAC) [25–28]; and the herpes virus system (HSV) [29–32].

Current vector requirements to support not only clinical needs, but also extensive pre-clinical toxicology and bio-distribution studies (a must-do to enable investigation new drug submission [IND] and approval by the American Food and Drug Agency [FDA]), approach scales in the range of 1 x 10^{15} to 1 x 10^{16} AAV vector genomes (vg). It is already foreseeable that these needs will soon commonly surpass 1 x 10^{17} while commercial-scale requirements will exceed capacity of 1 x 10^{18} vg. In other words, and to quote Dr Srivastava’s more emphatic terminology, we are looking at producing quadrillions (1 x 10^{15}) to quintillions (1 x 10^{18}) of AAV in the next 2 years.

Yields between 1 x 10^{15} to 1 x 10^{16} are currently achievable in a limited number of laboratories, often benefiting from high capacity in terms of equipment, space and labor, combined with processes that remain to be tailored for commercial scale.
The first commercially produced AAV was Glybera® (alipogene tiparvovec, UniQure), an AAV1-based drug for the treatment of lipoprotein lipase deficiency in adults [17,33]. To date, no AAV drugs have been approved nor submitted for approval in the USA, and it is not known which of the four methods mentioned above will support the first market approval of an AAV drug. The HSV packaging cell lines and transfection have been FDA approved and used in clinical trials in the USA. The capacity in terms of yields and the safety profiles may be giving the HSV system a slight lead over its other counterparts, yet several large biotech companies have invested in the BAC system to support their large-scale manufacturing and future market-scale production. It will take several years for the scientific community to gather sufficient data, both from pre-clinical and clinical studies, as well as from technical reports during the implementation phases of their method of choice, and to determine the feasibility, time constraints and cost of such implementation. As of today, it is foreseeable that several methods will be implemented and used, based not only on data-driven decisions but also, as we will discuss below, intellectual property and legal rights to such methods. As a result, the community, together with the FDA will need to establish clear standards to support bio-equivalency studies and demonstrate bio-compatibility across the different methods used.

Of the four main production methods, all have benefits and disadvantages. There is an overwhelming agreement that the transfection method in adherent format (HEK293 or HEK293T grown in multi-layer flasks) will not support large clinical and marketing scale production, since scalability is directly proportional to the surface area (i.e., the number of flasks) needed. A typical GMP manufacturing campaign requires more than 100 10-layer cellstacks for yields in the $1 \times 10^{15}$ vg of clinical product [19,34], and generating more than $1 \times 10^{16}$ vg would require greater than 500–1000 cell factories. Although technically possible, it is likely not a viable option for most if not all manufacturing facilities with regard to manpower, space and cost limitations.

The most appealing methods rely on suspension culture, whether using chemical (transfection) or biological reagents such BAC or HSV. Based on published yields, the two methods that seem the most comparable are BAC and tHSV with yields often more than $10^5$ vg/cell of purified material [25,31; Adamson et al., manuscript in preparation]. Both methods require relatively extensive upstream work with reagent development, either recombinant BAC or HSV to support AAV production, and include production and release testing of viral banks (raw material) or virus-infected cell banks. However, transfection still relies on a massive amount of plasmid to be produced at GMP-grade, and all things considered, could result in a higher cost based on lower yield per cell. Methods relying on biological raw materials like BAC or HSV also...
face challenges with regards to the viral stock genetic and infectious stability, as well as extensive safety testing to determine the amounts of residual viral materials (DNA, protein, wild-type or replication competent viral particles; recently reviewed in [19], and in this issue). Yet, as discussed above, the data currently available is not sufficient to determine the true technical challenges and cost of each method.

Another daunting aspect in the field is the complex yet unavoidable difficulty to consistently determine biological parameters of clinical AAV drugs based on the diversity and variability of quality control assays available [19,35]. Cross-referencing INDs would be the easiest, fastest and cheapest way to support clinical research of similar AAV products. Yet the inherent assay-to-assay or lab-to-lab variability makes it almost impossible to compare clinical doses from one trial to another, unless biological conversion rates are established between the different laboratories. Furthermore, with each new method, assays need to be developed to determine potential process-residual or impurities. Guidance on the level of impurities acceptable for increased human doses, especially in the context of systemic administrations, is still uncharted territory. Last but not least, stability of the purified particles may vary according to the methods used for production, purification and final formulation buffer, which will require formal stability studies to be initiated over several years.

Recently, a new type of challenge, neither biological, technical nor clinical, has plagued the scientific community and impacted decisions and research pace: intellectual property. Legal rights to use AAV reagents, sometimes created decades ago, have become a major roadblock to most academic and industry institutions, and often resulted in significant time, energy and cost investment. In many instances, it seems easier to re-invent the wheel than negotiate the rights to use the one available.

In conclusion, long-term clinical success of AAV is interwoven with making sure that the demand will be honored by implementing the necessary methods to produce large amounts of high-quality and high-potency AAV particles. Manufacturing capability not only requires the protocols in place, funding, legal rights, but also highly trained experts in both the academic and industry settings and access to manufacturing suites. Continued and substantive efforts to invest in research, training, develop robust and universal quality assays, increase the availability of manufacturing centers, both academic and industry-driven, streamline legal negotiations, and FDA approval, will pave AAV’s road to success.

Enjoy reading this exciting issue of Cell & Gene Therapy Insights!

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in the production of this manuscript.

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33. UniQure: http://www.uniqure.com


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In 2012, an adeno-associated virus (AAV) vector reached the market following its approval by the European Medicines Agency for the treatment of patients with lipoprotein lipase deficiency. This marketing authorisation represented an important step for the gene therapy field, moving from exploratory towards routine clinical use. However, a number of challenges remain in the production and manufacture of these therapies. In principle, four different production platforms (HEK293[T] transfection, stable packaging/producer cell lines, herpes simplex virus system and baculovirus system) have been developed and for some, scale-up to a 2000 L scale has already been performed. Despite these achievements and the well-advanced technological developments in manufacturing technology, further improvements in AAV technology are required to increase vector titers, and improve vector quality and potency. This article provides a review of the four vector production platforms, recent improvements and developments as well as perspectives on future requirements.

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The in vivo use of AAV vectors for the treatment of rare diseases has come of age, in particular, with the successful outcomes of several clinical trials, including the treatment of hemophilia B [1–4] and Leber’s congenital amaurosis type 2 [5–9], and obviously with the marketing authorization of uniQure’s Glybera® (an AAV1-based gene therapy product for the treatment of familial lipoprotein lipase deficiency) in the EU in Autumn 2012 [10]. There are many additional clinical applications in development that utilize AAV vectors for the treatment of various rare and acquired diseases and up to February 2016, 162 clinical trials using AAV vectors have been performed, which equates to 6.7% of all clinical gene...
therapy trials performed at that point (http://www.abedia.com/wiley/vectors.php). These promising developments and achievements were only possible because advances in bioprocessing and analytical development provided the necessary tools for the manufacture and quality control of clinical-grade AAV vectors.

Most of the AAV vector production to date has been performed at a small–medium scale using transfection-based production methods. These methods allow for the production of AAV vector at sufficient quantities for the treatment of diseases such as those of the retina, or for Phase I clinical trials, as in the case of the treatment of hemophilia B. In more advanced clinical trials or when moving to more routine use of AAV vectors for marketing purposes, larger vector amounts are required, which cannot be produced (or can only be produced with difficulty) using the traditional transfection method.

In order to design an adapted and scalable production method, it is important to know the vector quantity required for the clinical trial, which obviously depends on the dosing and the number of subjects to be treated. This is explained in more detail with the following two examples:

- A Hemophilia B trial carried out by Nathwani et al. [1,2] used an scAAV8 vector produced via bi-transfection of HEK293T cells grown in CS10 (10-stack CellSTACKS®, SigmaAldrich) [11]. In order to produce the required vector quantity for the treatment of the first six patients (two patients per cohort; in total, three different doses were assessed $[2 \times 10^{11} \text{ vg/kg patient weight; } 6 \times 10^{11} \text{ vg/kg; and } 2 \times 10^{12} \text{ vg/kg}]$) [1] and the subsequent four patients treated with the high dose [2], two production campaigns of a total duration of 18 months consisting of 432 independent 10-stack culture devices were performed [11].

- A preclinical study performed in GRMD (the dog model of Duchenne Muscular Dystrophy (DMD)) using an U7-based exon skipping approach vectorized by an ssAAV8 vector and produced with the insect cell/baculovirus system [12] indicated that an efficient dose should be beyond $5 \times 10^{13} \text{ vg/kg } (= \text{ high dose in that study). At this dose, approximately 40\% of the muscle fibers became dystrophin positive after locoregional administration [13]. If this vector amount should be produced with the traditional transfection system for a Phase 1 clinical trial with three cohorts of three patients (with dose progression, highest dose: $5 \times 10^{13} \text{ vg/kg}$), 1250–2500 10-stack devices would be needed.}

Both examples clearly demonstrate that either for advanced clinical trials as for hemophilia B or for the treatment of muscular tissues representing approximately 50\% of the whole body tissue such as in the case of neuromuscular diseases (e.g., DMD), huge amounts of AAV vectors will be needed, which have to be produced with adapted methods. It is obvious that the production of the scAAV8 vector as used in the hemophilia B study of Nathwani et al. [1-3] was limited with respect to the production system, and producing larger AAV quantities with that method cannot be readily envisaged. With respect to the preclinical trials assessing the exon skipping approach for the treatment of DMD [13], the choice of the insect cell baculovirus system was adapted because this production system is scalable.

On the other hand, both studies provided insights into the vector quantities required for the
transduction of liver or muscle tissues and it is obvious that for the treatment of muscular dystrophies, huge amounts of AAV vectors are needed, which can only be produced by optimized scalable production systems. However, another perhaps less obvious issue is that generally the efficiency and potency of AAV vector lots has to be increased, which will lead to the reduction in the effective doses.

This review presents an update on the available large-scale production and purification methods, their advantages and limitations, as well as challenges to overcome.

**BIOLOGICAL SYSTEMS FOR THE PRODUCTION OF AAV VECTORS**

AAV is a single-stranded DNA virus of the *Dependovirus* genus, part of the *Parvovirus* family. The name of the genus derives from the fact that AAV replication depends on the presence of a helper virus, such as adenovirus or herpes simplex virus (HSV), signifying that for vector production the required helper functions from one of these viruses have to be provided. Since the development of the baculovirus expression system for AAV production (see below), it has become apparent that the baculovirus can also provide the required helper function. Another important point to note is that the overall packaging capacity of AAV vectors is rather small (approximately 4.7 Kb including the two inverted terminal repeats [ITRs]). Its overall organization is presented in Figure 1A.

The first production system developed was based on the transfection of HEK293(T) cells with two or three plasmids. Although this system is very versatile and allows for the simple modification of the capsid and/or recombinant vector sequence, its main drawback is the limited scalability; therefore other systems have been developed to address this limitation: the use of stable packaging and producer cell lines; the recombinant HSV Type 1 expression system; or the use of the baculovirus/insect cell system. These systems will be discussed in more detail in the following paragraphs.

**Traditional methods for the production of AAV vectors: transfection of HEK293(T) cells**

**Use of adherently growing cells**

The traditional production methods for recombinant AAV (rAAV) are based on transient Calcium-phosphate based transfection of HEK293(T) cells. This typical laboratory approach is based on the delivery of the AAV rep and cap functions, of the adenoviral helper functions E2a, E4orf6 and VA [14,15] and of the recombinant rAAV transgene construct (in which the two ITRs flank the recombinant vector construct of a maximum size of 4.5 Kb) to the HEK293(T) cells (Figure 1B). These cells constitutively express the adenoviral E1a/E1b functions, which are also involved in the synthesis of rAAV vectors.

While the original system is based on the use of three plasmids (pAAV-rep-cap, pAd-helper and pAAV-transgene Figure 1B), a streamlined dual system was developed by combining the two helper plasmids into one plasmid [17]. The main advantage is that the cells only have to be transfected with two different plasmids. This system requiring only a double hit cell transfection
(A) AAV-2 genome organization. Shown are the general organization of the genome and the genetic elements of AAV type 2. A scale of 100 map units is used, 1 map unit being equivalent to approximately 47 nucleotides. The general organization of the other serotypes is similar. T-shaped red boxes indicate the ITRs. The horizontal arrows indicate the three transcriptional promoters. The solid lines indicate the transcripts, and the introns are shown by the broken lines. A polyadenylation signal present at map position 96 is common to all transcripts. The first open reading frame (orf) encodes the four regulatory proteins (Rep 78, Rep 68, Rep 52, Rep 40) for which transcripts arise from the promoters p5 and p19 in combination with alternative splicing. The second orf driven by promoter p40 encodes the three capsid proteins (VP1, VP2, VP3) from two transcripts. VP1 is initiated from the first cap transcript, and VP2 and VP3 are translated from two different start codon sites from the second cap transcript. At right side of the bars representing the three VP proteins, the frequency of these proteins in a wild-type AAV capsid is provided. The translation initiation sites of the viral proteins are indicated. An alternative ORF of the cap gene encodes the Assembly Activating Protein (AAP), necessary for AAV capsid assembly. (B) rAAV design and production principle using transient transfection of HEK293(T) cells. In this production system, pAAV-‘helper’ (pAAV-rep-cap) and pAAV transgene plasmids along with pAd-‘helper’ plasmid are brought into the packaging cells by transfection. Without further optimization, this method leads to the generation of about $10^5$–$10^6$ particles per cell. IVS: Intervening sequence (e.g., intron); Prom: Promoter. Reproduced with permission from [16].
results in up to ten-fold higher titers than those obtained with the conventional protocols [17].

Traditionally, transfection-based production is performed in 15-cm cell culture plates, which allow the production of $10^{11}$ vg/plate. Since adherently growing cells are used, an increase in vector production is performed by an increase in the cell culture surface, which in practice is performed via the addition of parallel culture plates (a scale-out approach).

Further increases in production scale can be performed via the use of larger culture devices including CellFactory™ (ThermoFisher) or CellSTACKs® as used by Allay et al. [11] for the production of scAAV8 vectors for the hemophilia B study, or roller bottles as used for the production of ssAAV2 in the Leber’s congenital amaurosis type 2 trials [18]. In the example of the roller bottle process, a sub-batch of 100 roller bottles consisted of $0.5-1 \times 10^{15}$ vg (purified vector) signifying that cell-specific vector production was approximately $2 \times 10^{5}$ vg/c [18]. Similar specific vector production rates have been reported for a CellSTACK process generating up to $10^{15}$ vg/lot of six stacks [19].

As already mentioned, this production method allows for the generation of vector quantities sufficient for a Phase I clinical trial as in the case of the scAAV8-based clinical trial for the treatment of hemophilia B for which 432 parallel CellSTACKs had to be used; however, this example also demonstrates the limitation of this approach when envisaging such a gene therapy approach for late-stage clinical trials or routine clinical use.

A recent development in vector manufacturing is the use of fixed bed reactor systems (iCELL-Lis®, PALL – disposable system) for the production of viruses and viral vectors and in this context the production of AAV vectors via a transfection-based method can be achieved, although the application of a fixed bed reactor system is much simpler when stable producer cell lines are used (e.g., as with the production of retroviral vectors [20,21]). Nevertheless, it was demonstrated that HEK293T cells grown in a 2-cm fixed bed (iCELLis® Nano, 0.53 m²) could be transfected homogenously and generated in average $3.6 \times 10^{14} \pm 7.1 \times 10^{13}$ viral particles, which was approximately a quarter of the quantity generated with 15-cm culture dishes (at equal surface) [22]. In comparison, the iCELLis® Nano system produced two-times lower rAAV levels than a 10-stack CellFactory™ system: $2.2 \times 10^{10}$ and $4.7 \times 10^{10}$ vg/cm², respectively [23]. An iCELLis Nano unit thus produced $1.14 \times 10^{14}$ vg per run (without optimization). Our own studies confirmed the utility of the iCELLis® system for AAV vector production and also showed that the 10-cm bed system could be relatively homogenously transfected [24]. The advantage of the iCELLis® system is obviously its scalability up to the iCELLis® 500 system, which provides a surface of 500 m² at the high compaction version, meaning that for adherently growing cells, the iCELLis® system represents a real breakthrough with respect to scale up. Further advantages are the control of the culture with respect to pH, pO₂, agitation and aeration, as well as the fact that much less operator involvement is required when compared to the use of roller bottles or CellFactory™ systems.
However, several issues are related to scale-up of the iCELLis 500 system that are yet to be resolved. In the case of obligatory adherent cells, cell amplification can only be performed using adapted systems such as roller bottle or CellFactories or more sophisticated systems such as Pall’s Xpansion® Bioreactor system. In the case of cell lines that can also grow in suspension, cell expansion is simply performed by suspension culture using wave type reactors, for instance. In contrast to Emmerling et al. [23] we have used a serum-free culture medium (a modified F17 medium for cell growth and DMEM for virus production in order to boost AAV vector production), which renders the process adapted for the production of clinical material [24]. Furthermore, the harvest of AAV vectors can be simplified by using a detergent containing harvest buffer for cell lysis [12], meaning that under these conditions, the process is easily scalable for routine manufacturing of AAV vectors for clinical use.

Use of suspension culture

Another and more flexible approach to the scale-up of transfection-based production processes is the use of suspension culture for which scale-up is much easier to perform than for surface adherent cell cultures. However, in this context, the use of the classical Ca-phosphate transfection protocol had to be replaced because of the huge impact on transfection efficiency resulting from environmental variations in pH, Ca\(^2+\) and phosphate concentrations. Furthermore, Ca-phosphate is cytotoxic whereby serum or albumin must therefore be present, and second, a complete medium exchange is required prior to transfection [25]. Since this is not really feasible at a large suspension culture process, other transfection agents such as polyethylenimine (PEI) or cationic lipids are used that, in addition, provide much more reproducible results because they are independent of environmental/culture conditions.

Durocher et al. showed that AAV2 vectors could be produced in suspension cultures of HEK293E via triple transfection using PEI [26] and some years later the same group demonstrated that using the same process, the production of AAV serotypes 1–9 was possible, yielding product levels of \(10^{13}\) vg/L [27]. These production levels could be confirmed at a 3 L reactor scale. Further scale-up of a suspension process protocol using 10- and 20-L WAVE reactors (GE Healthcare) was performed by Grieger et al. leading to vector yields in the range of \(6.7 \times 10^{12}\) to \(3.5 \times 10^{13}\) vg/L, depending on the AAV serotype and vector construct [28]. Total yields of up to \(5.5 \times 10^{14}\) vg could be achieved for 20 L cultures.

Although published data only represent production scales of up to 20 L [28], it is evident that scales as large as 200 L (or even larger) could be possible, allowing ten-times higher vector yields. However, it should be kept in mind that the costs of the GMP-grade plasmids (2 or 3) are much higher, meaning that they represent the most important production cost factor. A further drawback is the fact that transfection-based production methods and their yields are impacted to a larger extent by environmental factors than other production methods (see below).
Further developments & optimization

Although transfection-based large-scale production methods have been developed, further improvements are still possible and necessary in order to increase vector yields, vector quality (in particular, the full-to-empty-particle titer) and vector potency. These improvements include the choice of the cell line/cell clone, the plasmid construct, the choice of the medium and culture conditions.

Cells

Although the traceability of the HEK293(T) cells is relatively limited and incomplete [29], the routine use of this cell line for GMP production of different viral vectors is well established because of their advantageous growth behavior, transfectability and good capacity for vector production. Nevertheless, improvements are possible, for instance via the selection of better producing cell clones as carried out by Grieger et al., who selected a HEK293 clone capable of growing in suspension and characterized by high transfection efficiency and AAV vector production (>10⁵ vg/c, >10¹⁴ vg/L) [28]. Although it can be inferred that HEK293T cells should be the more efficient cell line because of their superior proliferation capacity, their greater transfectability and higher vector production rate compared with HEK293 cells [29], the presence of the SV40 T-antigen might present an increased safety risk and therefore it might be judicious to favor the use of the HEK293 cells for AAV vector production.

Plasmids/plasmid constructs

Today, the two or three plasmid systems most commonly used are produced using traditional bacterial production systems [30]. Plasmids contain backbone sequences, which contain motifs, which are recognized by the cell-autonomous immune system and are therefore prone to induce gene silencing and inflammatory responses [31,32]. Furthermore, the sequence of antibiotic resistance genes might be encapsidated into the AAV vector particles and thus might be transferred to the target tissue [33]. The frequency of the presence of AAV particles containing a rAAV vector coding for the resistance gene, can be 2.9 and 26.1%, respectively, for single-strand AAV (ssAAV) and self-complementary AAV (scAAV) vector preparation [34]. In order to avoid the transfer of plasmid-derived sequences into the target tissue, Schnödt et al. have adapted Plasmid Factory’s DNA minicircle technology to the generation of the plasmids used for the AAV production using a two plasmid transfection-based approach [34]. The evaluation of this plasmid system could show that the total particle yield was comparable to the classical plasmid system but the vector preparations contained higher numbers of transgene sequence containing particles. Furthermore, the use of the minicircle technology resulted in vector preparations with superior transduction efficiency, in particular, in the case of the production of scAAV vectors. With respect to the encapsidation of residual plasmid sequences, only 1.3% of the vector capsids still contained the short residual prokaryotic non-coding SCAR sequence (213 bp), which contains one recombination sequence and a tag for affinity purification [35,36]. Ideally, this sequence should also be removed in order to generate AAV vector preparations devoid of particles with foreign DNA sequences derived from the plasmids.
On the other hand, the helper plasmids (as used in a double or triple transfection approach) were also improved [23]. With regards to the adenoviral helper construct (pUC AdV), unnecessary sequences were removed, leading to a size of 10236 bp instead of 15263 bp. The rep/cap AAV helper plasmid was split into two separate plasmids with the advantage of reducing the risk of the generation of rcAAV particles (split packaging approach). Furthermore, these plasmids have been optimized by inactivating dispensable promoters and deleting potential start codons in the cap open reading frames. This led to a rep plasmid (pUCrepoptΔrep78/Δcap) for the expression of rep68 and rep52/40 proteins via separate sequences (in both sequences, the p40 was inactivated) and a cap plasmid (pUCcapoptp5p19p40cap) for the expression of the VP proteins. In this construct, the p5 and p19 promoters had been inactivated. Side-by-side comparison of this improved plasmid system consisting of four plasmids (the rAAV vector plasmid combined with the optimized rep/cap split-packaging plasmids and the helper adenoviral plasmid) with the widely used two plasmid system of the pDG family [37–39] showed that the optimized plasmid system led to a 12-fold increase in vector yield (2.7 x 10^5 vg/c) when using HEK293(T) cells grown adherently in serum-containing medium; however, under serum-free suspension conditions, the viral vector yield was in the range of 2 x 10^4 vg/c, probably due to the insufficient composition of the medium or lack of an essential nutrient [40].

Although this might be the case for suspension processes where we know that the cells normally grown in an attached mode are more sensitive to nutrient limitations, several authors have reported that for transfection-based processes using adherently grown HEK293(T) cells, the switch from a serum-containing medium to a simple DMEM 24 h after transfection leads to elevated release of AAV vectors into the supernatant: AAV2/5 [41,42], AAV2/8 [43,44], AAV2/1, AAV2/7 and AAV2/9 [44]. This release could be prolonged for several days when regular medium harvests were performed [42]. It should be indicated here that for many AAV serotypes the release of vector particles into the culture supernatant is also observed when serum-containing media are used (AAV1, 7, 8, 9) [41]. From a functionality perspective, it could be demonstrated that the behavior of the supernatant and the cell-derived AAV vectors was comparable. It is clear that only the use of supernatant-derived vectors will considerably facilitate purification because of the absence or the presence of rather low concentrations of cell-derived contaminants.

**Use of stable packaging & producer cell lines**

Stable producer cell lines are cell lines containing the AAV functions rep and cap as well as the rAAV transgene construct with the transgene expression cassette flanked by the two ITRs. These cells are often based on HeLa cells [45], although A549 cells have also been used [47]. The development of these producer cell lines includes an intermediate step of a packaging cell line, which contains only the rep and cap functions of AAV, but not the rAAV transgene construct. Such cell lines have been developed either using HeLa or A549 cells leading, for
instance, to the B-50 [48] and C12 [49] or K209 [50] cell lines, respectively. Finally, in order to establish the producer cell line, the packaging cells have to be transfected or transduced with a rAAV transgene plasmid or with a recombinant AAV vector, respectively. However, it should also be noted that some authors have developed the producer cell lines without the intermediate development of a packaging cell line. This can be achieved by the transfection of cells with a plasmid containing the rep-cap functions as well as a recombinant AAV transgene sequence [45,51,60].

Packaging cell lines

Since these cells contain the AAV functions rep and cap, they are also able to produce AAV vectors; however, in this case, they have to be sequentially infected with an adenoviral helper virus with functional genes of the E1 region and subsequently with an adenovirus—AAV hybrid virus (Ad-AAV Hyb. virus) containing the rAAV transgene sequence in the E1 region of the adenovirus [52]. Concerning the adenoviral helper virus, it is preferable that it is replication deficient to avoid its production and contamination of the final AAV vector product, although purification protocols have been developed and validated for the removal and inactivation of the adenoviral helper virus [45]. This replication deficiency was achieved by using an adenovirus with a functional E1 gene required to activate p5, p19 and p40 transcription units of the rep-cap genes and, in addition, containing a temperature sensitive mutation in E2B, for instance, for precluding contamination of the final rAAV preparation by the adenoviral helper virus.

**FIGURE 2**

Packaging and producer cells of rAAV vectors.

HeLa or A549 cells are transfected with a plasmid containing the rep2 (from AAV2)-capX (from any AAV serotype) sequences (Rep-Cap), which leads to establishment of packaging cells. When these cells are infected with adenovirus and 24h later by an E1-deleted adenovirus–AAV-Hyb(rid) virus (providing the rAAV transgene sequence), rAAV as well as adenovirus is produced. If the rAAV transgene sequence is stably integrated via plasmid transfection (pAAV-transgene) or vector transduction (rAAV-vector), the packaging cells become stable producer cells, which upon infection with adenovirus start to produce rAAV vector and adenovirus.
Based on the understanding of underlying biological processes and factors influencing the generation of rAAV vectors, the following optimal production protocol has been developed [48,50,52]: the packaging cells are infected with a conditionally replication defective helper virus at a non-permissive temperature with an multiplicity of infection (MOI) dependent on which packaging cell line (HeLa- or A549-cell-based packaging line) is used. After 24 hours, the cells are superinfected with the adenovirus–AAV hybrid virus containing the gene of interest flanked by both ITRs at an MOI of 10 and the harvest is performed 48 hours later followed by purification. However, since the temperature-sensitive adenovirus mutants are prone to reversion and thus difficult to produce and to characterize, they have not been implemented for large-scale AAV production.

This production system is characterized by several advantages: in addition to five- to ten-fold improved levels in cell-specific vector production [54], it provides the real possibility for scale-up to industrial reactor scales when compared to the traditional transfection method. Another feature is its ability to generate replication-competent AAV (rcAAV)-free rAAV preparations (<1/10^9) [48,52]. In addition, the system’s versatility by having to use one single cell line allows one to produce AAV vectors with different transgenes by only modifying the adenovirus–AAV hybrid virus; however, this might also be a disadvantage for routine productions at very large scale for which stable producer cell lines requiring only a mono-infection would be more adapted than the use of rep-cap containing packaging cells with their requirement for a sequential infection with two different viruses. A very comprehensive review providing more insights on this specific production system has been published by Zhang et al. [54].

**Stable producer cell lines**

The insertion of the rAAV vector genome (transgene cassette flanked by the two ITRs) into a packaging cell line leads to a producer cell line requiring only the infection with an adenovirus to induce rAAV vector production. The rAAV transgene construct is either inserted via transfection of the vector transgene plasmid [53,57,58] or transduction with a rAAV vector containing the transgene construct [47,56]. The former strategy is more convenient because a resistance gene either on the same plasmid or a separate plasmid co-transfected with the vector plasmid allows selection of rAAV containing cell clones. In the case of transduction using an rAAV vector, the selection process based on PCR screening and AAV vector production capacity induced by Ad infection is rather cumbersome. After obtention of cell clones able to produce rAAV vectors, they have to be screened for elevated specific productivity under serum-free suspension conditions since the final production system is a stirred tank reactor. This is followed by further screening for cell line performance and vector quality [45].

The use of wild-type adenovirus is the most straightforward way for induction of AAV production because high rAAV vector titers are obtained; however, this approach is characterized by co-production of high amounts of adenovirus, which have to be removed and inactivated during downstream processing.
This approach had been chosen by Targeted Genetics [45] who have validated the efficient inactivation and removal of co-generated adenovirus. In this context, the two very efficient steps chosen were heat inactivation of adenovirus using 52 ± 1°C and nanofiltration leading to 5–6 and >6 log10, respectively, adenovirus clearance [46]. Obviously, the use of replication-deficient adenovirus can alleviate this inconvenience [47]. However, these adenoviruses are sometimes characterized by instability [57] and lower rAAV vector titers in comparison to wild-type adenovirus. This was, for instance, observed by Jenny et al. when using a protease-deficient adenovirus (AdDPS) [59]. In the case of wild-type adenovirus, the optimal MOI ranges between 10 and 100 for ensuring amplification of rep and cap genes [55] as well as of the recombinant rAAV vector genome [53]. In this context, it should be noted that rAAV production levels are highly impacted by robust amplification of the AAV rep/cap genes upon adenovirus infection and not by the integrated vector plasmid copy number per se [60].

Optimal producer cell lines show cell-specific production levels in the range of 5 x 10⁴ to 2 x 10⁵ vg/cell [45,60] under serum-free suspension conditions. Furthermore, scale-up has been performed up to a 2000 L scale using stirred tank reactors [61] with the potential for generating up to 8 x 10¹⁶ vg (purified) per run. This production system is characterized by an absence of detectable rAAV (below 0.0002% = below limit of detection), by a ratio of full-to-empty vector particles of ≥70% and a potency equivalent to rAAV vector produced via transient transfection [60].

For further information, in particular, on the underlying biology of AAV vector production in context of packaging and stable producer cell line, the discussion section of the article by Martin et al. [60] is recommended; in addition, the paper by Thorne et al. [61] has more information on issues of large-scale GMP production of rAAV vector and related regulatory issues when using stable producer cell lines.

Use of the recombinant HSV Type 1 expression system

HSV is another helper virus that can rescue and induce replication of AAV; thus this virus has also been envisaged for the production of rAAV vectors. This AAV production system is a pure infection-based system in which the non-modified production cells are infected by two different recombinant HSVs providing the AAV functions rep and cap as well as the rAAV vector with the transgene flanked by the two ITRs. In order to reduce or completely avoid the production of recombinant HSV (rHSV), which is highly pathogenic, this expression system is based on the use of ICP27-deficient rHSV helper virus, which is replication incompetent in normal cells. The ICP27-deficient rHSV virus can be propagated in V27 cells (recombinant Vero cells containing the essential UL54 gene encoding for and thus supplying ICP27 in trans) [62]. Furthermore, the two required AAV sequences (rep2/capX and the rAAV vector construct with the transgene cassette flanked by the two ITRs) are inserted into the (TK) locus of the ICP27-deficient HSV vector, each sequence cloned into a separate HSV vector [63] (Figure 3). Although both HSV
vectors can be produced in T-flasks or CellFactories™, for routine large-scale use, a fixed bed reactor system (NBS, Celligen, working volume 3.5 L) had been developed and optimized [64]. This was of particular importance for the generation of the rHSV-Rep2CapX vector, because of the apparently slight toxicity of the rep protein. This reactor process is a prerequisite for the use of the herpes simplex expression system because high MOIs are required for AAV production. The rHSV vector preparations are further concentrated and formulated leading to seed stocks titering beyond 1.5 x 10⁹ PFU/ml [64].

Initially, adherently grown HEK293 cells have been used for AAV production; however, to obtain the highest cell-specific AAV vector production (6500 ip/c), an MOI ratio of 12:2 (rHSV-rep2/cap2:rHSV-GFP) was required [65]. Staying with a CS10 system, infection conditions have been improved for the generation of AAV9 vectors. Although not a scalable process, this improvement allowed the generation of up to 2 x 10¹⁴ rAAV9 per CS10 due to a specific productivity of approximately 2 x 10⁷ vg/c [66], which represents an increase of two- and five-fold, with respect to the non-optimized conditions and the HEK293-based transfection process, respectively.

Despite these improvements, the production conditions represent a drawback for large-scale production, because real scalability is ensured by suspension culture processes and low MOI is required to reduce the HSV vector amount per production run. This was improved by switching to BHK cells grown in suspension for which the MOI ratio could be

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**FIGURE 3**

Herpes simplex virus (HSV) and rHSV for generation of rAAV vectors [63]

Genomic organization of wild-type HSV-1 (top) virus is shown with major genetic elements (not to scale); genomic organization of recombinant HSV vectors thereof (middle – rHSV-Rep2CapX, bottom – rHSV-GOI); resulting rHSV vectors (right).

Cap: AAV structural protein open reading frame; GOI: gene of interest in the rAAV backbone; ICP: Infection cell protein; IR: Inverted repeat; ITR: Inverted terminal repeat; L and S: long and short; Rep: AAV nonstructural protein open reading frame; rHSV-GOI: rHSV carrying rAAV-GOI (rAAV-transgene); rHSV-Rep2CapX: rHSV carrying the Rep2CapX-AAV cassette; TK: Thymidine kinase open reading frame; U: unit; X: AAV serotype 1–10.
reduced to 4:2 (rHSV-rep2/cap2 to rHSV-GFP) [67]. Optimal cell density for infection was established to be in the range between 1.6 x 10⁶ and 3.2 x 10⁶ c/ml and the optimal harvest time is 24 hours post-infection [67]. This process generates between 69000 ± 7500 and 11,300 ± 16,400 DNase Resistant Particles (DRP = equivalent to vector genome [vg])/c at a 10L scale (using WAVE reactors), which is equivalent to production yields obtained at spinner scale; average productivities of 2.4 x 10¹⁴ vg/L have been reported [67]. Scale-up has been performed to a 100 L reactor scale [68]. Moreover, it was shown that practically all assessed AAV serotypes could be produced with the HSV expression system: AAV1, AAV2 [65,67], AAV5, AAV8 [67], AAV9 [65].

Although replication-deficient HSV vectors are used for the production of AAV vectors, the generation of replication-competent HSV due to recombination events during production cannot be excluded wherefore the downstream processing protocol consisting of orthogonal purification principles must be validated for inactivation as well as removal of HSV. The established purification regime consisting of a detergent step, CIM Q followed by AVB affinity chromatography was able to clear at least 14.04 log10 of HSV [69]. Further advantages of this expression system is the absence of rcAAV generation and the in vivo vector potency of AAV1 in mice (DRP/IP ratio) being approximately five times higher when compared to AAV1 vectors generated by transient transfection [65].

More details on the development of the HSV expression system for the production of AAV vectors can be found in Clément et al [65].

Use of the baculovirus/insect cell system

A second infection-based production system for AAV vectors is the non-mammalian insect cell/baculovirus system, which is based on the use of mainly Sf9 cells (or their derivatives) grown in serum-free/animal-free medium in suspension culture allowing thus a very simple scale-up. This expression system was first developed by Urabe et al. [70]. They proposed the use of three different baculoviruses in which the three required functions were inserted into the classical polyhedrin locus. One baculovirus provided the cap-sequence under control of the very late baculoviral polyhedrin promoter in which the VP1 start codon ATG had been replaced by the less efficient ACG to reduce translational efficiency and thus allow the ribosomal machinery to scan down to the next low efficiency start codon ACG for VP2 expression and further down to the start codon ATG of VP3 for expression at high efficiency. The second baculovirus provided the cap-sequence under control of the truncated version of the early promoter E1 of the baculovirus and rep52 under control of the late baculoviral p10 promoter, both in a head-to-head orientation. And finally, the third baculovirus provided the recombinant rAAV construct with the transgene cassette flanked by the ITRs (Figure 4A). Production yields of 5 x 10⁴ vg/c have been reported for Sf9 cultures infected at a cell concentration of 1-2 x 10⁶ c/ml (MOI 1.6/baculovirus) and harvested at 72h pi [70]. Nevertheless, this first-generation baculovirus system was characterized by a main drawback – the instability of the rep-baculovirus because of the homologous recombination events.
between the rep52 and the rep78 sequence (the rep78 sequence contains the complete rep52 sequence) and loss of this part within five passages [71]. Furthermore, VP1 protein indispensable for the infectivity of AAV vectors [72] was expressed at suboptimal levels. Thus, three strategies were used for solving these problems:

- Smith et al. created the two baculovirus systems with the advantage of the reduction of the number of baculoviruses (to two) and tackling the instability issue of the rep-baculovirus by generating a unique sequence coding for rep78 and rep52 (Figure 4B). They mutated the start codon of the rep78 sequence into ACG and performed codon optimization for mutating the following nine in-frame ATGs into non-start codons; however, the ATG start codon of rep52 was retained [73]. With respect to the cap sequence, the only modifications consisted in codon optimization as carried out for the rep sequence. The rep and cap sequences driven by polyhedrin and p10 promoter, respectively, were put into a single baculovirus in a head-to-head position, with the rep cassette positioned in the clockwise lecture sense. Production levels of $7 \times 10^4$ vg/c have been reported; however, the expression levels of VP1 were still lower than for rAAV produced with the transient transfection system approach. Productions have been performed at a 200L scale using the dual baculovirus system [74].

- Chen kept three different baculoviruses; however, the rep and cap baculoviruses have been improved. He inserted an artificial intron harboring the polyhedrin promoter into the rep78 coding sequence at the p19 promoter region in order to express both rep proteins (78 and 52) from a single rep coding sequence. A similar solution was used for the cap baculovirus, meaning that an intron as used for the rep construct was inserted into the VP1 sequence upstream of the VP2 start codon. The original start codon (ATG) of VP1 was kept and ensured high expression levels of VP1, which transduced to two-fold higher AAV vector potency than when using ACG as start codon for VP1 (Figure 4C). This improvement was shown for

![FIGURE 4A-B](https://example.com/figure4a-b.png)

**FIGURE 4A-B**

Different rAAV and baculovirus constructions created in context of optimization of the baculovirus expression system.

(A) First-generation baculovirus system (adapted from [70]) makes use of three different baculoviruses: BacRep harbors Rep78 and Rep52 expression cassettes. Rep78 is under control of a truncated promoter for the immediate-early 1 gene of Orgyia pseudotsugata nuclear polyhedrosis virus (DIE1) and the expression of Rep52 is under control of the AcNPV polyhedrin (polh) promoter. BacCap expresses capsid proteins VP1, VP2 and VP3 under the transcriptional control of the polyhedrin promoter. The ATG codon of VP1 is mutated to ACG, enabling the expression of all three VP polypeptides from one transcript without splicing of mRNA. BacGFP carries a rAAV-GFP vector genome. The CMV or p10 promoter drives GFP expression in mammalian cells or insect cells. The whole expression cassette is flanked by AAV ITRs. pA, Polyadenylation signal. (B) The second-generation baculovirus system makes use of two different baculoviruses: the Rep and Cap proteins of AAV are expressed from two different baculovirus late promoters. Messenger RNA transcripts are represented by wavy lines. All pertinent codons are indicated. The modified AAV2 rep gene is under the transcriptional control of the baculovirus polyhedrin promoter ($P_{polh}$). The bifunctional rep mRNA transcript utilizes a CUG triplet embedded within a Kozak consensus sequence to direct synthesis of Rep78 polypeptides. A portion of ribosomal subunits does not initiate translation at the nonstandard start codon and scans to the next AUG start codon to initiate translation of Rep52 polypeptides. The AAV cap gene is under the transcriptional control of the baculovirus p10 promoter ($P_{p10}$). The three capsid proteins (VP1, VP2, and VP3) are translated from a single mRNA species, as described in A. The polyA sequences are derived from simian virus 40 (SV40 pA) and the herpes simplex virus thymidine kinase gene (HSV tk pA), respectively, as indicated. (B) Reproduced with permission from [73].
AAV serotypes 1, 2, 6 and 8, and vector yields ranging from $3.53 \times 10^{13}$ to $1.58 \times 10^{14}$ vg/L culture were obtained. This system has been evaluated at a 25L (working volume) scale using WAVE reactors [76]. These recombinant baculoviruses (Bac-inCap, Bac-inRep) were shown to be stable for at least seven passages [75].

Hermens et al. used a single rep-construct by replacing the original ATG start codon of rep78 by the suboptimal initiation ACG effecting partial exon skipping and thus expression of downstream rep52 protein [77]. Expression was controlled by a polyhedrin promoter. This construct stayed stable for at least five passages. As also carried out by Chen, the suboptimal expression of the VP1 protein was increased by the modification of the start codon (CTG instead of ACG) leading to VP1 expression levels as found for the traditional plasmid transfection system [78]. As for Chen [75], the triple baculovirus system was kept.

Production issues & further improvements

Although the insect cell baculovirus system is scalable, can produce in principle unlimited amounts of rAAV and is routinely used for the GMP production of vector lots for clinical as well as for commercial use, further improvements are required to ameliorate vector titer, quality and potency:

Increase in AAV vector titer

In the originally developed production system, Sf9 cells are infected at a cell density of 1–2 $\times 10^6$ c/ml [70]. In principle, it can be stated that at constant specific production rate the final vector titer is directly related to the cell concentration in a given cell culture system. This would signify that a simple increase in the cell concentration should lead to a corresponding increase in vector titer. However, as for many other viral systems, the baculovirus expression system is also characterized by the so-called ‘cell density effect’ [79–83], which implies that beyond a certain cell density, a further increase in cell concentration does not lead to further increase in vector titer. This can be explained by lack of certain essential medium components, because Mena et al. could show that the feeding of high-density cultures 24 hours before, at and 24 hours after virus infection with a medium concentrate could maintain the
specific vector production rate up to an infection cell density of $10 \times 10^6$ c/ml [84]. However, it should be noted that at these cell numbers the infections can only be performed with low MOIs (e.g., 0.05 per baculovirus) because at high MOIs, the culture would be too much diluted with spent medium from the baculovirus production culture. However, it is important to note that only 60–70% of the cells will be infected with the two different viruses [85], meaning that part of the cells will not produce functional rAAV vectors.

### AAV vector quality & potency

Different biological parts of the insect cell/baculovirus system could probably be optimized for enhancing vector quality and potency. With respect to the choice of the ITRs, Dickx et al. [86] could show that the use of complete wild-type ITRs (FLIP – FLOP) instead of the use of truncated versions of FLOP ITRs (SUB201-ITRs) largely used without negative impact in context of the transient transfection-based production system [87-89] led to an increase in the full-to-empty particle ratio (increase from 10 to 40%) and a reduced (approximately ten-fold) encapsidation of baculovirus DNA sequences. Noordman et al. showed that the encapsidation of baculoviral DNA sequences could be reduced via mutation of the rep-DNA sequence when producing rAAV5 vectors [90]. These mutations led to three- to five-fold, five- to 13-fold, and 25–52-fold reduction in encapsidation of left ORF (603), right ORF (1629) and HR3 residual baculoviral DNA, respectively. In parallel, the use of some of these mutated rep proteins led to increased vector titers.

Baculoviruses contain the cathepsin gene (a cysteine protease), which works in tandem during baculovirus infection of lepidopteran larvae to achieve dissemination of the occluded form of the baculovirus progeny [91,92]. Though this is important for the wild-type virus, the presence of an active protease during the production of recombinant proteins can be a drawback. In this context, Galibert et al. demonstrated that the VP1 of certain AAV serotypes (1, 6, 8) is cleaved during vector production whereas this was not observed for other serotypes (2, 9, rh10) [93]. Either use of the cathepsin-specific protease inhibitor E64 [91] or the disruption of the cathepsin gene led to AAV8 vector preparations with intact VP1 capsid proteins. It was shown that the in vitro and in vivo infectivity of the AAV8 vectors was two- to four-fold increased in comparison to those produced using baculoviruses with an intact cathepsin gene.

Although these are only some of the recent developments for improving the Sf9/baculovirus system for AAV production, there are a lot of further possible improvements to be made; this concerns in particular, the timely regulation of the expression of the AAV rep and cap genes and the baculovirus helper functions.

### An additional reduction in the number of baculoviruses leads to a single baculovirus system

The initial baculovirus system as published by Urabe et al. (2002) was based on three different baculoviruses [70], which could be reduced to two baculoviruses [73]. However, the ultimate improvement and in the same time simplification from a user’s point of view consists in the
use of a single baculovirus for inducing AAV vector production (at low MOI all cells would receive the whole set of genes for producing AAV vectors – compare with Rivollet et al. [85]). Two approaches have been developed for achieving this objective:

**MONOBAC system**

The first approach is the MONOBAC system, which is based on the fact that several of the genes of a baculovirus bacmid are transcriptionally very active but not essential for in vitro use of the baculovirus. Thus we have decided to place the rep-cap construct developed by Smith et al. [73] into the EGT locus and kept the classical polyhedrin locus for the insertion of the recombinant AAV vector cassette (the transgene cassette flanked by the two ITRs) (Figure 4D). The combination of the rep-cap functions and the rAAV vector construct in one single baculovirus was only possible because the expression of the rep proteins is driven by the very late polyhedrin promoter, which is only active after replication of the baculovirus DNA precluding thus the eventual excision of the vector sequence from the baculovirus genome. The replacement of the dual baculoviruses by the monobaculovirus for AAV vector production led to a two- to four-fold increase in vector titers [95].

**OneBac system**

The second single baculovirus system is the OneBac system whose first generation was published by Aslanidi et al [96]. The principle of this system is relatively simple. The producer cell line (Sf9) harbors stably integrated the rep and cap functions, which are amplified and expressed upon infection with the recombinant baculovirus providing the rAAV genome. The rep and cap expression cassettes have been constructed using an identical approach: they consist of homologous region (hr) 2 followed by the AAV rep binding element (RBE) a stuffer sequence and finally the rep or cap sequence, the expression of both driven by the very late baculovirus polyhedrin promoter. The expression is only induced after infection with baculovirus via the binding of the immediate-early trans-regulator 1 (IE-1) to the hr2 target sequence inducing expression via a combination of the amplification of integrated resident genes (up to 1200 copies per cell) and the enhancement of the expression. Furthermore, the expression of rep78 leads to the boosting of the integrated genes (via integration with the RBE). This system leads to a ten-fold increase in AAV vector production in comparison to the original baculovirus system – up to a specific production of 5 x 10^5 vg/c (rAAV2) exceeding typical yields of current rAAV production systems. However, it could also be established that probably due to this amplification a considerable percentage of AAV particles containing rep and cap sequences were detected (our own unpublished results, [97]). Thus, this first generation could not be further improved for an eventual GMP application. Mietzsch et al [97] developed the second-generation OneBac system (OneBac 2.0), in which in the first line the RBE element responsible for the encapsidation of high frequencies of rep and cap sequences into AAV capsids was removed (Figure 4E, left panel) leading to a functional production system characterized by a reduced but sufficient amplification of the
rep and cap genes (up to 65-fold in comparison to 200–500-fold in the clones containing the RBE sequence). Mietzsch et al. showed that these second-generation cells generate AAV vectors with a frequency of 0.001 and 0.02% of AAV particles containing rep and cap sequences, respectively [97]. This is equivalent to values for encapsidated cap sequences reported in the case of a transfection-based production system [98]. A further modification with respect to the first-generation OneBac system was the insertion of an intron into the cap construct (as already carried out by [75]) and the use of the original AUG start codon (instead of ACG) for VP1 for increasing the insufficient VP1 expression in the first-generation OneBac system (Figure 4E, center and right).

**FIGURE 4D**
Different rAAV and baculovirus constructions created in context of optimization of the baculovirus expression system.

MONOBAC – bacmid, with the rAAV vector sequence (vector cassette flanked by two ITRs) inserted into the polyhedrin locus of the bacmid, and the rep-cap construct (as presented in Figure 4B) inserted into the EGT locus. Adapted from [94].
This modification led to increased VP1 expression levels in the range of 10% of VP3 expression levels. This was of particular importance for the production of high-potency AAV5 vectors, which showed insufficient VP1 expression levels in the case of the first-generation OneBac system [99]. AAV5 vector yields of approximately 10⁵ vg/c have been reported [97]. This second-generation OneBac system should now also be developed for the other AAV serotype as it had been carried out for the first-generation OneBac system [99].

General ‘pseudotyping’ issues

In the vast majority of studies a pseudotyping strategy has been adopted for simplicity reasons, meaning that only the cap sequence was from a desired serotype, whereas the ITRs as well as the rep sequences were derived from AAV2 [100]. Although this represents a considerable simplification of production of different AAV serotypes because of the simple switch from one capsid sequence to another one, it is not necessarily the best way to produce high vector amounts of elevated quality. In the context of the optimization of the quality and quantity of AAV vector production, Ling et al. generated AAV3b vectors using a completely homologous expression system based on the use of rep protein and ITRs sequences derived from AAV3b [101]. They could show that when using this homologous expression system (in a transient transfection context) four-fold higher titers as well as four-fold higher transduction efficiencies of a human hepatocellular cell line in vitro were obtained in comparison to the traditional heterologous system characterized by the use of rep and ITR sequences derived from AAV2. Thus it could be inferred that the use of rep and ITR sequences homologous to the capsid serotype might also be a way to improve vector titers and efficacy of other AAV serotypes.
Comparison of different production systems

Relatively few comparisons have been performed between AAV vectors produced with the different production systems. Based on published data, Table 1 compares the different production systems (excluding the packaging cell line-based system because up to now no real scale-up has been performed for this system) with respect to specific production rate, the largest production scale established up to now, the vector amount produced per culture litre using standard conditions as well as some safety parameters, including the generation of rcAAVs, the percentage of full vector particles before purification and the encapsidation of rep and cap sequences as far as communicated.

With respect to the specific vector yield per cell, all expression systems as well as the transfection system-based on a selected HEK293 cell clone adapted to suspension growth are comparable and the specific yield ranges between 5 x 10^4 and 2 x 10^5 vg/c. For all production systems it can be stated that real scale-up is only possible when using a suspension culture. However, this has to be relativized for the HEK293(T) cell-based transfection process for which also an adherent production process using a fixed bed reactor (e.g., iCELLis® reactor) might be used. Furthermore, it has to be indicated here that fixed bed reactors are also well adapted to viral infection based production systems [20,102] although not yet shown for AAV production.

In principle, all production systems generate vector titers ranging from 5 x 10^13 to 2.4 x 10^14 vg/L or DRP/L. An exception in this context seems to be the baculovirus system, for which different production levels have been reported for various AAV serotypes. While for the production of AAV2 and AAV7m8, similar production levels have been reported for the baculovirus system and the HEK293 cells-based transfection system; production levels for AAVrh10 were approximately two times lower for the baculovirus system [103]. In the specific case of AAV8, the baculovirus system underperformed by a factor of 5–10x [104] or generated non-functional AAV8 vector capsids devoid of VP1 when produced at large scale (200 L) [105]. These observations clearly infer that in the case of the baculovirus expression system no generalized view is permitted, and production and functionality issues have to be assessed for a chosen serotype.

Attention has to be paid to the fact that the differences in the production levels (when comparing different production systems) can be explained by different production cell densities and specific production rates. However, it should also be kept in mind that the titration methods were not harmonized/standardized making a real comparison challenging.

With respect to genetic stability, the used biological raw materials have to show stability exceeding that required for the whole process. This concerns the producer cells as well as viral seed stocks. In this context, genetic stability of HeLa-based stable producer cell lines has been demonstrated for more than 60 population doublings [45] and the recombinant viruses for the HSV and the baculovirus-based production systems have been shown to be stable for 13 [106] and seven [73] successive passages, respectively. Furthermore, no
**TABLE 1**

Comparison of different rAAV production systems with respect to specific vector production rate, volumetric production level, largest scale established as well as several safety aspects (generation of rAAV, ratio of full to empty particles, level of encapsidated rep, cap, and HSV DNA).

<table>
<thead>
<tr>
<th>Production system</th>
<th>Biological system used</th>
<th>Cell specific production rate (vg/c)</th>
<th>Volumetric production (vg/L)</th>
<th>Largest scale used</th>
<th>rcAAV production</th>
<th>Percentage of full particles (%)</th>
<th>Encapsidated rep/cap, HSV sequences (%)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid transfection</td>
<td>Selected suspension adapted HEK293 clone</td>
<td>1-2x10^5 (AAV2)</td>
<td>10^14</td>
<td>20L (WAVE)**</td>
<td>+*</td>
<td>5–50</td>
<td>Rep: 0.3–1.5 [33] Cap:0.4–1 [130]; 0.016–0.024 [98]</td>
<td>[28]</td>
</tr>
<tr>
<td>Stable cell line</td>
<td>HeLa (rep-cap/AAV vector), infection with wt adenovirus 5</td>
<td>&gt;5x10^4***</td>
<td>&gt;5 x 10^13</td>
<td>250L (STR) → 2000L (STR)</td>
<td>Below detection limit</td>
<td>&gt;50</td>
<td>0.02–0.05 (rep, cap) HSV: 0.007-0.012</td>
<td>[45], [61], [60]</td>
</tr>
<tr>
<td>Herpes simplex type 1</td>
<td>Suspension adapted BHK cells, infection with 2 viruses (rHSV-rep2cap/rHSV-RAAV vector), MOI=4/2</td>
<td>6.9 x 10^4 to 1.13 x 10^5</td>
<td>2.4 x 10^14 (AAV1)</td>
<td>25L (WAVE) → 100L (AAV1)</td>
<td>Below detection limit</td>
<td>&gt;70</td>
<td>97.55 ± 0.24% [131]</td>
<td>[63], [67], [64]</td>
</tr>
<tr>
<td>Baculovirus system</td>
<td>Sf9, infection with 2 viruses (Bac-rep2/capX/Bac-RAAV vector), MOI=0.05/0.05 – 1.6/1.6</td>
<td>10^4 to 10^5</td>
<td>9.4 x 10^13 (AAV1)</td>
<td>200L (STR, WAVE)</td>
<td>Below detection limit</td>
<td>10–40%*** (AAV8) [86] &gt;70% (AAV2) [103]</td>
<td>Cap: 0.016 Rep: 0.019-0.024 [76]</td>
<td>[12], [74]</td>
</tr>
<tr>
<td>OneBac 2.0 system</td>
<td>Stable Sf9 cell line (rep-cap), infection with 1 virus (BAC-RAAV vector), MOI=5</td>
<td>~10^5 (AAV5)</td>
<td>1.4 x 10^15</td>
<td>Small scale**</td>
<td>Below detection limit</td>
<td>No information</td>
<td>Cap: 0.02 Rep: &lt;0.001</td>
<td>[97]</td>
</tr>
</tbody>
</table>

* Depending on the plasmid system used (compare with Emmerling et al [23]).
** Large production scale possible.
*** Selection criteria.
**** Depending on the ITR construct.
generation of rcAAV has ever been reported for all expression systems (below limit of detection of the assay system used) except for the transfection-based production process for which recombination events can generate rcAAV [107].

From a practical point of view, all suspension-based productions can be performed either in WAVE-type or stirred tank reactors, with a certain advantage for the stirred tank reactor due to the easier scale-up (which is theoretically unlimited) as well as the possibility to infect cultures at higher cell densities than $10^6$ c/mL due to the better mass transfer features of stirred tank reactors. While the maximal scale of the WAVE reactor is limited to 200–250 L, because at larger volumes, mass transfer becomes critical and thus limiting, stirred tank reactors for animal cells have been scaled up to reactor sizes with 20,000L working volume [108]. Today reactor scales of 200–250 L are routinely used for the production of clinical-grade AAV vectors and the use of a 2000 L reactor scale is under implementation [45,61]. It should be added here, that the HSV and the baculovirus-based production systems should in principle also be scalable although the modification of the physico-chemical parameters (e.g., shear stress) and their impact on cellular metabolism during scale-up to larger scales merits consideration wherefore WAVE type reactor systems might be more adapted.

Side-by-side comparisons of different AAV expression systems with respect to in vivo transduction efficiency are rarely performed. In this context, Zhang et al. compared the efficiency of transduction of striatum and cortex tissue by AAV2 vectors produced with the herpes simplex, the baculovirus system (as developed by Chen [75]), and the HEK293-based transfection process [109]. Similar transduction efficiencies as well as cellular tropism were observed for the AAV2 vector particles produced with the three expression systems, signifying that the different production systems are apparently able to generate AAV vectors of comparable efficiency. By comparing different AAV serotypes (2, 7m8, rh10) Ramirez et al. could also show that these serotypes produced with the baculovirus expression system (as developed by Chen 2008) or via triple transfection of HEK293 cells had comparable in vitro and in vivo potency [103]. With respect to AAV vector particles generated using the HSV expression system, Kang et al. and Adamson-Small et al. showed that the potency of HSV expression system derived AAV particles was superior to that of AAV particles produced via a transfection-based process [65,66]. This concerned AAV serotypes 1 and 9, respectively.

These comparisons infer very clearly that at this moment it is impossible to provide a clear statement on the quality and capacity of a given AAV expression system versus another system because insufficient data available for comparisons and in most cases a direct comparison is not possible because different serotypes and vector constructs have been used not allowing a real comparison.

Purification

There are several means for purifying AAV vectors, starting with traditional density gradient ultracentrifugation using CsCl or iodixanol [110,111] going up to the novel affinity chromatography gels initially developed by BAC/GE Healthcare (AVB chromatography) and further
improved by Invitrogen for specific AAV serotypes (POROS CaptureSelect AAV8 Affinity Matrix and POROS CaptureSelect AAV9 Affinity Matrix). Different aspects of AAV purification have been described previously (e.g., [111,112]) and do not need another repetition. Therefore only two issues concerning downstream processing of AAV vectors are discussed here.

Availability & implementation of novel affinity chromatography matrices

The appearance of novel affinity matrices specific for AAV8 and AAV9 have allowed the development of a highly efficient and vector specific purification protocols consisting of clarification, affinity chromatography, tangential flow filtration and eventually a gel filtration step for formulation purposes. AVB column chromatography was initially developed for the affinity purification of AAV2 [125] and was reported by the manufacturer to capture AAV serotypes 1, 2, 3 and 5. Recently, Mietzsch et al [99] communicated that AAV1-8, AAVrh.10 and AAV12 could be purified from crude cell lysate, whereas this was not possible for AAV9 and AAV11. Later, Wang et al. established that AVB affinity chromatography column had a high affinity towards AAV3B, AAVrh.10 and AAVhu.37 and a low affinity/absence of affinity towards AAV8, rh.64R1 and AAV9 [128]. Since in particular for the purification of AAV8 and AAV9 vectors, AVB chromatography was not or only partially adapted and since both serotypes are of high interest for clinical use, two new matrices with specific llama antibody ligands (POROS Capture Select Affinity Matrix) have been developed. In the case of purification of AAV8 vectors, this novel support required an approximately ten-fold reduced support volume due to the increased ligand density and led to an increase in the overall yield from 20–30% to approximately 60%. A similar overall recovery yield was communicated for AAV9 vectors [126]. This represents an interesting improvement with respect to cost of goods and operator time per run.

One issue related to the use of affinity chromatography based on the use of these new affinity ligands is the fact that full particles cannot be separated from empty particles, meaning that additional steps, including ion-exchange chromatography or ultracentrifugation, are required when empty AAV particles have to be removed (see below).

Full–empty AAV particle issue

A specific issue is the presence of empty particles in vector preparations, which are almost always co-produced. In the case of the transient transfection-based production system, empty capsids are produced at a high frequency going up to 50–95% of the total particle count [113]. In order to separate empty from full AAV particles, the Center for Cellular and Molecular Therapeutics at the Children Hospital of Philadelphia used a downstream processing protocol including an ion-exchange chromatography as capture step followed by a CsCl ultracentrifugation for manufacturing of GMP vector lots for clinical studies [114]. For instance, this protocol was used for generation of vector preparations used for the different clinical trials performed in Philadelphia: ssAAV2-FactorIX for hemophilia B treatment [115] or AAV2-hRPE65v2 for treatment of Leber’s congenital amaurosis [116,117]. On the other
hand, the vector preparation used for the scAAV8-FactorIX trial contained an elevated level of empty particles, which was estimated to exceed the full particles ten-fold [1]. In both cases, efficient vector transduction was observed although only transiently for the hemophilia B trial performed with ssAAV2 vectors free of empty capsids. Thus to date, vector preparations containing only full vector particles on one side and preparations with mostly empty particles (10% of full particles) have been used for clinical studies. With respect to the presence of a percentage of empty AAV vector capsids it could be shown that the presence of empty vector particles was beneficial for neutralizing anti-AAV antibodies present, which would completely block transduction. This absorbance happened in a dose-dependent manner [118]. However, on the other hand, Gao et al. showed in two mouse models, that the presence of empty vector particles had a negative impact on liver transduction in a dose-dependent manner [119]. The conclusions from both studies are the following: the vector preparation should be devoid of empty particles for avoiding the competitive situation of empty and full particles for the same target. However, as shown by Mingozzi et al. [118] the presence of empty capsids can be beneficial for absorbing eventually present anti-AAV antibodies. Thus in order to avoid this competitive situation, they could show that use of a mutant AAV capsid able to interact with the anti-AAV antibodies but unable to interact with the cellular receptor could be a solution: removal/absorption of anti-AAV antibodies without competing for the cellular target sites of the functional AAV vector with the transgene to be transferred. In principle, such an approach can be developed via the use of AAV preparations, which contain only full particles to which a certain percentage of these AAV particles with a mutant capsid have been added.

As mentioned, at research scale, the separation of full and empty AAV particles is easily performed via CsCl [110,111] or iodixanol [120,121] gradient centrifugation; whereby iodixanol is preferred because it is non-toxic – thus no extensive dialysis is required as when CsCl is used, and iodixanol prevents aggregation of rAAV particles and does not reduce infectivity [120,127]. Although this separation step can be used at later stages of purification schemes, it is not a scalable purification step. Apart from the use of production methods reducing or avoiding the production of empty particles, the only other way to separate full from empty particles is the use of column chromatography. Since it could be established that the isoelectric point of empty particles is significantly higher than of full particles, probably due to the absence of DNA in the empty particles [124], it is possible to separate them using ion-exchange chromatography. This was shown by Davidoff et al. [123] and by Okada et al. [124] who developed a two stage ion-exchange chromatography process based on a strong cation-exchange column (Mustang S), which retains only empty particles whereas full particles are found in the flow through, this is followed by a strong anion-exchange column (Mustang Q), which retains full AAV particles, which can then be eluted using a salt gradient/step gradient. Using this method, Davidoff et al. [123] generated AAV8 vector preparations with only 5–10% of residual empty vector capsids, whereas Okada et al. [124] used a
similar protocol for the purification of AAV1 vectors contaminated only by 0.8% of empty particles. Finally, based on the same principle – differences in the isoelectric point of full and empty particles – Qu et al. also used a two-step protocol with a first cation-exchange chromatography, however, which retains full as empty particles [122]. The eluted material was charged to a Q-Sepharose anion exchanger to separate full from empty particles at elevated pH using a very shallow salt gradient. Using this protocol, the empty particle load could be reduced by 86-fold with approximately 20% of residual empty particles in the final vector preparation. This method was also able to separate empty particles of AAV6. The separation of empty form full particles of different serotypes is feasible but has to be optimized by selecting different elution conditions, choice of salt and type of resins. It should also be mentioned here that to date, nobody has shown a large-scale application of this separation method.

TRANSLATIONAL INSIGHT
Different scalable manufacturing systems have been developed for the large-scale production of AAV vectors for research, development, clinical evaluation and routine use for the treatment of rare diseases. Although most of the production-related issues have been settled today (e.g., large-scale production is possible using different biological systems), there are still several issues that need solutions. These are the large-scale production of vector preparations free of empty AAV particles including scalable separation methods for removing empty AAV particles, the question of the treatment of patients who are positive for anti-AAV antibodies because of a previous AAV infection or previous treatment with AAV vectors, as well as the increase of vector potency, which is generally low. An improvement in vector potency will have a direct impact on the vector dose to be administered and thus also on the vector quantities needed as, for instance, could be shown for AAV vectors with an improved percentage of functional VP1 protein (e.g., [93]). A further improvement of the AAV production will consist in the establishment of the routine use of homologous AAV systems since it has been shown that the use of such an homologous system leads to titer and potency increase [101]. Furthermore, the implementation of next-generation sequencing will provide a great deal of information on the packaged vector genome, as well as on the encapsidated DNA sequences from different origins (cellular origin, helper virus origin) (e.g., [129]) and will be a crucial tool for process development as well as optimization purposes. At the end of the day, these and further improvements will lead to more efficient production processes and a high-quality vector system with improved biosafety.

FINANCIAL & COMPETING INTERESTS DISCLOSURE
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Advances and challenges in the use of recombinant adeno-associated virus vectors for human gene therapy

Arun Srivastava

Recombinant vectors based on a non-pathogenic parvovirus, the adeno-associated virus (AAV), have taken center stage in the past decade. The well-established safety of AAV vectors in 162 Phase I/II clinical trials (and one recent Phase III trial) in humans to date, as well as their clinical efficacy in several human diseases, are now well documented. Despite these remarkable achievements, it is becoming increasingly clear that the full potential of AAV vectors composed of the naturally occurring capsids is unlikely to be realized. In this Expert Insight article, I will describe the advances that have been made, and the challenges that remain, in the optimal use of AAV vectors in human gene therapy applications. I will also attempt to provide additional avenues of research and development that could be pursued in order to further ensure both safety and efficacy of AAV vectors in targeting a wide variety of human diseases, both genetic and acquired, in the not-too-distant future.

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Adeno-associated virus 2 (AAV2), the most extensively studied prototype, is a small, naked icosahedral virus, which was first discovered in 1965 [1]. Approximately 90% of the human population is seropositive for AAV2 antibodies [2], implying that most humans have been exposed to the wild-type AAV2 (WT AAV2), yet there is no conclusive evidence that AAV2 infection leads to any known disease in humans, although there has been a recent report claiming that the WT AAV2 is the etiologic agent of hepatocellular carcinoma (HCC) [3], a claim that has been seriously questioned by us and others [4,5]. Recombinant vectors based on AAV2 (rAAV2), on the other hand, have been, or are currently being, used in a number of Phase I/II clinical trials, and thus far, no serious adverse events, much less cancer of any type have ever been observed.
or reported [6]. Furthermore, the use of rAAV2 vectors has led to clinical efficacy in the potential gene therapy of at least three human diseases: Leber’s congenital amaurosis (LCA) [7–10], aromatic L-amino acid decarboxylase deficiency (AADC) [11] and choroideremia [12]. In the past decade, at least 12 additional AAV serotype vectors, some derived from non-human primates, have also become available [13–21]. rAAV1 vectors have successfully been used in the gene therapy of lipoprotein lipase deficiency [22], and rAAV8 vectors have shown clinical efficacy in the potential gene therapy of hemophilia B [23,24].

Despite these remarkable achievements, I have argued that the first generation of rAAV vectors are unlikely to reach their full potential until we gain a better understanding of how rAAV vectors interact with the target cell, and have also posited that the WT AAV did not evolve to be used as a vector for the delivery of therapeutic genes [25]. A brief historical account follows.

VECTOR–HOST CELL INTERACTIONS: DISCOVERY OF THE CELLULAR RECEPTOR & CO-RECEPTORS FOR AAV2 & ITS IMPLICATIONS IN GENE THERAPY

AAV2 was discovered in 1965 [1], but for nearly three decades, it was generally assumed that infection by AAV2 was non-specific, because all cell types across the species barrier could be infected by AAV2. However, in 1996, we identified a human megakaryocytic leukemia cell line, MB-02, that could not be infected by the WT AAV2, or transduced by recombinant AAV2 vectors [26]. This observation prompted us to suggest that AAV2 infection of human cells is receptor-mediated. Indeed, Summerford and Samulski identified heparan sulfate proteoglycan (HSPG) as the first cellular receptor for AAV2 in 1998 [27]. The identification of HSPG as a cellular receptor provided an explanation as to why AAV2 infects all cell types across the species barrier since all cells express HSPG. Interestingly, we documented that MB-02, and a second human megakaryocytic leukemia cell line M07e, lack HSPG expression [26]. The discovery of the cellular receptor for AAV2 also provided the explanation as to why the very first Phase I clinical trial with AAV2 vectors for the potential gene therapy of cystic fibrosis, performed by Flotte and colleagues [28], did not show clinical efficacy since human airway epithelial cells express HSPG predominantly on the baso-lateral surface, rather than on the apical surface, and as a consequence, are not efficiently transduced by AAV2 vectors [29]. Thus, these observations further reinforced the value of basic science of AAV biology, with direct implications in the use of AAV vectors in human gene therapy.

Soon after the discovery of HSPG as the cellular receptor for AAV2, which is required for binding of AAV2 to the cell surface, we observed that HSPG alone was insufficient to mediate viral entry into cells. In 1999, we reported the identification of human fibroblast growth factor receptor 1 (FGFR1) as the first cellular co-receptor, which AAV2 utilizes to gain entry into cells [30]. In addition, Summerford et al. identified αVβ5 as yet another co-receptor for AAV2 [31].
However, when Chen et al. isolated AAV sequences from various tissues, predominantly tonsils, from children, they observed that although 7% of these 'AAV2-like' sequences shared ~98% identity with the WT AAV2, they lacked the HSPG-binding site, and consequently, failed to bind to the cellular receptor [32]. These authors concluded that AAV2 either utilizes other putative cellular receptors as well in vivo, or the use of HSPG is a consequence of long-term propagation of AAV2 in tissue culture in vitro. Regardless, the use of rAAV2 vectors, from which the HSPG-binding domain has been deleted, transduce murine brain and retinal tissues more extensively than their unmodified counterpart [33,34]. Furthermore, AAV2 has been shown to utilize at least four additional cellular co-receptors – hepatocyte growth factor receptor (HGFR) [35], α5β1 integrin [36]; laminin receptor (LamR) [37]; and CD9 [38] – in addition to FGFR1 [30] and αVβ5 [31], for viral entry. Thus, these studies have yielded a much clearer picture of AAV2–host cell interactions, none of which was available when the first clinical trial for the potential gene therapy of cystic fibrosis was pursued in 1996 [28]. In addition to AAV2, a number of additional AAV serotypes have since become available [13–21]. To date, at least 13 distinct AAV serotype vectors (AAV1 – AAV13) have been described, and it is highly likely that this number will continue to grow. The ten most commonly used AAV serotype vectors are depicted schematically in Figure 1. Although the precise mechanism of transduction by these AAV serotype vectors in vivo remains unknown, in general, it has been observed that AAV1 – AAV6 serotype vectors transduce tissue culture cells to various degrees of efficacy in vitro, and by and large, AAV7 – AAV10 serotype vectors transduce various tissues and organs efficiently in experimental animal models in vivo. There are only limited data on the transduction efficiency of AAV12 and AAV13 vectors.

Although it is clear that attachment to putative cell surface receptors is the initial step for successful transduction by each of the AAV serotype vectors, and the following 23 different glycan receptors have been identified: α2-3 and α2-6 N-linked sialic acid (SIA) for AAV1 [39,40]; HSPG for AAV2, AAV3 and AAV13 [21,27,41]; α2-3 O-linked and α2-3 N-linked SIAs for AAV4 and AAV5, respectively [42–44]; HSPG and α2-3 and α2-6 N-linked SIA for AAV6 [40,45,46]; and terminal N-linked galactose (GAL) of SIA for AAV9 [47,48]. The primary cellular receptors for AAV7, AAV8, AAV9, AAVrh10, AAV11, AAV12 and AAV13 serotypes have not yet been identified. More recently, a trans-membrane protein, designated as an essential receptor for AAV2 infection (AAVR) was identified, which was shown to bind directly to AAV2, and was found to be a critical factor for infection by several AAV serotypes [49]. Thus, AAVR was reported to be a universal receptor for AAV infection, but what role, if any, AAVR plays in large animal models, and especially in humans, remains to be evaluated. It is clear, however, that binding to the primary cellular receptors is unlikely to be sufficient for AAV serotype vectors for gaining entry into cells, and most likely, additional cell surface as co-receptors are required. The following cellular co-receptors have been identified thus far: FGFR1 [30], αVβ5 [31]
and α5β1 integrins for AAV2; a putative integrin for AAV9 [50]; FGFR1 for AAV3 [51]; HGFR for AAV2 [35] and AAV3 [52]; platelet-derived growth factor receptor (PDGFR) for AAV5 [53]; epidermal growth factor receptor (EGFR) for AAV6 [54]; and laminin receptor (LamR) for AAV2, AAV3, AAV8 and AAV9 [37]. Based on these studies, the tissue-tropisms of AAV serotype vectors have been determined, which are also indicated in Figure 1. However, for the most part, a large body of our current knowledge of AAV vector tropism has been derived from studies with rodent models, which, in my opinion, are poor surrogates for humans [55]. Here, I will provide one specific example to corroborate my contention.

In 1997, we first reported the liver tropism of rAAV2 vectors, following intravenous administration, in a murine model in vivo [56], an observation that was subsequently replicated by other groups [57,58]. Based on those studies, a Phase I clinical trial for hemophilia B was carried out with rAAV2 vectors expressing the human clotting factor IX (h.FIX) [59]. Even though in

FIGURE 1

The most commonly used recombinant AAV serotype vectors and their tissue-tropism.
pre-clinical studies with both hemophilic murine and canine models, rAAV2-FIX vectors provided complete phenotypic correction of the disease for the entire lifespans of these animals, the predicted dose of these vectors in humans did not express therapeutic levels of FIX in humans. Although the administration of a ten-fold higher vector dose did lead to expression of therapeutic levels of FIX in one patient, it was short-lived due to the host immune response to AAV2 capsid proteins [59]. The lesson learned from this first liver-directed gene therapy trial was that AAV2 serotype vectors, although effective in mice and dogs, were not optimal for humans.

On the basis of subsequent studies with rAAV8 serotype vectors, which established the far superior efficacy of these vectors in murine hepatocytes, compared with rAAV2 vectors, rAAV8-FIX vectors were used in a second Phase I clinical trial in patients with hemophilia B [23]. Although this landmark trial with rAAV8 vectors has been deemed highly successful [24], we raised the issue of whether AAV8 is really the optimal serotype for human hepatocytes. Nearly a decade ago, we identified the AAV3 serotype (which was largely ignored by the AAV community because it fails to transduce any cell/tissue/organ in mice) as the most efficient vector for transducing human hepatocytes, both malignant and primary [60].

We later discovered the basis of the selective tropism of AAV3, which was due to the use of HGFR as a co-receptor by AAV3 [52]. Although human and mouse HGFRs share 88% identity, there are four amino acids in the extracellular domain of human HGFR that AAV3 recognizes and binds to, which are different in the mouse HGFR. These studies were subsequently extended to include murine xenograft models to establish the remarkable specificity and efficacy of AAV3 vectors [61,62].

Interestingly, human and non-human primate HGFRs share 99% identity, and in our recent studies, we were able to achieve selective and high-efficiency transduction of NHP livers, both short-term (7 days) and long-term (91 days), following intravenous delivery of rAAV3 vectors, with no apparent toxicity at a relatively high dose of $1 \times 10^{13}$ vgs/kg [63]. These studies were corroborated by Wang et al. [64], which further established the remarkable specificity, efficacy and safety of AAV3 vectors [63,64].

In our more recent studies with humanized mouse models, we have reported that rAAV3 vectors are approximately eight times more efficient than rAAV8 vectors, and approximately 82 times more efficient than rAAV5 vectors (the two serotypes that are currently being used in liver-directed gene therapy in humans), in transducing primary human hepatocytes [65]. Thus, my prediction is that, compared with rAAV8 and rAAV5 vectors that are currently being used (Table 1), rAAV3 vectors will prove to be far more efficacious in targeting human liver diseases in general, and gene therapy of hemophilia in particular.

THE WILD-TYPE VERSUS RECOMBINANT CAPSIDS: IMPLICATIONS IN HOST IMMUNE RESPONSE & GENE THERAPY

In 2001, I had emphasized not only the importance of gaining a better
understanding of underlying mechanism of AAV–host cell interactions, but also need to develop the more efficient, next generation of AAV vectors [66]. Whereas the former appeared to be relatively straightforward to achieve eventually, it was not readily apparent precisely how the latter could be accomplished. As it turned out, the first clue was available from our studies published in 2000 [67], in which we observed that only ~20% of the input rAAV2 vectors gain entry into the nucleus, whereas ~80% fail to escape the endosome in the cytoplasm. Duan et al. subsequently reported that AAV2 capsids become ubiquitinated in the cytoplasm, where they are targeted for degradation by the host cell proteasomal machinery [68].

In 2006, the results of the first Phase I clinical trial for the potential gene therapy of hemophilia B with the first generation of rAAV2 vectors were reported [59]. As described above, at low (8 x 10^{10} vgs/kg), and medium (4 x 10^{11} vgs/kg) vector doses, rAAV2 vectors failed to express FIX in two patients. At the high dose (2 x 10^{12} vgs/kg), rAAV2 vectors did lead to expression of therapeutic levels of FIX in one patient, but it was short-lived due to the host immune response to AAV2 capsid proteins. Following uptake of AAV2 vectors by dendritic cells, and then proteasomal degradation of capsid proteins, led to activation of AAV2 capsid-specific CD8+ memory T cells, which in turn, led to the destruction of transduced hepatocytes and consequently, the loss of FIX levels in this patient [69]. Thus, to a certain extent, these studies provided an explanation as to why a high dose of rAAV2 vectors induced a host immune response against the capsid proteins.

Since it appeared that the observed immune response correlated directly with the AAV2 vector dose, we pondered the following two questions: (i) Why is such a high vector dose needed to achieve therapeutic levels of FIX? and (ii) What can be done to reduce the vector dose at least ten-fold, and yet achieve therapeutic levels of FIX? As stated above, the answer to the first question came from our studies published in 2000, where we documented that ~80% of the input rAAV2 vectors fail to gain entry into the nucleus [67], as they are targeted for degradation by the host cell ubiquitination/proteasomal machinery [68]. The answer to the second question was predicated on our hypothesis that if we could circumvent the ubiquitination/proteasome pathway, it might be feasible to achieve more efficient nuclear transport of rAAV2 vectors, which, in essence, would allow for a reduction in the vector dosage. Serendipitously, we had previously observed that inhibition of the host cell EGFR protein tyrosine kinase (EGFR-PTK) resulted in a significant increase in the transduction efficiency of rAAV2 vectors [70]. Thus, we hypothesized that following infection, the AAV2 capsid protein becomes phosphorylated at surface-exposed tyrosine residues by EGFR-PTK, and that tyrosine phosphorylation leads to ubiquitination, followed by proteasomal degradation of rAAV2 vectors in the cytoplasm [71]. Indeed, we obtained experimental evidence to support this hypothesis, which we reported in 2007 [72]. These studies provided the impetus to mutagenize the surface-exposed tyrosine residues in the AAV2 capsid to circumvent this barrier.
There are seven tyrosine (Y) residues in the AAV2 capsid that are surface-exposed (Y252, Y272, Y444, Y500, Y700, Y704 and Y730). Each of these Y residues was mutagenized to phenylalanine (F) residues to generate seven single-mutants (Y252F, Y272F, Y444F, Y500F, Y700F, Y704F and Y730F), the transduction efficiency of three of which (Y444F, Y500F and Y730F) was significantly higher than their WT counterpart. The Y730F single-mutant rAAV2 vector was the most efficient, the use of which resulted in the expression of therapeutic levels of hFIX in three different strains of mice following intravenous or portal vein administration at ten-fold reduced vector doses [73].

In subsequent studies, seven double-mutants (Y252 + 730F; Y272 + 730F; Y444 + 730F; Y500 + 730F; Y700 + 730F; Y704 + 730F; and Y444 + 500F), one triple-mutant (Y444 + 500 + 730F), one quadruple-mutant (Y272 + 444 + 500 + 730F), two pentuple-mutants (Y272 + 444 + 500 + 704 + 730F and Y272 + 444 + 500 + 700 + 730F), one sextuple-mutant (Y252 + 272 + 444 + 500 + 704 + 730F), and one septuple-mutant (Y252 + 272 + 444 + 500 + 700 + 504 + 730F) were also generated, and the triple-mutant (Y444 + 500 + 730F) rAAV2 vector was found to the most efficient, and provided a long-term therapeutic and tolerogenic expression of hFIX in hemophilia B mice [74]. Interestingly, the triple-mutant rAAV2 vector was also shown to minimize in vivo targeting of transduced hepatocytes by capsid-specific CD8+ cells [75].

Although it appeared that the next-generation tyrosine triple-mutant rAAV2 vector, which circumvented the problems associated with the first-generation rAAV2 vectors, could potentially be used successfully in patients with hemophilia B, Nathwani et al. [23] reported that the use of rAAV8 serotype vectors, which had previously been shown to be far more efficient than rAAV2 serotype vectors in transducing murine hepatocytes [18,76,77], led to phenotypic correction of hemophilia B in two patients who received the highest vector dose, which appears to be sustained for more than 3 years [24], but with some diminution in hFIX levels (Table 1). Despite these highly encouraging results, I would reiterate, as stated above, that rAAV8 vectors might not be the panacea, especially for patients with severe hemophilia B, since based on our studies with non-human primate and humanized mice models [63,65], rAAV8 vectors are approximately eight times less efficient than rAAV3 vectors. Table 1 also illustrates additional AAV serotypes and their variants that have been used, or are currently being used, in the potential gene therapy of both hemophilia B and hemophilia A. It should be noted, however, that most, if not all, of these vectors are composed of naturally occurring capsids, which are likely to induce host immune responses, especially when used at astronomically high doses in some instances. Thus, I was prompted in 2016 to also posit that the WT AAV did not evolve for the purposes of delivery of therapeutic genes [25]. In other words, rAAV vectors composed of naturally occurring capsid are unlikely to be optimal in human clinical trials.

In our quest to develop more efficient and potentially less immunogenic AAV vectors, we also extended our studies to include...
two additional amino acid residues in the AAV capsid that are surface-exposed, and can also be phosphorylated by cellular serine/threonine protein kinases. For example, in addition to seven tyrosine (Y) residues, the AAV2 capsid also contains 17 surface-exposed serine (S) and 15 surface-exposed threonine (T) residues, each of which has been mutagenized, and rAAV2 vectors containing various permutations and combinations thereof, have been generated [78], and a quadruple-mutant (Y444+500+730F+T491V) has been identified to be the most efficient rAAV2 vector to date, at least in the murine liver. In addition, since ubiquitination occurs on lysine (K) residues, all seven surface-exposed residues in the AAV2 capsid have also been mutagenized, and limited numbers of Y+S+T+K-mutant rAAV2 vectors have been generated [79]. Although there is circumstantial evidence that these modifications lead to reduced degradation of AAV vectors in the cytoplasm, and therefore improved intracellular trafficking to the nucleus, and consequently efficient transgene expression [73], it should be noted that Douar et al. [80] observed a lack of direct correlation between the fold increase in intracellular trafficking with the fold increase following treatment with inhibitors of cellular proteasome. Thus, it remains possible that additional mechanisms, such as induction of p53 expression, activation of stress kinases and induction heat-shock gene expression, postulated by Douar et al. [80], might also play a role.

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### TABLE 1

First generation of recombinant AAV serotype vectors used/being used for the potential gene therapy of hemophilia.

<table>
<thead>
<tr>
<th>Investigators/sponsors</th>
<th>Vector</th>
<th>Dose</th>
<th>Expression level</th>
<th>Total dose*</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hemophilia B</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>High/Kay</td>
<td>ssAAV2</td>
<td>8 x 10^{10} vgs/kg</td>
<td>0%</td>
<td>5.6 trillion</td>
<td>[59]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 x 10^{10} vgs/kg</td>
<td>0%</td>
<td>28 trillion</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 x 10^{12} vgs/kg</td>
<td>11%</td>
<td>140 trillion</td>
<td></td>
</tr>
<tr>
<td>Nathwani/Davidoff</td>
<td>scAAV8</td>
<td>2 x 10^{11} vgs/kg</td>
<td>2%</td>
<td>14 trillion</td>
<td>[23,24]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 x 10^{11} vgs/kg</td>
<td>2–4%</td>
<td>42 trillion</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 x 10^{12} vgs/kg</td>
<td>8–12%</td>
<td>140 trillion</td>
<td></td>
</tr>
<tr>
<td>Baxalta/Shire**</td>
<td>scAAV8</td>
<td>2 x 10^{11} vgs/kg</td>
<td>2–5%</td>
<td>14 trillion</td>
<td>[113]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 x 10^{12} vgs/kg</td>
<td>20–25%</td>
<td>70 trillion</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 x 10^{12} vgs/kg</td>
<td>50–7%</td>
<td>210 trillion</td>
<td></td>
</tr>
<tr>
<td>Spark Therapeutics***</td>
<td>Undisclosed</td>
<td>5 x 10^{11} vgs/kg</td>
<td>20–44%</td>
<td>35 trillion</td>
<td>[113]</td>
</tr>
<tr>
<td>uniQure</td>
<td>scAAV5</td>
<td>5 x 10^{12} vgs/kg</td>
<td>3–7%</td>
<td>350 trillion</td>
<td>[113]</td>
</tr>
<tr>
<td><strong>Hemophilia A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BioMarin</td>
<td>ssAAV5</td>
<td>2 x 10^{12} vgs/kg</td>
<td>2–5%</td>
<td>1.4 quadrillion</td>
<td>[113]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 x 10^{12} vgs/kg</td>
<td>50–200%</td>
<td>4.2 quadrillion</td>
<td></td>
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* Based on an average patient’s weight of 70 kg (estimated number of cells in a 70 kg ‘reference man’ = 3.0 x 10^{13} or 30 trillion) [139].

** This trial has now been stopped since the expression levels of F.IX were inconsistent among different patients, and in some patients, the level of expression decreased with time.

*** One patient manifested an immune response to AAV capsid proteins 12-weeks post-vector administration, accompanied by a drop in F.IX activity level.
Interestingly, however, most, if not all of the surface-exposed Y, S, T and K residues are highly conserved among all ten commonly used AAV serotype vectors, and most of these residues have also been mutagenized in each of the ten AAV serotype vectors. Although further extensive studies would be needed to identify the most efficient combination of these mutations for a given serotype, cell or tissue type, and the host species, it has become abundantly clear that the use of the capsid-modified next generation of AAV vectors, as schematically illustrated in Figure 2, is likely to overcome some of the limitations associated with the first generation of AAV vectors. In this context, it is important to point out that three Phase I/II clinical trials with the tyrosine triple-mutant rAAV2 vectors have been initiated (Table 2), and that the initial results appear very promising in that two patients with Leber’s hereditary optic neuropathy (LHON), who were administered a medium dose of the vector intravitreally, showed improvement in visual acuity at 90 days follow-up, without any loss of vision or any serious adverse events [81]. Thus, my

<table>
<thead>
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<th>FIGURE 2</th>
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<td>The capsid-modified next generation of recombinant AAV vectors.</td>
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</table>

Surface-exposed, specific tyrosine (Y), serine (S), and threonine (T) residues on AAV capsids can be phosphorylated, which is a signal for ubiquitination. Surface-exposed, specific lysine (K) residues on AAV capsids can be ubiquitinated, and subsequently degraded by the host cell proteasome machinery. Site-directed mutagenesis of these residues leads to the generation of AAV vectors that are more efficient at reduced vector doses, and consequently, less immunogenic. Specific examples of the most efficient rAAV2 [78], rAAV3 [62,63,65], and rAAV6 [141–143] serotype vectors generated thus far, are also depicted.
The prediction is that the capsid-modified next generation of rAAV serotype vectors will prove to be far more efficacious than their WT counterparts in human gene therapy.

SINGLE-STRANDED VERSUS SELF-COMPLEMENTARY RECOMBINANT AAV GENOMES: IMPLICATIONS IN TRANSGENE EXPRESSION & GENE THERAPY

The genome of the WT AAV is a single-stranded DNA of 4,680 nucleotides [82], but single-stranded DNA of both [+] and [-] polarities are encapsidated into separate mature virions with equal frequency [83]. While advantageous for the WT AAV, which prefers to remain latent in host cells, the single-stranded nature of the genome in a rAAV vector is problematic, since single-stranded DNA is transcriptionally-inactive, and viral second strand-DNA synthesis is a rate-limiting step during rAAV vector-mediated transgene expression in tissue culture cell lines, as originally described by Fisher et al. [84] and Ferrari et al. [85] in 1996. However, since the AAV genome most likely exists as double-stranded circular episomes and concatemers, especially in post-mitotic cells and tissues, it appears unlikely that its single-stranded nature contributes to its latency. In 1997, we [56] and others [57] reported that following intravenous administration of rAAV2 vectors in a murine model in vivo, up to 95% of the mouse hepatocytes were transduced, but transgene expression occurred in ~5% of the hepatocytes [86]. However, the mechanism underlying the lack of viral second-strand DNA synthesis in ~95% of the hepatocytes remained unclear. There was robust debate among three groups of investigators, two groups favoring the viral second-strand DNA synthesis model [84,85] and the third group favoring the DNA strand-annealing model [87]. A preponderance of evidence suggested that the former was the predominant mechanism underlying rAAV vector-mediated transgene expression [88–95].

Using tissue culture cell lines as a model, we identified that a cellular protein, phosphorylated at tyrosine residues, binds specifically to the
single-stranded sequence of 20 nucleotides, termed the D-sequence, within the AAV inverted terminal repeat (ITR) at the 3’-end of the viral genome, and that this phospho-protein strongly inhibits the viral second-strand DNA synthesis, resulting in impaired transgene expression [96]. We subsequently identified this cellular protein to be a 52 kDa protein that binds the immunosuppressant drug FK506, and hence the designation, FKBP52, a well-known cellular chaperone protein [97]. A number of strategies were developed to circumvent the barriers that hinder AAV second-strand DNA synthesis [90–94,98,99], the most significant of which was the generation of double-stranded, self-complimentary AAV (scAAV) vectors by McCarty et al. [88]. The use of scAAV vectors was shown to easily overcome the rate-limiting step of viral second-strand DNA synthesis, leading to early onset and robust transgene expression, both in tissue culture cell lines in vitro, and in murine models in vivo [89]. This observation was further validated by the successful clinical trial for hemophilia B by Nathwani et al. [23,24], who used scAAV8 vectors and achieved sustained levels of expression of hF.IX in ten patients. It is tempting to speculate that since AAV8 vectors transduce human hepatocytes less efficiently than mouse hepatocytes [63–65,100], the use of the scAAV vectors, rather than the AAV8 serotype, was largely responsible for the successful outcome.

It is intriguing, therefore, that with a few exceptions, nearly all clinical trials reported thus far have been performed using ssAAV vectors, and yet clinical efficacy has been observed in the potential gene therapy of several human diseases, such as LCA, lipoprotein lipase deficiency, aromatic L-amino acid decarboxylase deficiency and choroideremia. If the expression cassettes of each of the therapeutic genes used in these trials were within the limited packaging capacity of ~2.5 kb for scAAV vectors, it is reasonable to suggest that the levels of the transgene expression would be significantly higher.

However, since it is unlikely that expression cassettes of all therapeutic genes can be encapsidated in scAAV vectors, it is clear that additional strategies to achieve higher levels of transgene expression from rAAV vectors containing single-stranded DNA genomes are warranted. Since, as stated above, we had observed that binding of FKBP52 to the D-sequence at the 3’-end in the AAV2-ITR strongly inhibits the viral second-strand DNA synthesis, and consequently, transgene expression, we hypothesized that deletion of the D-sequences from the viral genome would allow us to achieve that objective, but we observed that deletion of the D-sequences from the AAV genome resulted in failure of the viral progeny DNA strands to undergo genome encapsidation. Thus, we learned that the D-sequences are indispensable, as they serve as the ‘packaging signal’ for the AAV genome [101–103]. Interestingly, however, when only one of the two D-sequences was deleted from the AAV genome, successful encapsidation of the progeny viral DNA ensued, but depending upon which D-sequence was deleted, the resulting vectors contained either [+] or [-] polarity strands [104]. More interestingly, the transduction efficiency of these single-polarity ssAAV vectors was observed to be significantly higher than that of their unmodified counterpart in both established cell
lines in vitro and in murine hepatocytes in vivo [104].

Further detailed studies revealed that the D(-)-sequence at the 3'-end in the viral inverted terminal repeat contains the binding site for a cellular protein, FKBP52, phosphorylated forms of which bind to the proximal end of the D(-)-sequence, and strongly inhibit the viral second-strand DNA synthesis [96,97]. The D(+) -sequence at the 5'-end in the viral inverted terminal repeat contains the binding site for a cellular NF-κB repressing factor (NRF), which inhibits the viral transgene expression [105]. Whereas removal of both D-sequences is incompatible with vector genome encapsidation [102], removal of the D(+) -sequence leads to the generation of either the [+] or the [-] polarity ssAAV vectors, which mediate more efficient transgene expression due to the loss of the NRF binding site [104]. These one D-sequence-deleted genome-containing vectors are depicted schematically in Figure 3. Thus, the use of genome-modified rAAV vectors appear to partially overcome the limitation associated with the conventional ssAAV vectors, and expression cassettes of therapeutic genes of up to ~4.5 kb can easily be encapsidated to achieve improved transgene expression.

As expected, when the modified AAV genomes were encapsidated into the most efficient quadruple-mutant (Y444F+T492V+T491V) AAV2, or the double-mutant (S662V+T492V) AAV3 capsids, the resulting optimized vectors were documented to transduce cells and tissues significantly more efficiently at 20–30-fold further reduced vector doses [106]. These optimized AAV serotype vectors circumvent the problems associated with the first generation of AAV vectors. Thus, my prediction is that, in contrast to the enormously high vector doses that are currently being used, particularly for the potential gene therapy of hemophilia (Table 1), the optimized AAV serotype vectors, in addition to being far more efficacious, will also offer the potential advantages of being less immunogenic, and more cost-effective.

TRANSLATION INSIGHT

Gene therapy has had its ups and downs, but now there is little doubt that it is here to stay, and it is likely to cure a number of human diseases in the near future. Perhaps Dr Philippe Leboulch said it best in 2013 [107]: “The development of the field of gene therapy shares many similarities with the history of aviation. Each is based on deceptively simple principles: the introduction of a therapeutic gene into cells and the flow of air over an aircraft’s wing. Each field was marred by shortcomings and adverse events early on. But in spite of naysayers lacking vision, both fields continued their quest, and now there is firm hope that gene therapy will soon do for medicine what airoplanes did for transportation.” As for rAAV serotype vectors and their successful use in a number of Phase I/II/III clinical trials, this sentiment has clearly been validated.

Commercia lly viable therapies

The AAV1 vector expressing the gene for lipoprotein lipase (LPL) was approved as a drug in Europe in 2012 [108]. It is designated as Alipogene tiparvovec, and marketed...
under the trade name Glybera™. Based on the recent successful Phase III trial for the potential gene therapy of LCA, it is likely that the AAV2 vector expressing retinal pigment epithelium-specific 65 kDa protein (RPE65), also known as retinoid isomerase, will soon be licensed as a drug in the USA. Licensing of various AAV serotype vectors and their variants, which have shown clinical efficacy in a number of gene therapy trials for hemophilia B and A [109], will soon follow as well. A number of additional clinical trials in which AAV vectors have already shown efficacy, such as hemophilia B with AAV8 vectors [23,24], aromatic amino acid decarboxylase deficiency and cho-roideremia with AAV2 vectors [12], and additional Phase I/II clinical trials are currently being pursued with AAV1 vectors for the potential
gene therapy of α1 anti-trypsin deficiency [110], AAV1 and AAV9 vectors for Pompe disease [111,112], and AAV8 and AAV5 vectors for hemophilia B and hemophilia A [113], respectively, and once their efficacy has been established, commercial viability will certainly be pursued. Thus far, AAV1, AAV2, AAV5, AAV8 and AAV9 serotype vectors have been, or are currently being used, in 162 Phase I/II clinical trials in humans to date [6,114], which will eventually lead to commercially viable therapies.

The fact that several Big Pharma companies, such as Baxter, Bayer, Biogen, BioMarin, Bristol-Myers Squibb, GlaxoSmithKline, Novartis, Pfizer, Sanofi and Shire, among others, have invested well over $3 billion since 2014 [115], bodes well for the commercial viability of AAV vector-mediated gene therapy in humans.

Challenges

Despite the remarkable progress that has been made in the use of rAAV vectors for human gene therapy, and the future prospects that appear very promising, several challenges also remain. One of the major challenges is pre-existing antibodies to AAV. A significant proportion of humans are sero-positive for one or more of the AAV serotypes, and studies have documented that anti-AAV antibody titers as low as 1:10 are sufficient to neutralize systemically administered rAAV vectors [116,117]. Cross-reactivity of these pre-existing antibodies against one AAV serotype to many other AAV serotypes is also a significant barrier [118].

The second challenge is the inability of the currently available rAAV vectors to selectively target a given cell type, tissue or organ following systemic administration. Highly regulated transgene expression restricted to a given cell type, tissue or organ also remains a desirable goal.

The third challenge with the currently available rAAV serotype vectors is the lack of standardization of vector titers and potency, although reference standards for at least two serotypes (AAV2 and AAV8) are now available [119,120]. However, reproducibility among different production methods also remains a challenge.

The final challenge is the inherently limited packaging capacity of ~5 kb for the conventional ssAAV vectors and ~2.5 kb for scAAV vectors. Although there was a lone report [121] claiming that AAV genomes of up to 8.9 kb could be packaged in rAAV5 serotype vectors, at least three independent groups failed to reproduce those results [122–124]. Several groups have reported the use of dual vectors to achieve the delivery and expression of oversized genes [125–128].

Research requirements

The value of basic science research on rAAV vector biology cannot be overstated. As I have emphasized previously [55], it was entirely due to the sustained efforts of a very few investigators who continued to pursue basic science research on AAV for nearly three decades despite the complete lack of interest of the scientific community at large, that was instrumental in the development of rAAV vectors. In my opinion, detailed molecular studies on every aspect of the AAV lifecycle – attachment and entry, intracellular trafficking, nuclear transport, viral uncoating, second-strand DNA synthesis, and transgene expression – must continue to be pursued.
In this context, it is important to reiterate that, as stated above, and illustrated in Figure 3, although removal of the D(+) -sequence from the inverted terminal repeat (ITR) at the 5′-end leads to generation of either the [+ ] or the [- ] polarity ssAAV vectors, which mediate more efficient transgene expression due to loss of the NRF binding site [104], it has thus far not been possible to generate ssAAV vectors that lack the D(-)-sequence at the 3′-end in the ITR since ssAAV genomes lacking the D(-)-sequence fail to undergo encapsidation [103]. Thus, the development of additional strategies are warranted to generate ssAAV genomes that lack the FKBP52-binding site, and yet can be packaged, such that efficient viral second-strand DNA synthesis can ensue, leading to robust transgene expression.

Unless and until the astronomically high vector doses that are currently being used in human clinical trials [Table 1], which clearly trigger the host immune response, can be reduced to achieve clinical efficacy, detailed studies on the intricacies of the AAV vector immunology must also be pursued.

For the most part, rAAV vector genomes have been shown to remain episomal for extended time periods lasting years and decades, but thus far, those studies have been carried out with post-mitotic cells, tissues and organs. There is clearly a need to develop rAAV vectors that can also stably transduce actively dividing cells. This would necessitate that rAAV vector genomes undergo integration into the host cell chromosomal DNA. In order to circumvent the possibility of insertional mutagenesis due to random integration, efforts must also be made to achieve site-specific integration of rAAV vector genomes, akin to what has been observed with the WT AAV [129,130].

It has become increasingly clear that despite the extensive use of mouse and rat models in biomedical research in general, for the most part, these rodent models are poor surrogates for humans, as well as poor predictors for evaluating the efficacy of rAAV serotype vectors for human diseases [55]. Thus, at the very least, the use of non-human primates, or humanized mouse models, should be considered as more reliable model systems. In addition, individual difference among humans may also significantly influence the reproducibility of AAV vector-mediated gene therapy, as was illustrated by differences in transduction efficiency of rAAV3 and rAAV8 vectors in hepatocytes from various donors [63–65,100]. In this context, it is also important to note that despite the limited lifespan of primary hepatocytes, sustained transgene expression mediated by rAAV vectors lasting decades, warrants further mechanistic studies.

Manufacturing needs

There are currently two common rAAV vector production protocols that are being used: human embryonic kidney cell line, 293 (HEK293) and plasmid triple-transfections; and insect cell line, sF9, and baculovirus vector infections. Although both systems have their advantages as well as disadvantages, additional systems, including suspension cultures, would need to be refined to achieve scalable production of various rAAV serotype vectors to meet the ever-growing need as additional clinical trials for a wide variety of human diseases are contemplated and pursued.
As pointed out above, there is an urgent need to develop standardized protocols not only to produce high-quality rAAV serotype vectors, but to also determine their titers and potency accurately, such that vectors produced and clinical trials performed at various geographical locations can be compared consistently and in meaningful ways. During the process of vector packaging, a large amount of empty capsids are produced. A better understanding of the underlying mechanism of vector assembly might also lead to strategies that significantly increase vector production. It should also be noted that thus far, all recombinant AAV vectors are generated using AAV2-ITRs and AAV2-Rep proteins, regardless of the AAV serotype capsid, which is not optimal. We have suggested the use of the homologous ITRs and Rep proteins, specific for each serotype, which, at least for rAAV3 serotype vectors, appears to significantly improve the titers as well as the potency [131].

The cost associated with large-scale rAAV vector production, especially for clinical grade vectors, is not insignificant. With the steadily shrinking research support from the National Institutes of Health, it is becoming prohibitively expensive for academic investigators to pursue clinical trials. Thankfully, as mentioned above, the Big Pharma companies have stepped in and begun to fill this void. The establishment of additional Clinical Manufacturing Organizations (CMOs) would also go a long way to meet this critical need.

As stated above, although the very first rAAV vector as a drug, Glybera®, was approved in Europe, nearly 64% of all gene therapy clinical trials have been, or are currently being performed in the USA, and ~20% in Europe [6]. Yet, a large population of patients in dire need of life-altering and life-saving treatments lives in the third world. Thus, all efforts should be made to make gene therapy not only cost-effective, but also available to eligible patients worldwide.

**Regulatory framework**

The US Food and Drug Administration (FDA) has recently begun to grant expedited review and approval, termed Breakthrough Therapy, for specific gene therapy trials, among Priority Review, Accelerated Approval and Fast Track, to facilitate such treatments as rapidly as possible, which is highly commendable. However, the current emphasis, both by academia and Big Pharma, is still on targeting the orphan diseases. Now that the safety of rAAV vectors has been well established in 162 Phase I/II clinical trials, and one Phase III clinical trial, one would hope that the regulatory agencies, including the FDA, would consider granting approval for diseases where the rates of incidence is higher, and the life expectancy is shorter.

These regulatory agencies should also consider granting approval for gene therapy clinical trials with rAAV vectors for diseases where the end of life is imminent.

Although at least two Phase I clinical trials with modified AAV vectors were approved by the FDA, one contained five amino acid substitutions from AAV1 to AAV2 (AAV2.5) [132], and the second in which three surface-exposed tyrosine residues were mutagenized and replaced with phenylalanine residues (AAV2-Y444+500+730F) [81], it remains to be seen what regulatory hurdles, if any, additional AAV variants, both rationally
designed and shuffled, might face in the future.

**Next steps**

In addition to pursuing various research avenues outlined above, the quest for the isolation of novel AAV serotypes from other species, both vertebrates and non-vertebrates, and their development as vectors must also continue. Although several approaches, including directed evolution [133], DNA shuffling [134], rational design [73,78,135–137], dual vectors [124–127] and chemical modifications [138] are currently being used, further optimization of not only the capsid, but the vector genomes should be pursued as well [104].

Finally, efforts towards the development of site-specific integrating vectors; tissue- and organ-specific vectors; vectors capable of escaping pre-existing antibodies; and vectors capable of repeat administrations, should also continue, in order to realize the full potential of these remarkable biological entities that were once considered a 'biological oddity' [139].

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**FINANCIAL & COMPETING INTERESTS DISCLOSURE**

The author holds several issued patents on recombinant AAV vectors that have been licensed to various gene therapy companies.

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Continuous flow centrifugation: importance in vector scale up

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Q Can you provide some background information on what continuous flow centrifugation is?

SM: Continuous flow centrifugation technique is widely known for its use in the clarification of bulk materials, for example disc stack centrifuges. But as a tool for the purification of viruses, viral vectors, or particulate materials, it’s not so widely known. This centrifuge, or ultracentrifuge, can substitute for a chromatography or filtration step in a purification protocol.

In the laboratory tube rotors are often used and offer an excellent purification option for small quantities of material, but are limited when it comes to scale up. The solution to this is to employ a continuous flow ultracentrifuge, where sample size is not limited by rotor volume, as the feed stream...
will continually enter the rotor during high-speed operation. In this way, a sample load for example of 50 fold can be loaded, concentrated and purified to over 95% purity in one step. The way this works is that the ultracentrifuges use rotors known as zonal rotors, which are one hollow tube per centrifuge divided into segments. Within these segments a density gradient can be formed, within which viral vectors and virus of interest can be purified.

Q As more gene therapies move into clinical trials, there’s an increased need for scalable, efficient vector production. What do you perceive to be the biggest opportunities within vector manufacturing that can help us achieve this?

SM: In order to move to more commercial-scale manufacturing, your equipment and process methodology need to be suitable for scale up. Where our centrifuge can support this is that they are truly scalable. There is a perception in the market that ultracentrifugation is not scalable; when looking at tube rotors in the laboratory setting, then clearly to scale those up you would need either a different centrifuge or 10 to 20 of the same centrifuge and that simply is not efficient or cost effective.

What we can provide are truly scalable centrifuges. In this current market adeno-associated virus (AAV) is a very important vector which has entered into many clinical trials, with over 42 trials currently open. These trials are phase 1 and phase 2, and eventually they will need to reach a manufacturing scale. Downstream processing can be a bottleneck if you thought you were going to employ 10 small centrifuges. Because of this perception, a lot of people choose chromatography instead of centrifugation. This prompted us to carry out some studies to demonstrate our continuous flow centrifugation can be that downstream processing step of choice.

SG: As Sandra mentioned, we certainly have identified a market need for scalability and of course cost is always a key consideration when looking at process scale up. When you have the opportunity to go from research production to full scale production, and not have to start over again at each stop along the way, that’s a very important benefit and can be incredibly cost effective. That’s where our systems, our process, come into play.

Alfa Wasserman have been in this business for over 45 years and we’ve been involved in process development and industrial-scale manufacturing, predominantly in vaccine production. We are totally home-grown, we never third party any service or support. There are a range of ultracentrifuges each with the linear process scalability which is a significant benefit, as well as our systems being incredibly robust.
**SM:** I think it’s also important to clarify what we mean when we talk about scale up. We have a rotor of a fixed size, but as it scales up as a continuous flow centrifuge – while it’s spinning at the operating speed – fluid is continually pumped through that rotor. So that means for any of our rotors, you can load more than 40 times your actual rotor volume of your process fluid, which allows concentration at the same time as purification.

**Q** You mentioned you’d recently undertaken a study to look at the potential use of centrifugation as a purification technique for viral vectors. Can you describe the rationale and methodology?

**SM:** We’re always looking at viruses, viral vectors, nanoparticles, exosomes, all sorts of particles that can be purified in a centrifuge. As this is an ultracentrifuge, it’s not a low speed machine, it’s high-speed used for purifying very small particles from background material. It can accommodate anything up from 50 nanometers right up to 1000 nanometers. AAV falls into this range of particles that can be purified. As we’ve already identified there is a need in the market for scale up, particularly since AAV is one of the most commonly used vectors at the moment in clinical trials, we decided to undertake a study to demonstrate our centrifuge’s capability in purifying this vector.

We collaborated with Virovek, a Hayward, California-based company who produced the material for this study and with our centrifuge installed in their facility we carried out a number of test runs to try and establish the best protocol.

Currently there are no efficient large-scale separation methods. Small-scale methods use caesium chloride density gradients in tubes, or iodixanol density gradients in small tubes. We decided to use a wave bioreactor to grow S9 cells and use a baculovirus system that is proprietary to Virovek, to generate the AAV particles.

This material was then analyzed, clarified with low-speed centrifugation to remove large particles, and processed through the Alfa Wasserman centrifuge.

**Q** What were the main outcomes and how did these prepare with your expectations?

**SM:** On a small scale, caesium chloride density gradient is very commonly used. In fact that’s the process Virovek were using in their normal processes. So we first tried to establish caesium chloride as a continuous flow density gradient inside the rotor but found this was not possible. The main reason is there’s a degree of stability required in continuous flow density gradient centrifugation. This is established through the viscosity of the medium and caesium chloride has very low viscosity.
That led us to then try iodixanol, which was found to have sufficient viscosity to form a stable gradient. We then performed some test runs with a background buffer, PBS, some sodium citrate, which is well established as a standard medium for using in the background of AAV purifications, and we achieved get a good capture rate.

Therefore, we loaded lysate, if you’ve taken your cell culture, collected your cells, lyzed those cells to release the AAV, then you have quite high protein material. That can then be loaded directly into the centrifuge without any further concentration step. In our first profiles we got a very clear peak of AAV in the gradient, which was round about the 2.8 x 10^{13} particles in that peak, which is quite substantial.

We also established a very nice density gradient in the rotor. The peak of the product was round about the 42/43% iodixionol, which is where you would expect it to be. When we analyzed that with SDS PAGE, we obtained a very pure sample. There were a few extraneous proteins lurking in the lower molecular weight levels of the SDS PAGE. So at first we thought that maybe we were overloading the gradient, with too much lysate going in, and too much total protein. Therefore we performed a second run of that material peak and then diluted it back down to reduce density. What we actually saw was that although we recovered a substantial amount of AAV, we did have a single protein that seemed to be tracking with the particle. Our conclusion was that this was associated with the surface of the particle and wasn’t just in the background material.

This led us to see if we could disassociate that single protein. When tube gradients are made with caesium chloride, this strange protein is not seen, but using iodixanol then this protein is often seen associated with the particle. We decided to add a small amount of caesium chloride back into that iodixonal mix in the buffer, as a background modifier. That changed the ionic strength of the solution, which led to a further result, and in that second SDS PAGE we could see in fact we had purity equal to that of a caesium chloride gradient. In that purification, when we loaded that lysate to the centrifuge, even with that extra band associated with the particles, we were achieving about a 55% yield, which is fairly good for a single step protocol. Utilizing this process you can go from lysate to pure product in one step. Now that’s a very significant process advantage as typically 3 or 4 rounds of chromatography might be required to achieve a similar yield and reported yields have been down at about 20%.

In a single step, with 55% yield, we feel we have a pretty robust method for collecting AAV particles. This research was all undertaken on our research size machine, the AW Promatix™ 1000. This is a small unit we can put into research facilities, it has a very small footprint, and is a mobile machine. We also have large-scale machines used in the vaccine manufacturing industry. This is called KII and has 27-fold larger rotor than what we used for this Promatix study.

Therefore, it’s possible to extrapolate that peak of 2.8 x 10^{13} particles we collected that was about 20 ml, could scale up then to 540 ml, from a KII rotor. That’s a substantial amount of pure AAV vector and supports the use of ultra-centrifugation as part of a commercially viable scale up process. Furthermore, this purification of AAV as a physical separation means you can
have different constructs in AAV, different therapies and they will all purify in a similar fashion thus removing the need to keep redefining the test protocol.

This study was focused on the purification of AAV vectors, whether they are full or empty capsids was not investigated as the size differential between the two is very small; however, we are keen to look into this in the near future.

Q: What relevance can we draw from this in terms of large scale manufacturing of vectors? The commonly held perception is that centrifugation is not a scalable process. Is that correct?

SM: Centrifugation on a laboratory scale, using tube rotors, is not a scalable method. But here we’re talking about a specific piece of equipment for which we have linear scale up. What I mean by that is the experiment we performed on a 120 ml rotor will directly translate to a 3.2-liter rotor. The only difference is the volume of product you load and the flow rate you use would be multiplied.

This means you can process a 10 liter batch on a Promatix at small scale and move to a 200 liter batch on a large scale. With scalable rotors we retain their linear scale by having the same maximum: minimum radius.

It’s also the case that within a centrifuge, for example the PK, our pilot scale centrifuge, we have different rotor cores that will establish different volumes within one rotor. For example a 400 ml rotor, an 800 ml rotor and a 1600 ml rotor can all be created using the same rotor assembly, by simply changing the inserts inside that rotor. This means inside one machine you can process 10 and 50 liter batches.

One factor that is critical for scale up to clinical trial stage is that your production process is compliant with the cGMP requirements. This is central to the Alfa Wasserman manufacturing and testing of centrifuges. We come from a background of large-scale GMP-compliant centrifuges. The laboratory centrifuge, the Promatix, is a unique product that enables people to do test work at volumes of less than 10 liters but retain the same critical separation parameters of the cGMP KII ultracentrifuge. Laboratory centrifuges, although they may be valid at small scale, often lack this cGMP compliance in terms of the validation and test package and can therefore present a compromise when scaling up.

Q: While the study looks at AAV, do you feel this is applicable to additional vectors, including perhaps next generation non-viral vectors?

SM: Absolutely. The centrifuge can be used as a purification tool for any particle between 50 and 1000 nanometers. That could be
exosomes, nano-particles, nano-structures etc. There’s a lot of work at the moment looking at exosomes as part of cells that also need purified. These seem to provide a good gene therapy, because they are recognized as cells, so they can go into tissues where other systems might be rejected.

This centrifuge came initially from influenza vaccine manufacture, but has been used for all sorts of things, including hepatitis B, rabies, adeno and vaccinia vectors. So centrifugation is a physical separation method that is not affected by the chemistry of the particles, therefore it’s going to separate lots of different constructs a company might be making, all in the same way, in a very generic process. This means you can use this process across your pipeline for more than one vector or vector construct that is being purified.

Q Have you run this process with lentivirus and other viral vectors?

SM: We've certainly looked at purification of lentivirus and it tends to be a little bit more delicate. Similar work has been done with respiratory syncytial virus (RSV) which some people say you can't centrifuge because it will fall apart. However, other groups have centrifuged RSV and had great successes. Some of this seems to be related to the shear factor caused when liquid coming into the stationary machine suddenly starts to swirl. What we see on our centrifuges is we have quite a long transition zone from the stationary to the turning phase and this seems to reduce the shear factor which can be a problem on laboratory scale continuous flow machines.

SG: Whilst AAV was selected for this particular study, given the 42 ongoing clinical trials using this vector, we feel confident that our continuous flow ultracentrifugation will achieve similar unsurpassed levels of purity, and yield, for additional viral vectors and with the added benefit of facilitating the move from research scale to production.

You can access the poster here for a full overview of the study and outcomes discussed here.

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Dr. Chetan (Chet) Tailor is the Founder and CEO of Tailored Genes. He has over 20 years of experience in molecular biology, cell culture and generating retrovirus/lentivirus vectors. Dr. Tailor obtained his Ph.D in the field of Virology from the University of London UK in 1995, under the supervision of Prof. Robin A. Weiss and Prof. Mary K. Collins, two of the top Scientists in the field of retroviruses/lentiviruses and gene therapy. He then spent 5 years (1996-2001) as a post-doctoral Fellow at the Oregon Health Sciences University in Portland, Oregon, USA under the supervision of Prof. David Kabat. Dr. Tailor next established his own research laboratory at The Hospital for Sick Children Research Institute, Toronto, Canada from 2001-2012. From 2012-2014, Dr. Tailor was Scientific Director of the Vector Core Facility at the University Health Network, Toronto, Canada generating custom-made lentivirus vectors for Canadian and US customers before finding Tailored Genes.

Q What are the key considerations when assessing which viral vector platform to use for your potential therapy?

Target cell and the size of gene you want to introduce are the two key factors for determining the vector you want to use. Another factor includes the duration of gene expression. There are different types of virus vectors currently being used, predominantly lentivirus, adeno-associated virus (AAV), adenoviruses (AV), and retroviruses but also a number of other types. So, looking at the first consideration, the target cell: depending on what cell you’re going to target will determine which type of vectors you want to use as they all have different affinities and expression in different tissue/cell types. For example, if you want to target lung...
cells, AVs would be a good vector of choice simply because you can make it as an inhaler/aerosol to get it down to the lungs. If you want to target brain cells, AAVs and herpes simplex virus vectors are good vectors of choice. And if you want to target stem cells or blood cells, a good vector to use would be the lenti viral vectors.

The second consideration is the gene itself. Some viruses, such as AAV can only incorporate small pieces of genes, with the maximum capacity being around 4–4.5 Kb. For lentiviruses, it is 8–9 Kb but for AV you can insert up to almost 30 Kb of DNA.

Duration of gene expression is also an important consideration for virus vector choice. Lentiviruses and retroviruses vectors provide long-term gene expression as they integrate the transgene into the target cell genome. For shorter-term expression, AAV and AV vectors can be used as the transgene is not integrated in the target cell genome.

And then there's the issue of whether the target cells are dividing or non-dividing cells. Retroviruses can only infect dividing cells, which can be limiting factor to any gene therapy utilizing these viruses as a delivery tool. As such, a lot of researchers have reduced the use of retroviruses and are now using lentivirus, AAV and AV so they can infect dividing and non-dividing.

Q You specialize in the production of lentiviral vectors. Could you describe the main differences between 2nd and 3rd generation LV?

Lentivirus vectors are a class of retroviruses that are derived predominantly from a modified version of HIV where the disease-causing genes, and replication ability, are removed thus enabling it to become a viral vector for delivering genes to target cells.

There are different generations of lentivirus vectors that have been produced, and the whole reason for this was to improve their safety profile.

The 1st generation lentivirus vectors involved taking the full genome of HIV, deleting non-essential genes for virus production, in addition to removal of a signal called the packaging signal. A second plasmid with your gene of interest – the transgene – containing the packaging signal is also required, therefore creating a 2-plasmid system.

One safety consideration with viral vectors concerns the frequency of recombination events. Recombination happens between DNA that have similar sequences. So certain sequences such as a packaging signal on the gene of interest will get...
transferred to the viral plasmid. So in first generation vectors only one recombination event is required to obtain a fully replication-competent virus. Therefore, later-generation lentiviral vectors were created that involved splitting up these plasmids in order to reduce the chances of developing replication-competent lentivirus vector.

Second generation lentivector production involves a 3-plasmid transfection: the main viral plasmid, which produces all the viral proteins to make the virus particle; a second plasmid required to make the envelope of the virus vector (usually the vesicular stomatitis virus [VSV] G envelope), and the third plasmid contains the transgene.

A 3rd generation vector was then generated to increase the safety profile further by using a 4-plasmid approach. Basically the first plasmid that encodes the viral genes is split, resulting in 2 plasmids that have the viral genes, the 3rd plasmid expresses the VSV-G envelope gene, and the 4th plasmid is the transgene plasmid. That’s the big difference between the 2nd and 3rd vectors.

There’s also a 4th generation lentiviral vector that involves a 6-plasmid transfection. For that you need to amplify and transfet 6 plasmids which I believe leads to loss in efficiency. If you consider the efficiency factor, to get virus produced using a 4th generation system you have to make sure the packaging cell gets all 6 plasmids, which is much more difficult than the 4 plasmid required with 3rd generation lent vectors. I’ve utilized the 2nd, 3rd and 4th generation lentivirus vectors and my preference is 3rd generation. In my hands I have found that the viral titer is not high enough in the case of 4th generation vectors and there are safety issues for 2nd generation vectors.

So that’s the difference between 2nd and 3rd generation vectors. In terms of what is better, most researchers are going with 3rd generation now, because of the safety element. Moving to the 4th generation has been slow because there are too many plasmids involved.

Q What are the main production steps in manufacturing clinical-grade lentiviral vector?

The first steps in manufacturing research-grade and clinical-grade vectors are very similar: You take a plasmid, transfet the cells and produce a virus. It’s the next step, the downstream processing and purification, that’s the key difference in the case of clinical-grade vectors.

The first requirement is removing the plasmid DNA in the viral preparation. Plasmid DNA is added to the packaging cells to make the virus vector, and so in your virus medium you’re going to have plasmid DNA freely floating around. Removal of the free DNA requires treatment of the virus containing medium with enzyme (Figure 2).

The second requirement is the elimination of cellular debris or host cell proteins. The cells you use for virus production are human embryonic kidney cells, HEK293 cells. You’re going to get cell debris, and protein as well, that could be toxic to the individual when you administer to the patient.

There are standards set by each health agency, which has the limits you can have within a preparation for clinical grade lentivirus vector, whereas for research grade you don’t have to go through all these purification steps.
Q Why do we use HEK293 cells for virus production?

HEK293T cells are a cell type that are easily transfectable with plasmids and can express plasmids/proteins at very high levels and that's the key advantage. There are additional advantages like the HEK293T have a hexamer protein, called the SV40 large T antigen, which actually helps increase the overall plasmid expression and hence protein expression. This results in really robust expression of proteins, which is an advantage if you want to make high quantity of virus.

Another quality of the 293T cells is that there are certain components in HEK293T that help are essential in making adenovirus and AAV vectors. The HEK293T cells contain E1A and E1B, two genes with helper function for making adenovirus vectors and AAV’s.

Q There are a number of bottlenecks within the manufacture of cell and gene therapies including vector production. What do you see as the main production challenges behind these?

If we take it stepwise, the first challenge is one of scalability. On a small scale you're making maybe 1-2 litres of virus. When you scale up to say...
50 litres, the amount of plasmid you need is much higher and you have to make sure it’s clean and doesn’t contain contaminants such as endotoxins.

One way to get around it, is to make stable cell lines. This requires the expression plasmids to be stably introduced within the packaging cell, and the way you express those plasmids is you induce them with a drug, and they will start expressing the specified viral genes/proteins.

In this case, you still have to amplify and transfect the transgene plasmid each time you are making lentivirus vector. Another way to make a stable cell line that has all your plasmids and so each time you want to make a scale-up version of the virus, you don’t have to do additional transfections, you just scale up the cells.

A downside is you’re adding drugs to the stable cells to induce expression of plasmids. So when you go through the purification process you need to make sure you get rid of the selectable drug so that there’s no trace of it or it meets the requirements set by health agencies.

Another key challenge is that you lose a lot of the virus during the whole downstream purification process, potentially up to 70%. That’s a huge problem in manufacturing.

The key to this is make sure you make a really good batch of virus, because you’re going to expect some losses in the purification process. You can do a sample test during your downstream purification to see how much virus is there and estimate your loss within a batch, but obviously from one batch to the next there will be variations.

This is definitely a crucial bottleneck within manufacturing and I know the industry is trying to improve the equipment used for downstream processing to reduce this inefficiency.

Q One possible approach to reduce vector manufacturing costs is to create stable cell lines as you mentioned. What does this process involve?

It’s not too difficult and in fact there are some labs that have already produced stable cell lines and are going through the process now of testing the stability. For example if you want to make a stable cell line using a third-generation vector, you take the 4 plasmids, introduce them individually to HEK293T cells, and then you select for the cells that express all 4 plasmids at a very high rate.

The first plasmid will have a drug resistance gene encoded in its sequence. Addition of drug will kill off HEK293T cells that don’t have the plasmid and will maintain the cells that do have it. Each cell will express the drug-resistant gene at different rates and therefore a screening process needs to be performed where you can select for the cells that have the high rate of plasmid expression. Following this, the cells need to be transfected with the second and third plasmid to make a stable packaging cell line. Although it is time consuming to make this initial stable packaging cell line, once you have it, you can freeze it down and thaw it to use it whenever you want. And when you do need to use this cell line, you now only have to transflect one additional plasmid – your transgene.
Some groups are now moving towards making a stable packaging cell line that has all the four plasmids: 2 viral plasmids, VSV-G envelope plasmid, and the transgene plasmid. So if someone wants a clinical-grade lentivirus, you just need to thaw out the cells and transfer them to a bioreactor to start obtaining the virus. Even after a few years, if you want to scale up and make more of the same lentivirus vector you don’t have to go through the whole transfection process. You would just simply thaw out the cells and start producing virus vector, and that’s going to be a crucial advancement to improving manufacturing efficiencies and therefore cost.

In Europe, the group headed by Luigi Naldini in Italy, are now manufacturing clinical-grade lentivirus and have successfully utilized it in two disease indications. There are more companies that are coming on board now to manufacture clinical-grade lentivirus as demand for virus vectors increases. In fact, the demand is outstripping current manufacturing volumes. One of the goals we are trying to achieve here in Canada is to build up manufacturing capabilities and capacity to produce clinical-grade lentivirus and other virus vectors.

And what about the move to closed systems and automation in tackling some of the challenges and bottlenecks in vector manufacturing?

In the context of vector manufacturing, closed system refers to the use of bioreactors. Right now a lot of what we do is based on adherent cell cultures. For example, on a small scale we use a tissue culture flask and the cells stick to the surface. In some larger scale processes you can use bioreactors that have what’s called surface membrane that the cells attach to.

One of the advancements moving forward is to utilize suspension cell cultures technologies. This involves the use of large flask/container that have stable packaging cells swirling around. You add nutrients and media to grow the cells. The process results in a high production rate with increased efficiency and reduced cost as well, because you don’t have to make these bioreactors with multiple surface areas.

What opportunities are on the horizon in terms of new developments in vector manufacturing?

I think some exciting developments in lentiviral and viral vector use in general is gene editing. You can package the CRISPR gene editing elements into a lentivirus vector and target those cells that you want to correct. This is the beauty of lentiviruses and whilst there is still a lot of work to be done in increasing CRISPR gene editing efficiencies, it’s going to be exciting to see how this CRISPR-lentivirus vector and other CRISPR virus vector technologies pushes the field forward.

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Mouse mammary tumor virus-based vector for efficient and safe transgene delivery into mitotic and non-mitotic cells

Stanislav Indik

Retroviruses are cellular parasites that have evolved to deliver and integrate their nucleic acids to the host chromosomes. Therefore, they have been converted into gene delivery vehicles (vectors) and used for the effective transduction of genes in tissue culture cells as well, as for therapeutic gene transfer approaches. Until recently, vectors derived from mouse mammary tumor virus (MMTV) were inefficient in gene delivery. However, recent advances in betaretrovirology revealed the underlying obstacles that prevented the conversion of MMTV to an efficient gene delivery vehicle and allowed the construction of a high-titer MMTV-based vector system with a split genome design. The vector combines several desirable properties of retroviral vectors such as random integration into the host chromosomes and the capability to transduce genes into non-dividing cells. This article will discuss the latest developments and the remaining challenges in utilizing MMTV-based vectors in gene delivery.

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Detailed knowledge of retrovirus biology, including an understanding of the retrovirus life cycle, genome structure and virus particle organization, paved the way for the development of various retrovirus-based technologies for the delivery of gene or protein delivery into cells in vitro as well as in vivo [1-3]. Due to the unique characteristics of retroviruses, retrovirus-based means of gene or protein delivery are superior (with respect to efficiency) to non-viral transfer methods.
genes or proteins to target cells. This includes the generation of: integration-competent retrovirus vectors; integration incompetent episome transfer vehicles; mRNA delivery tools; and finally protein transfer vector particles. The different technologies can also be combined to achieve co-delivery of a protein of interest, together with an RNA or a donor DNA template.

Retrovirus-based vectors have been developed from members of all seven genera of the Retroviridae family. The most commonly used vectors in the laboratory and clinical practice were developed from gammaretroviruses (mostly derived from murine leukemia virus [MLV]) and lentiviruses (mostly derived from human immunodeficiency virus type-1 [HIV-1]). They are, in most cases, used for long-term transgene expression and permanent cell modification. Historically, gammaretroviral vectors were the first vectors to be used in gene therapy clinical trials. Due to a simple genome organization, knowledge of the complete MLV sequence and the establishment of a stable packaging cell line for the production of replication-incompetent retroviral particles, MLV-based vectors were used to correct genetic defects of immunocompromised patients [4–6]. Stably corrected immunological phenotype in the vast majority of the patients confirmed applicability of retrovirus-based gene delivery for gene therapy applications. However, these clinical trials also uncovered drawbacks of the early generation MLV-based vectors; namely, their potential genotoxicity leading to adverse effects that culminated in tumorigenesis in some human patients [7]. Since then, the safety profile of MLV-based vectors has been markedly improved. The long terminal repeats (LTRs) have been modified such that after vector integration two promoter- and enhancer-less LTRs flank the transgene of interest whose expression is driven by a heterologous promoter [8]. Furthermore, insulator sequences have been inserted into the residual U3 regions of the 3´LTR to further reduce any undesired effects of insertional mutagenesis. Great effort has been invested into the characterization of the undesired propensity of the gammaretroviral vectors to integrate in the vicinity of the promoter and enhancer regions in the host chromosomes. Cellular BET proteins were found to interact with MLV integrase and direct the virus integration into the transcription start sites [9–11]. Understanding of the underlying mechanism responsible for the non-random promoter-directed MLV integration profile may pave a way to re-directing integration to safer genomic loci. Another limitation of gammaretroviral vectors is their inability to transduce genetic material to non-dividing cells. This stems from the inability of the MLV pre-integration complex (PIC) to traverse the nuclear pores [12]. Thus, breakdown of the nuclear membrane during mitosis is essential for entry of the MLV DNA into the host genomes. Most tissues consist of differentiated, non-dividing cells, therefore MLV-based vectors are not applicable for gene delivery to these tissues.

This restriction does not apply to vectors derived from lentiviruses. They can cross the intact nuclear membrane via the nuclear pore complexes and access the host cell chromatin of non-mitotic cells. The ability of lentiviral vectors to
transduce non-dividing cells is given by the fact that HIV-1 and other lentiviruses can replicate in certain quiescent or terminally differentiated cells such as macrophages or microglia [13,14]. The lentiviral PICs carry karyophilic signals mediating interaction with the components of cellular nuclear import machinery such as TNPO3 and RANBP2 proteins leading to translocation of PICs across the nuclear membrane [15,16]. Due to this property, lentivirus-based vector have been developed to transduce genes into hard-to-transduce cells including neurons and hematopoietic stem cells [17,18], and have thus become the most popular vectors for use in retrovirus-based gene therapy trials. Apart from their ability to transduce cells regardless of cell cycle status, lentiviral vectors are also commonly used due to their integration pattern, which is distinct from that of gammaretroviruses. Specifically, lentiviruses do not preferentially integrate near enhancers, transcription start sites, CpG islands and DNase I hypersensitive regions. Instead, their integration is targeted to the body of active transcription units [19,20]. Up to 80% of the newly integrated proviruses can be found in genes. This targeting is achieved through interaction of the HIV-1 integrase with a transcriptional coactivator – lens epithelium-derived growth factor (LEDGF/p75) [21–23]. Although the integration within genes is considered to be less genotoxic than integration directed to promoter/enhancer regions, it still represents a considerable risk of causing malignant transformations. Thus, vectors exhibiting a neutral integration pattern may represent an alternative to current commonly used platforms. One of the recently developed vectors integrating randomly into the host genome is a vector derived from avian sarcoma-leukosis virus (ASLV) – alpharetroviral vector (AVV) [19,24]. Another recently developed vector with a random integration profile is a vector derived from the prototypic betaretrovirus, mouse mammary tumor virus (MMTV) [25,26].

MOUSE MAMMARY TUMOR VIRUS BIOLOGY

MMTV was discovered in the late 1930s as a virus transmitted from infected mothers via milk to newborn mice [27]. During milk-borne infection, the virus first infects dendritic cells (DCs) in the small intestine and Peyer’s patches and then spreads to T and B cells [28]. A MMTV accessory protein, superantigen (Sag), is presented by the MHCII proteins on the surface of the infected B cells and DCs to T cells. The Sag-activated T cells divide, secrete cytokines that stimulate the infected B cells for proliferation and recruit additional T and B cells to the lymphoid compartment. The amplification of pools of the infected lymphocytes is critical for the virus to spread to its ultimate target – the mammary gland [29,30].

The genome of MMTV, much like the genome of other retroviruses, encodes the structural components of virions (matrix/capsid/nucleocapsid), the enzymes required for virus replication (reverse transcriptase/integrase) and the envelope protein responsible for virus binding to the cell surface and its entry (Figure 1). Additionally, MMTV encodes at least three accessory non-structural proteins that facilitate virus replication in the
host. The MMTV genome is 9 kb in length and like all retroviruses is flanked by the 5´- and 3´ LTRs. The LTRs are exceptionally long (1.4 kb) because they contain an open reading frame encoding the viral accessory protein Sag. In addition, the LTR carries a number of transcription factor binding sites that determine tissue-specific and hormone-dependent (glucocorticoid; progesterone) virus gene expression.

At least five transcripts are generated from the MMTV genome. A full-length, unspliced RNA serves either as the virus genome that is packaged into viral particles or as the transcript translated into Gag, Gag-Pro and Gag-Pro-Pol polyproteins (Figure 1). The production of the latter two polyproteins is dependent on a frameshift suppression during which the ribosome must back up by one base to continue translation in the alternate frame. The efficiency of frameshifting seems to be higher for MMTV RNA than for RNAs from other retroviruses (e.g., ASLV), because the equivalent amount of the Gag-Pro-Pol precursor is synthesized even though two frameshift events are required [31].

As with other complex retroviruses, MMTV encodes several accessory proteins that affect the immune system of the host (e.g., Sag protein) and facilitates

**FIGURE 1**

Schematic representation of the MMTV proviral genome and the MMTV RNAs.

The full length RNA (9 kb) serves as the virus genome and mRNA. It contains a 5´ cap and a 3´ poly-A tail and can be translated to Gag, Gag-Pro and Gag-Pro-Pol polyproteins that are processed by the virus protease (PR) to matrix (MA), capsid (CA), nucleocapsid (NC), nucleocapsid-dUTPase (NC-DU), reverse transcriptase (RT) and integrase (IN) proteins. The singly spliced mRNA encodes gp73/Env proteins that are processed by furin protease to surface (SU, gp52) and transmembrane (TM, gp36) subunits. The doubly spliced mRNA encodes the regulator of MMTV expression, Rem that binds to RmRE and facilitates nuclear export of the unspliced RNA. The superantigen protein (Sag) is encoded by the 3´ end of the genome.
replication of MMTV in cells (Figure 1). A deoxyuridine-triphosphatase (dUTPase [DU]) is encoded by the full-length transcript and translated in the pro frame. The enzyme, which catalyzes hydrolysis of dUTP to dUMP and diphosphate, is produced following proteolytic cleavage of the Gag-Pro and Gag-Pro-Pol polyproteins. The dUTPases remove dUTPs from the deoxynucleotide (dNTP) pool thereby reducing the probability of incorporation of this base into DNA during reverse transcription. It is believed that the role of the enzyme is especially important in cells with a low concentration of dNTPs such as non-dividing cells (macrophages, DCs).

MMTV also encodes an accessory protein analogous to the HIV-1 Rev: Rem. Rem is encoded by doubly spliced mRNA and, like Rev, facilitates the transport of unspliced viral transcripts containing the Rem responsive element (RmRE) from the nucleus to the cytoplasm. Thus, Rem is a crucial regulator of the expression of viral genes.

MMTV entry into cells is mediated by the Env protein encoded by a singly spliced mRNA. The synthesized Env (gp73) polyprotein precursor is cleaved by cellular furin protease into surface (SU, gp52) and transmembrane (TM, gp36) subunits. The gp52 binds to the mouse transferrin receptor 1 (TfR1) that is then used as a virus entry receptor [32]. TfR1 is a ubiquitously expressed carrier protein used for the import of iron into the cell. Importantly, entry of MMTV to cells, mediated by gp52-TfR1 interaction, is rather inefficient [32,33]. Thus, the usage of the MMTV Env in virus vectors is limited.

DEVELOPMENT OF MMTV-BASED VECTOR SYSTEMS

The above described attributes led to the development of the third generation of MMTV-based vectors. It differs from the early generations in that it carries a number of safety features. Additionally, the third-generation vectors can be produced at markedly higher titers comparable to lentiviral vectors.

Historically, the first described MMTV vectors were derived from a complete molecular clone. They were replication deficient and carried a marker expression cassette either in the Pol/Env coding regions or in the 3’LTR. The vector particles were produced by transfecting the vector construct into MMTV-infected helper cells or, in case of the complete molecular clone carrying the transgene in the 3’LTR, into cells lacking MMTV sequences [34–37]. A split-genome design (second generation) was for the first time used by Salmons and colleagues who developed a packaging cell line expressing Gag-Pro-Pol and Env from a heterologous RSV promoter [38]. Further improvement of the split genome system was achieved by introducing a hybrid CMV-R/U5 promoter into the vector construct to completely overcome the need for hormonal stimulation that is required for the RNA synthesis from the authentic MMTV promoter [39]. In the early generation vectors, the authentic MMTV Env protein was used to mediate virus entry. However, as mentioned above, the MMTV Env-mediated vector entry resulted in low virus titers in murine as well as non-murine target cells. The titer did not markedly improve when an MMTV Env mutant, optimized for
entry to human cells, was used [33]. Thus, the development of a high-titer vector necessitated the usage of pseudotyped vector particles [Unpublished Data]. Another drawback of the first- and second-generation vectors was insufficient transport of viral RNAs to the cytoplasm. The viral proteins were not produced at high levels resulting in a low infectivity of the MMTV vectors (titer $\leq 10^3$ transduction units [TU/ml]) [35]. This resulted from the fact that MMTV was considered to be a simple retrovirus analogous to MLV. Discovery of Rem and its function opened up new possibilities for vector improvements [40–43].

High-titer vector preparations (>10$^5$ TU/ml) were obtained by developing the third-generation MMTV vector system. The vector genome and helper functions (Gag-Pro-Pol, Env, Rem or Rev) were expressed from four separate plasmids containing a strong constitutive promoter (CMV or RSV) (Figure 2). To produce vector transcripts, the U3 region of the 5'LTR was replaced with the RSV promoter such that transcription initiates at the authentic transcription start site. This modification resulted in a vector where high-level transcription is independent of the presence of glucocorticoid hormones. To further improve vector efficiency and safety, the cis-acting sequences required for efficient gene transfer were mapped and nonessential regions

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**FIGURE 2**

Schematic representation of the four plasmid expression system for generation of a pseudotyped MMTV-based vector by transient transfection of HEK293T cells.

Transfer vector

RSV U3 → R → U5 → gag → RRE → CMV → EGFP → WPRE → RmRE → SIN LTR

Packaging construct

CMV → gag → pro → pol → RRE → bg pA

Rev-coding plasmid

RSV → rev → BHG pA

Env-coding plasmid

CMV → env → BHG pA

A constitutive promoter (CMV or RSV) drives the expression of vector RNA and helper proteins. A large portion of the 3'LTR has been deleted resulting in a self-inactivating (SIN) vector that loses the capacity to drive the expression of vector RNA following reverse transcription. The expression of transgene (EGFP) is driven by an internal constitutive promoter. Stability of the vector RNA is enhanced by the woodchuck hepatitis post-transcriptional element (WPRE) and the nuclear export is facilitated in the presence of Rev by a cis-acting element - Rev-responsive element (RRE). The expression of the structural and enzymatic components of the MMTV vector is also facilitated by the Rev-RRE interaction. The Rev and Env (VSV-G) proteins are expressed in trans.
were removed. This included the enhancer region in the 3’LTR, resulting in a self-inactivating (SIN) vector lacking the sag gene. Special attention was paid to preserving signals involved in the regulation of mRNA polyadenylation, such as CPSF, CstF, CF Im and U-rich region, as it is generally known that some SIN vectors suffer from leaky transcriptional termination [44,45].

To stabilize vector RNA and facilitate export from the nucleus, two post-transcriptional regulatory elements were inserted into the vector. The woodchuck hepatitis virus post-transcriptional regulatory element was inserted upstream of the 3’LTR and the Rev responsive element (RRE) was placed downstream of the packaging signal (ψ) that has been mapped to a region spanning the 5’UTR/gag junction [46,47].

As mentioned above, the MMTV Env is not efficient in mediating vector entry. Therefore, a heterologous envelope was used to enhance vector infectivity. The vesicular stomatitis virus glycoprotein (VSV-G), previously shown to support the infectivity of MMTV cores, was employed to pseudotype vector particles [39]. The pseudotyping combined with the implementation of post-transcriptional regulatory functions resulted in a marked increase of vector titers relative to the previous generations (100–1000-fold increase; titer >10⁵ TU/ml). Additionally, the pseudoparticles were stable and they could be efficiently concentrated by ultracentrifugation leading to further increase in vector titers (titer >10⁷ TU/ml) [26].

A high-vector titer is essential for clinically relevant applications as well as to gain deeper insight into the virus biology. We used the third-generation vector to investigate whether MMTV, like HIV-1, is capable of infecting non-dividing cells. In vivo, MMTV infects terminally differentiated dendritic cells and uses dUTPase to avoid misincorporation of dUTPs into the nascent DNA during reverse transcription. This evidence suggests that MMTV infects non-mitotic cells. We used cell cycle-arrested tissue culture cells (a high dose of γ-irradiation) and unstimulated hematopoietic stem cells (human CD34+, mouse Lin) to test if the virus can infect cells regardless of cell cycle progression. Importantly, the MMTV vector transduced the eGFP gene into the non-dividing cells with the same efficiency as the control HIV-1 vector. Thus, we concluded that MMTV infects non-dividing cells [26].

However, in contrast to HIV-1 vector, monitoring of integration sites showed that the vector derived from the prototypic betaretrovirus has a more favorable integration pattern. Specifically, the vector exhibited in dividing and non-dividing cells a close-to-random integration site distribution [26]. This result confirmed previously observed unbiased integration profile for the wild-type virus [25,48]. Thus, in contrast to lentivirus- and gammaretrovirus-derived vectors, most of the integrations occurred in non-coding regions of the genome. The underlying mechanism responsible for this integration pattern is currently unknown. MMTV seems to use a different nucleus entry pathway than HIV-1. This pathway is independent of a nuclear import factor for serine/arginine-rich proteins (TNPO3) that was shown to interact with HIV-1 CA and mediate the HIV-1 nuclear import
Moreover, MMTV does not interact with another cellular factor that directs localization of the HIV-1 PICs in the host nucleus, LEDGF/p75 [Data not shown]. Work to identify a cellular factor that tethers the MMTV PICs to the host chromatin is currently ongoing, as characterization of this chromatin tethering mechanism may be beneficial for the improvement of the integration profile, and thus may lead to targeted integration.

**TRANSLATIONAL INSIGHT**

The unique properties of MMTV have been exploited for the development of a new platform for efficient gene delivery to target cells. The vector combines several safety features such as: split genome design that minimizes the possibility of reconstitution of a replication-competent virus; deletion of a large portion of the 3’LTR, resulting in a SIN vector that has a low potential to transactivate nearby genes and does not contain <i>sag</i> gene; and the random integration profile that decreases the chance of unwanted integration into genes that is commonly observed with other vectors.

Additionally, MMTV vector does not require mitosis for efficient transduction. This property makes the vector system superior to many other vectors that require mitosis and their therapeutic application is thus limited to mitotic cells. The ability of MMTV to transduce long-term repopulating cells has been demonstrated, providing the basis for <i>ex vivo</i> applications such as hematopoietic stem cells gene therapy. To date, no <i>in vivo</i> experiments have been performed with the new MMTV vector system. These trials are needed to demonstrate lower genotoxicity of the vector compared to other vectors. Such studies will hopefully establish MMTV vector as an efficient and safe alternative to gammaretroviral and lentiviral vector systems.

**FINANCIAL & COMPETING INTERESTS DISCLOSURE**

The author has no relevant financial involvement with an organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock options or ownership, expert testimony, grants or patents received or pending, or royalties. No writing assistance was utilized in the production of this manuscript.

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Repurposing lentiviral vectors for delivery of genome editing tool kits

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Viruses are natural carriers of genetic information and enzymes that operate in infected cells. For years, gene therapists have exploited the gene-carrying capacity of virus-derived vectors, and lentiviral vectors based on HIV-1 have become key tools in biomedical research and genetic therapies. Inspired by the capability of the virus to package and transport its own enzymatic tools for reverse transcription and integration, we and others have engineered lentiviral particles incorporating and delivering foreign hitch-hiking proteins. Here, I describe attempts to repurpose lentiviral vectors for delivery of genome editing tool kits. Administration of protein by lentiviral particles results in effective and short-lived protein activity in virus-treated cells even with low concentrations of protein, supporting further development of virus-based protein delivery strategies for safe genome editing applications.

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For years the gene therapy community has been striving to engineer gene vehicles facilitating persistent, preferably life-long, expression of therapeutic transgenes. With the development of stem cell genetic therapies using human immunodeficiency virus 1 (HIV-1)-derived lentiviral vector systems [1–4] and therapy of hemophilia B exploiting vectors based on adeno-associated virus (AAV) [5], just to mention a few among many emerging treatments, it is fair to say that the perseverance of vector engineers has paid off. Much is still to be learned; whether expression of virally delivered transgenes can persist for decades in patients is not known, but current vector systems featuring optimized promoters, insulator elements, codon-optimized transgene cassettes, and the unique capacity of viruses to deliver genetic cargo with efficacy and specificity may support long-term gene expression, lending support to further clinical translation.

Who would have thought, after all these years, that delivery approaches allowing high, but only short-term, gene expression would be suddenly a key interest to the
gene therapy community? As classical gene therapy approaches continue to develop, it will not have escaped anyone’s attention that genome editing and tools to engineer the genome are currently revolutionizing genetics and are quickly moving toward clinical use. Unique molecular tools that operate within the genome, like Cre and Flp recombinase, have been around for a while, but these are relevant as research tools only and not for clinical purposes due to the lack of compatible sequences in mammalian genomes. With the discovery of transposases capable of mobilizing genes with high efficacy in human cells, it became possible to integrate transgenes into genomes using nonviral delivery approaches [6,7], essentially what was until then achieved by retroviral vectors carrying their own integrase proteins. Although the integration profiles of the most popular transposon vector systems based on the Sleeping Beauty and piggyBac transposons were found to differ markedly from the gene-based integration profiles of gamma-retroviral vectors and lentiviral vectors [8,9], this type of intervention lacks control and does not allow targeted insertion or engineering of the genome at predetermined positions. The game changed with the invention of zinc-finger nucleases (ZFNs) [10], consisting of a DNA-binding domain, designed to recognize a double-stranded DNA duplex with a certain sequence, fused to the endonuclease domain of the restriction enzyme FokI. Only by co-administration of two ZFNs recognizing two neighboring sequences a FokI dimer is formed upon binding of the ZFNs to the DNA, leading to creation of a double-stranded break (DSB) at this genomic position. It is now textbook knowledge that cellular DSB repair processes based on non-homologous end-joining (NHEJ) introduce indels at the cleavage site or that homology-directed repair of the damaged DNA using homologous DNA as a sequence donor may restore the sequence and potentially correct nearby mutations. This opened a world of opportunities, but it turned out to be challenging to fulfill the full potential of the ZFN technology due to difficulties related to engineering of ZFNs, leading to high costs and reduced accessibility and availability of effective ZFN pairs. Some of these challenges were circumvented with the invention of Transcription activator-like effector nucleases (TALENs) containing customizable array of repeats that can be assembled using easy-to-follow design guidelines [11,12]. However, with the discovery of CRISPR-directed adaptive immune responses in bacteria [13] and the implementation of RNA-guided endonucleases in human cells [14], targeted genome editing and introduction of knockout mutation with the speed of light has become a standard technique in molecular genetics research. Hence, cellular delivery of the endonuclease (typically Cas9 from Streptococcus pyogenes) guided by a single guide RNA (sgRNA) molecule facilitates firm studies of causal linkages between genetic variation and cellular phenotypes, but numerous other applications of the CRISPR system, far too many to discuss here, appears with constant pace. Although far from trivial, one of the ultimate goals is to deliver components of the CRISPR system to individuals aiming at treating disease by genomic editing directly in the patient.
From old-school, but still highly relevant recombinases and transposases, to ZFNs and a wide variety of advanced and user-friendly RNA-guided DNA and RNA endonucleases, the genomic tool box is rapidly expanding in the hands of genome entrepreneurs. Common for the use of all these tools is that efficient delivery across cell and nuclear membranes is essential for efficacy. As new tools appear, they are traditionally tested in proof-of-principle studies showing efficacy after delivery of plasmid DNA or viral vectors encoding the particular protein. Using methods based on intracellular production of the relevant tool, the genomic engineering community has directly benefitted from years of accumulated experience with gene therapy vectors aiming at establishing persistent and highest-possible levels of gene expression. However, long-term expression of recombinases or DNA endonucleases may potentially be harmful to the genome.

Intuitively, an optimal vehicle for delivering genome editing tools results in a short-lived boost of enzyme activity, long enough to do the job and short enough not to cause unwanted genomic modifications or off-target effects. At the same time, the short boost should not compromise specificity and safety due to momentary far too high protein and activity levels. These goals have an inherent contradiction in terms, since high-efficacy delivery is likely to cause unnecessary high levels of protein that may last longer than required for the desired genomic modification to occur. Therefore, editors of the genome are facing a new set of challenges calling upon new ways of delivering nucleic acids and proteins to cells or tissues.

Transfection of in vitro-transcribed mRNA encoding transposases or endonucleases is a well-established approach, which results in short-term intracellular expression due to the short half-life of mRNA. This method has been successfully used for delivery of transposases, ZFNs, and TALENs to primary T cells [15–17] and hematopoietic stem cells [18] and is clinically applicable [19]. Despite evidence of in vivo applicability based on transfection of mouse liver by hydrodynamic injection [20,21], administration of mRNA seems to have limited potential for in vivo use. The same may be true for delivery of recombinant protein, although delivery of in vitro-synthesized spCas9 protein in ribonucleoprotein (RNP) complexes consisting of recombinant spCas9 and in vitro-synthesized sgRNA is now well established in cultured cells. Delivery of recombinant protein can be assisted also by conjugation with cell-penetrating peptides (CPPs) [22,23], but yet other proteins, like the Sleeping Beauty and piggyBac transposase do not seem to be compatible with in vitro production methods, and earlier attempts to deliver such proteins to cells have been unsuccessful [24,25].

In the past few years, a new protein delivery approach based on the incorporation and transport of heterologous proteins in the HIV-based lentiviral particles has emerged. As it seems, lentivirus particles are sufficiently flexible to allow packaging and processing of nonviral proteins, and proteins ranging from transposases and designer nucleases to RNA-guided endonucleases can be delivered to cells as cargo transported by lentiviral couriers. In this Expert Insight article, I review the repurposing of lentiviral vectors for delivery of proteins for genome engineering.
LENTIVIRAL DELIVERY OF VECTOR-ENCODED GENOME EDITING TOOLS

Lentiviral vectors based on HIV-1 were developed for delivery of gene expression cassettes using strategies that were basically adapted from gene vectors based on genetically simpler viruses like the gamma-retroviruses with murine leukemia virus (MLV) as one of the primary drivers in the field. Production of the first generation of lentiviral vectors was based on co-transfection of the transfer vector plasmid with plasmids encoding the vesicular stomatitis virus glycoprotein (VSV-G) and the viral proteins including all accessory proteins like Rev and Tat. The resulting vectors were found to transduce nondividing cells [26] and, thus, offered an attractive alternative to MLV-based vectors. Due to the risk of generating replication-competent HIV-1, both the transfer vector and packaging plasmids later underwent considerable modifications. Hence, for production of third-generation vectors, now widely used in laboratories worldwide, expression of full-length vector RNA from the transfer vector plasmid is driven by a CMV promoter rather than by the natural HIV-1 promoter. This means that transcription does not require the Tat accessory protein. Also, natural HIV-1 promoter sequences have been deleted from the U3 region of the vector, ensuring that the viral promoter is not present in the reverse-transcribed and eventually genomically inserted vector sequence [27,28]. This type of vector is referred to as a self-inactivating (SIN) vector. Equally important, the safety of the system was further improved by removal of sequences encoding the accessory proteins from the packaging construct. The gene encoding the Rev protein, which is required for proper nuclear export of the vector RNA, was moved to a separate plasmid, resulting in a production scheme based on co-transfection of four plasmids, (i) the SIN vector plasmid encoding the transgene of interest driven by an internal promoter, (ii) the GagPol-encoding packaging construct, (iii) a Rev expression plasmid, and (iv) a plasmid encoding VSV-G. Limited sequence similarity between these four plasmids and the absence of a subset of viral genes rendered production of replication-competent HIV-1 very unlikely [29]. Even with such modifications requiring co-transfection of several plasmids, production schemes are efficient, and co-transfection of an easy transfectable cell line, like HEK293T, results in standard production of millions and millions of virus particles each loaded with a vector RNA dimer. Due to the broad tropism and high gene transfer efficacy, VSV-G-pseudotyped lentiviral vectors have become a state-of-the-art tool for delivery of gene expression cassettes to cell lines and hard-to-transfect primary cells. It is beyond the scope of this article to cover the many aspects and applications of lentiviral gene delivery, but genetic therapies based on this technology are obviously attracting particular attention [30,31]. Emerging evidence supports clinical use of lentiviral vectors for correction of hematopoietic stem cells for treatment of primary immunodeficiencies, leukodystrophies, and hemoglobinopathies [3,32–34], and corrective ex vivo gene therapy was recently approved in Europe for treatment for ADA-SCID [35,36]. With big pharma moving into the
gene therapy area, one example being GlaxoSmithKline acquiring the commercialization rights to Strimvelis for ADA-SCID treatment, additional treatments are likely to gain approval in the near future. Notably, this first licensed gene therapy using an integrating vector system is based on a gamma-retroviral vector system. A comprehensive overview of current hematopoietic stem cell gene therapies in clinical development can be found in a recent review [37].

The capacity to transfer and genomically integrate genetic information makes lentiviral vectors powerful carriers of genome editing tools. However, in contrast to the goals of conventional gene therapies, prolonged expression of endonucleases is rarely desired for therapeutic genome editing purposes. Gene transfer by integrase-defective lentiviral vectors (IDLVs; reviewed in [38]), vectors that carry an inactive integrase protein, does not lead to gene insertion, and, thus, allows only transient transgene expression in dividing cells. We have previously shown delivery of recombinases and transposases using an IDLV-based platform [39–41]. Also, IDLV-based delivery of ZFN expression...
cassettes has been demonstrated on several occasions [42–45], whereas a similar strategy for delivery of TALENs is not applicable due to frequent recombination between repeated sequences in the TALEN gene [46]. However, this problem can be overcome by altering codon sequences [47] or by expressing the TALEN protein from RNA delivered in lentiviral particles that do not support reverse transcription due to lack of a functional reverse transcriptase enzyme [48]. Other potential RNA delivery methods exploit an MS2-driven packaging system, allowing lentivirus-based transfer of heterologous RNAs [49]. For delivery of Cas9 and sgRNA cassettes, transfer by lentiviral vectors is now well established [50,51] and can be harnessed for gene knockout or repair essentially as shown schematically in Figure 1. Exploiting the capacity to integrate the Cas9 gene as well as the sgRNA gene, such vectors are widely used for functional genomics and genetic screens based on genome-wide CRISPR-directed gene knockout [50–54]. However, data supporting effective IDLV-directed delivery of the CRISPR/Cas system is still lacking in the literature. Notably, episomal DNA intermediates formed in IDLV-transduced cells serve as effective templates for repair by homologous recombination, supporting the use of IDLVs as vehicles of genome editing repair substrates [42–44,55,56].

RE-THINKING LENTIVIRAL DELIVERY – NOW FERRYING PROTEINS

Try google ‘repurposing’ and somewhere on the Internet you will find that ‘repurposing is the art of finding new uses for old items’. That works for an old wheelbarrow carrying your garden flowers or a chair made of your old alpine skis. But does it work for lentiviral vectors? A few years back, we initiated work aiming at repurposing lentiviral particles for delivery of genome-modifying proteins like transposases and site-targeted endonucleases. Our ultimate goal was to explore the capacity of viruses to carry and transfer both scissors for genomic surgery and the donor patch for repair by homology-directed repair (Figure 2).

Early work aimed at tracking intracellular migration of the HIV core exploited fusion of a reporter protein, like GFP, to the Vpr accessory protein [57]. As previously reviewed in [58], a similar strategy was used for packaging of different types of proteins in lentiviral particles, but toxicity of Vpr in transduced cells is a potential problem [59]. Inspired by work of Jun Komano and coworkers, we decided to focus on protein incorporation strategies based on fusing proteins of interest to the lentiviral Gag and GagPol polypeptides. Initially, Aoki et al. fused β-lactamase to the N-terminal end of the Gag polypeptide and added the myristoylation signal of lyn to the N-terminal end of β-lactamase [60]. Enzymatic activity was detected in cells exposed to the resulting lentivirus particles, each loaded with about 5000 copies of β-lactamase. A similar approach was used subsequently to induce apoptosis in cells treated with lentiviral particles carrying caspase 3 protein fused to the matrix protein in the N-terminal end of Gag and GagPol [61].

Incorporation of genome-modifying proteins into lentiviral particles by fusion to the Gag and
GagPol polypeptides is based on the accumulation of Gag and Gag-Pol at the inner surface of the cell membrane during virus assembly. As depicted schematically in Figure 3, Gag and GagPol polypeptides, tagged N-terminally with a protein of interest, are expressed from a packaging construct transfected into virus-producing cells. Alongside this plasmid, the cells are transfected also with plasmids encoding the virus envelope protein (typically VSV glycoprotein) and the Rev protein (not shown). Also, plasmid DNA encoding packagable vector RNA carrying either a DNA transposon sequence or a donor sequence for homology-directed repair can be included in the transfection mix. Budding lentivirus particles consist of approximately 5000 Gag molecules and about 250 GagPol molecules [62], the latter generated only through a frameshift at the Gag-Pol junction. If vector RNA is expressed in the cell, a vector RNA dimer will be encapsidated in the particle through interactions between the nucleocapsid (NC) protein and the packaging signal located near the 5’ end of the RNA. Released virus particles undergo a maturation process, which is based on the cleavage of Gag and Pol domains into structural and enzymatic proteins, respectively. This process
FIGURE 3

Schematic representation of the basic principles of lentiviral protein transduction - from lentiviral particle production to protein release within transduced target cells.

POI refers to ‘protein of interest’ (e.g. transposase or endonuclease), which works in the genome to insert, knockout or repair genetic information. The figure illustrates production of protein-loaded virus particles, which also carry a dimeric vector RNA genome. Particles carry the D64V integrase variant, rendering them incapable of supporting integrase-directed insertion of reverse-transcribed double-stranded DNA into the genome of target cells. It is not known how virus-ferried proteins are delivered to the nucleus; a and b refer to trafficking mechanisms based on transport by the viral capsid and by diffusion, respectively. Virus particles can also carry a DNA transposon sequence or a transgene cassette flanked by appropriate homology arms for insertion by homologous recombination. We normally refer to such vectors as ‘all-in-one’ IDLVs. A similar strategy can be used to produce lentiviral particles, which do not contain vector RNA, but is loaded only with the protein of interest. Copyright BioInsights Publishing Ltd.
is catalyzed by the viral protease, which releases the foreign fusion protein as the core condenses in the maturating particle. Proper protein release is achieved by introducing a HIV-1 protease recognition site at the junction between the tagged protein and Gag. Upon uptake by endocytosis in transduced cells and subsequent endosomal escape, released proteins eventually enter the nucleus through unknown mechanisms that may involve migration as part of the virus core or diffusion through the cytoplasm.

**GENE INSERTION BY LENTIVIRAL PARTICLES LOADED WITH TRANSPOSASES**

With resurrection of the Sleeping Beauty DNA transposon back in the late 1990s [6] and evidence of effective piggyBac DNA transposition in mammalian cells [63], it became possible to insert transgenes into mammalian genomes using nonviral delivery platforms, typically plasmids encoding a transposon-embedded transgene cassette and the transposase. Others and we showed preclinical efficacy of gene delivery by DNA transposition in animal models [7,64–66], and efforts were later made to deliver the two-component vector system using viral vehicles [39,41,67]. Also, DNA transposition has been achieved by administration of in vitro-transcribed mRNA [15,20,21], but attempts to purify and administer recombinant Sleeping Beauty and piggyBac transposase protein have not been successful [24,25]. In proof-of-principle studies of lentiviral incorporation and delivery of protein, we therefore initially focused on loading of DNA transposases into lentivirus particles. Utilizing a third-generation packaging construct, as depicted in Figure 3, we produced virus particles carrying a hyperactive piggyBac transposase (hyPBase, originally described by Yusa et al. [68]) fused via a protease cleavage site to the N-terminus of Gag and flanked on the N-terminal side of the Lyn-derived myristoylation signal. Upon virus particle maturation, this stowaway protein was released from the polypeptide and induced high levels of DNA transposition in cells treated with the virus [69]. Notably, we also learned that virus particles carrying Gag/GagPol with an N-terminal fusion domain were not able to deliver and reverse-transcribe vector RNA. To circumvent this restriction, we generated mosaic viruses carrying wildtype Gag/GagPol as well as hyPBase-fused Gag/GagPol in each particle and demonstrated the capacity of such viruses to co-deliver vector RNA. In fact, by engineering vector RNA carrying the transposon sequence, which upon reverse transcription would become a substrate for DNA transposition, we observed effective mobilization of the transgene in cells treated with ‘all-in-one’ IDLVs carrying both hyPBase and the transposon. Such DNA transposition was evident in different cell types including a panel well-known cell lines and primary cells, like keratinocytes and dermal fibroblasts. By staining of virus-treated cells for the hyPBase (using an HA-tagged version of the transposase), we found only low levels of the transposase protein as opposed to the high levels detected in cells transfected with hyPBase-encoding
plasmid DNA. Still, levels of DNA transposition, as measured by colony formation after mobilization of a puromycin resistance gene, were equally high. Although still hypothetical, this could indicate that protein delivered by the virus does not rely on random diffusion but rather traverses the cytoplasm and the nuclear membrane through mechanisms that are supported by the intracellular movement of the viral core. If such mechanisms are indeed in play, this would argue that virus-delivered enzymes can be effective even at relative low intracellular concentrations.

GENOME EDITING BY LENTIVIRAL DELIVERY OF ZFNS OR CAS9 PROTEIN

Prior to the arrival of CRISPR on the genome editing scene, we investigated the potential of lentiviruses as carriers of designer nucleases [70]. Using a slightly modified strategy exploiting a packaging construct carrying the heterologous phospholipase C-δ1 pleckstrin homology (PH) domain for improved Gag/GagPol recruitment to the membrane, pairs of ZFNs were incorporated into lentivirus particles. ZFN-loaded virus particles were produced by cells transfected with two packaging constructs, each carrying a ZFN sequence fused to the gag gene via the PH domain. Incorporation and protease-dependent release of was verified for both ZFNs, and DNA cleavage leading to indel formation was obtained in cell lines and primary cells after delivery of ZFN pairs targeting a transgenic egfp locus, the CCR5 locus, or the AAVS1 locus. In case of the most effective set of ZFNs (targeting CCR5), targeted disruptions were detected in one fourth of the CCR5 alleles in a population of primary keratinocytes treated with ZFN-loaded lentiviral particles [70]. By co-packaging of vector RNA carrying a template for homologous recombination in ‘all-in-one’ IDLVs stuffed with egfp-directed ZFNs, repair of a mutated egfp report gene was achieved in more than 8% of the cells in a HEK293 cell-based model, indicating that protein and vector RNA could be effectively co-delivered. This approach was later utilized to achieve site-directed transgene insertion into the CCR5 and AAVS1-loci in hematopoietic progenitor cells and induced pluripotent stem cells (iPSCs) [71]. Notably, 34 out of 38 analyzed iPSC clones generated by treatment with ZFN-loaded lentiviral particles carried the reporter gene cassette inserted precisely by homologous recombination into the locus that was targeted by the ZFNs.

The optimal tool for genome work is immediately effective and short-lived. By administering the protein itself and not the source for production of protein, the genome-modifying activity will only last until the protein is diluted and degraded. Immunostaining of cells treated by lentiviral ZFN protein transduction showed detection of ZFNs already within the first hour after exposure to the virus and certified that the protein was gone (or at least not detectable) after twenty four hours [71]. Intuitively, such time restriction should improve the safety of the procedure and be beneficial as long as the activity is high immediately after delivery. In fact, comparing a transfection-based
ZN delivery approach and virus-directed ZFN delivery for targeted disruption of the CCR5 locus, we found reduced off-target cleavage within the neighboring CCR2 locus after protein delivery even under experimental conditions facilitating higher on-target rates than observed by plasmid transfection [70]. In support of this, next-generation sequencing of a number of potential off-target sites in cells harboring a targeted transgene insertion obtained through viral ZFN delivery did not show any sign of off-target cleavage [71].

Successful delivery of active protein by engineered lentiviral particles depends on proper cleavage of the Gag/GagPol polypeptides, leading to release of the protein during virus particle maturation. In case of both transposases and ZFNs, cleavage happens to occur specifically at the protease cleavage site between the fusion domain and Gag, but we have also seen examples of proteins that are cleaved internally by the viral protease, despite the apparent lack of a HIV-1 protease cleavage site at this particular position. Such internal cleavage was evident for egfp-directed TALENs, resulting in only few particles carrying the full-length protein and reduced targeted disruption rates in virus-treated cells [70].

The probability of proteolytic cleavage at positions inside the protein of interest supposedly increases with the size of protein, but we suspect that also the protein structure can be decisive for protease accessibility and potential cleavage. With the protein size in mind, we initially invested much effort in incorporating the Cas9 nuclease from Staphylococcus aureus (saCas9; [72]) into lentiviral particles using the above-described approaches. SaCas9 is 1053 amino acids long and, thus, 315 amino acids shorter than Streptococcus pyogenes Cas9 (spCas9). However, we only detected limited amounts of full-length saCas9 in mature lentiviral particles, resulting in low levels of targeted gene disruption in virus-treated cells expressing an appropriate sgRNA (Y Cai and JG Mikkelsen, unpublished observations). Surprisingly, however, spCas9 fused to the N-terminal end of Gag could be effectively packaged in lentiviral particles and was released from Gag primarily as a full-length 160 kDa protein during maturation of the particle [73]. It was estimated that 3 million particles contained a total of approximately 50 ng spCas9 protein. By transduction of primary T-cells first with a lentiviral vector encoding a CCR5-directed sgRNA and then with spCas9-loaded viral particles, indels in CCR5 were generated in 15% of the cells. Furthermore, co-delivery of spCas9 protein and a vector encoding a sgRNA directed against the CD4 gene in an ‘all-in-one’ format resulted in targeted CD4 disruption and reduced presentation of CD4 on the surface of HeLa-derived TZM-BL cells [73]. Also, by including a sgRNA targeting HIV long terminal repeats (LTRs), spCas9-loaded virus particles induced excision of the HIV proviral DNA in a T-cell line harboring a transcriptionally competent HIV-1 provirus [73].

**TRANSLATIONAL INSIGHT**

Over the last 20 years, lentiviral vector systems have been developed and optimized for gene
therapy applications. More than ever, it seems that therapeutic gene transfer using HIV-1-derived particles will benefit patients and therefore continue to attract attention of investors. For genome editing purposes, persistent expression of essential tools, like Cas9 and sgRNA, is attractive only for certain applications, like genome-wide CRISPR screens, and short-lived activity of site-targeted endonucleases is desired, at least for potential clinical use. For ex vivo applications, including editing in genomes of cultured stem cells, delivery of RNPs consisting of recombinant Cas9 protein and synthetic sgRNA stands out as a robust and relatively safe administration strategy [74,75]. For in vivo genome editing, however, it is not currently obvious how genomic tools are delivered effectively without compromising safety, although recent examples of delivering Cas9/sgRNA gene cassettes by use of adeno-associated virus-derived vectors show solid targeted gene disruption in mouse liver [72]

In attempts to repurpose lentiviral vectors as a delivery tool, others and we have studied the capacity of lentiviruses to encapsidate and transfer foreign protein of nonviral origin. In fact, the structure of the lentiviral particle seems more flexible than one should have thought considering the evolutionarily conserved structure and function of the enveloped capsid. In any case, the list of proteins successfully incorporated in lentiviruses is growing (see reference [76] for recent review), and different protein packaging strategies have turned out to be effective. Hence, proteins can be fused to the N-terminal end of Gag (as described above) or to the integrase protein in the C-terminal end of GagPol [77–79], or inserted between the matrix and capsid proteins in Gag [80]. Regardless of the configuration, solid and time-restricted protein activity is observed in cells exposed to protein-loaded virus particles. As a unique feature protein-loaded particles can carry also a template sequence for homologous recombination, allowing co-delivery of complete genome editing tool kits in cells that take up the virus.

Furthermore, by incorporating heterologous envelope proteins in the envelope surrounding the viral core particle, it is possible to pseudotype lentiviral vectors and alter the tropism [81]. Just like delivery of genes can be targeted to specific cell types, pseudotyping can be exploited also to target genome editing to specific tissues or subsets of cells. A recent report demonstrating delivery of ovalbumin into antigen-presenting cells using lentiviruses pseudotyped with measles virus glycoproteins (using Signaling lymphocyte activation molecule, SLAM, as the primary receptor) serves as proof-of-principle for cell-targeted protein delivery using protein-loaded lentiviral particles [80]. Such targeted delivery of genome-editing tool kits utilizing the capacity of virus particles to circulate and target cells may pave the way for in vivo engineering of the genome through processes that are both cell- and locus-specific. Hopes are that efficacy and safety can go hand-in-hand toward preclinical and eventually clinical translation of repurposed lentiviruses – now transferring protein.
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Foamy virus: an emerging viral vector for human gene therapy

Dr Jennifer E. Adair received her Bachelor of Science degree from Youngstown State University and her Doctor of Philosophy degree from Washington State University. She is currently an Assistant Member in the Clinical Research Division at Fred Hutchinson Cancer Research Center and a Research Assistant Professor in the School of Medicine at the University of Washington in Seattle. Her laboratory develops and translates gene therapy using hematopoietic stem cells as a treatment for genetic, malignant and infectious diseases. Specifically, her lab has translated successful drug resistance gene therapy to protect blood from otherwise myelotoxic chemotherapy used to treat solid tumors, functionally corrective gene therapy for Fanconi Anemia, X-linked severe combined immunodeficiency syndrome and Wiskott-Aldrich syndrome, and anti-HIV gene therapy in combination with drug resistance gene therapy for the treatment of AIDS-lymphoma. The lessons learned from these studies provide research directives to overcome remaining barriers to mainstream clinical application of hematopoietic stem cell gene therapy.

Q Foamy virus is more of a recent addition to the viral vector toolbox for gene therapy. Can you tell us a little about the biology of foamy viral vectors, and how they compare with other viral vectors?

The characterization of natural foamy virus vectors is largely described by the laboratories of Dr Maxine Linnial, Dr Ali Saïb and Dr Axel Rethwilm, as well as others. Foamy viral vectors are derived from the spuma retroviruses, an exogenous type of retrovirus. In nature, foamy viruses are endemic in non-human primates and other mammals. They are thought to be one of the oldest known retroviruses. They're...
believed to have co-evolved with their hosts over the last 60 million years. They can infect humans and induce lifelong persistent infections, but are apathogenic. Foamy viruses are a different subfamily compared to gamma retroviruses and lentiviruses, which are ortho-retroviruses.

In general, the viral genomes of gamma-retroviruses, lentiviruses and foamy viruses have a similar principle order: long terminal repeat (LTR), gag, pol, env, accessory genes, LTR. All three types of retroviruses share another characteristic feature, reverse transcription and integration into the host cell genome. However, there are differences between these vectors. Probably the biggest difference is that foamy viruses actually perform a very late reverse transcription of the RNA, or the pre-genome, into double-stranded DNA before the resulting virion buds from the producing cell membrane. The resulting double-stranded DNA genome in the virion is considered to be very stable.

Additionally, the foamy virus envelope glycoproteins are able to transduce almost any cell type, meaning they have a very broad tropism. The foamy virus receptor was identified by two different groups in 2012 as the ubiquitous heparan sulfate receptor [1,2].

Foamy viruses are considered to have a larger carrying load capacity than gamma-retroviruses or lentiviruses. Their current documented carrying capacity goes up to 12 or 13 kilobase pairs (kbp). At minimum, we know they can package about 9.2 kbp, which is approximately the maximum packaging capacity of gamma-retroviruses and lentiviruses.

The current strain of foamy virus in use for gene therapy was originally isolated from infectious clones of a foamy virus isolate from a human infection, but other simian and feline foamy viruses have also been developed. They generally carry a deletion in the U3 promoter region, as well as in most of the viral genome, with the exception of cis-acting sequences in the viral gag and pol genes, which are required for packaging. They all carry Pol protein encapsidation deletions in the transactivator and accessory genes. Thus, both the viral promoter and transactivator are deleted, rendering these foamy virus vectors as true self-inactivating (SIN) vectors. This SIN configuration has also been developed for gamma-retroviruses and lentiviruses.

Foamy viruses can also be produced using a three- or four-plasmid transfection system. Crude vector preparations can be concentrated by centrifugation or filtration to improve the vector titers about 100-fold without an observable loss of infectivity. Head-to-head experiments were performed in earlier studies with lentivirus, gamma-retroviruses and foamy virus in transduced human and canine cells. In these studies, foamy vectors performed as efficiently or better at lower multiplicities of infection than lentiviruses or gamma-retroviruses. This work was
conducted by the laboratories of Drs David Russell, Hans-Peter Kiem, Derek Persons and Helmut Hanenberg.

In terms of their integration profile within genomes, foamy viruses are generally considered to have a relatively more neutral integration profile, compared to lenti and gamma-retroviruses. They’re less likely to integrate into regions proximal to gene promoters or within genes compared to gamma retroviruses or lentiviruses, respectively.

To date, as far as I know, transgene silencing has not been observed with foamy viral vector-delivered transgene cassettes. This suggests that foamy viruses would be good for life-long expression of therapeutic transgenes. Specifically for gene therapy, another advantage of foamy virus vectors is a documented resistance to serum inactivation, probably due to the apathogenicity in humans. Thus foamy viruses are a good candidate vector for intravenous delivery of therapeutic transgenes. In fact, this has been demonstrated in a preclinical canine model of X-linked severe combined immunodeficiency (X-SCID) by the laboratory of Dr Hans-Peter Kiem. It’s not yet possible to pseudotype foamy virus particles with heterologous envelope glycoproteins, but it is possible to pseudotype other retrovirus vectors, such as lentiviruses, with foamy virus envelope glycoproteins to alter their tropism.

What makes foamy a favorable vector for gene delivery into HSCs?

In my opinion, the most prominent feature is the late reverse transcription of the viral genome prior to budding off the producing cell membrane during viral replication. This results in a foamy virus particle that already has a double-stranded DNA genome. Hematopoietic stem cells in particular are thought to be relatively quiescent, meaning these cells don’t have a lot of free nucleotides hanging around because they’re not actively trying to synthesize their DNA in cell division. Thus, a virus particle that has already completed the reverse transcription step into double-stranded DNA has an advantage. Additionally, it’s known that the foamy virus pre-integration complex will hang around the centrosomes of the cellular chromosomal DNA until the cell divides. Thus, foamy virus infection in HSCs results in a stable double-stranded DNA pre-integration complex, which can wait in the nucleus until the HSC begins proliferating in vivo.

This work to document the late reverse transcription during viral replication and cell cycle dependence for integration was primarily conducted by Dr David Russell’s, Dr Axel Rethwilm’s and Dr Ali Saïb’s groups. Their experiments demonstrated that foamy viruses transduced actively dividing cells at similar levels compared to gamma-retroviruses and lentiviruses, but foamy viruses transduced non-dividing cells more robustly than the other vector types.

Finally, depending on the target disease for gene therapy, the larger packaging capacity can also be very useful.
You mention X-linked SCID - what progress is being made in this particular disease?

There are many groups studying gene therapy for X-SCID, a genetic disorder caused by a mutation in the common gamma chain gene, which results in defective development of immune cells such as T cells and natural killer or NK cells. The disease can be cured with a bone marrow transplant from an unaffected person, but matched donors are not available for all patients and complications from donor bone marrow transplants can be fatal. For these reasons, gene therapy to provide a functional version of the common gamma chain gene into the X-SCID patient’s own blood stem cells is a beneficial alternative treatment. In both donor bone marrow transplant and gene therapy trials, it has been shown that adult and/or heavily treated X-SCID patients don’t do as well as younger patients that have had less intervention. Unfortunately, unless a family has a history of X-SCID, often the diagnosis is not made until the child is symptomatic and requires intervention. In 2010, X-SCID was added to the core Recommended Uniform Screening Panel for heritable disorders in newborns in the USA. In many states, newborns are screened and diagnosed with X-SCID very early, which in turn permits early intervention.

In terms of blood cell gene therapy-based interventions, invasive procedures that involve collecting bone marrow and processing stem cells outside the body, especially in a child that could potentially be prone to more infectious complications, becomes a little bit more worrisome. This is where we formulated a multi-institutional collaboration between the Fred Hutchinson Cancer Research Center, Seattle Children’s Research Institute and Washington State University to study the hypothesis that very early injection of a foamy virus vector, which is resistant to serum inactivation, encoding a functional common gamma chain gene could be a better approach. We postulated that because these children are very small at the time of treatment, smaller amounts of vector could be used successfully. To study this preclinically, we applied a canine model for XSCID originally developed at the University of Pennsylvania, which we now have here at the Fred Hutch in Seattle. Much of the disease pathology in these dogs is very similar to the human disease and breeding permits us to intervene in affected pups shortly after birth.

The ultimate goal of these studies is to hit a hematopoietic stem cell in vivo given that this disease doesn’t only impact T cells. However, from a treatment perspective, you could do a lot of good by correcting a T-cell precursor that is very long-lived. The first study published in Blood in 2014, we demonstrated that intravenous injection of a common gamma chain encoding foamy virus vector was feasible and safe [3]. Current work within this collaboration aims to improve the efficacy of this approach by optimizing the promoter regulating expression of the common gamma chain gene and also improving the likelihood that hematopoietic stem cells are targeted for transduction in vivo.
In terms of how complex it would be to translate our observations in the dogs to a gene therapy trial for human X-SCID patients, we have to think about feasibility, safety and efficacy.

In regard to feasibility, the pups we treated were about 1kg in size and each received approximately $4 \times 10^8$ infectious units/kg. The average baby, at least in the USA, weighs about 3.5 kg. Thus to directly translate this approach we’re talking about 3.5 times the number of vector particles required for injection or we need to demonstrate some clinically acceptable way of transducing a target number of hematopoietic stem and progenitor cells safely with less vector particles.

In terms of safety, we consider short- and long-term risks. For short-term, there is obviously the immediate response to the vector administered and the potential for adverse events associated with the actual injection, such as an increased risk of immediate infection. We did not observe any adverse events associated with the foamy virus particle injections in any of the pups treated in the 2014 study, and have since followed-up with additional animals for up to 2.5 years without adverse events. Again, the apathogenicity of the foamy virus in humans is likely an advantage in this approach. For long-term risks, we consider both off-target effects and the potential for insertional mutagenesis. For example, off-target effects include tissues aside from the blood are transduced in vivo. In particular, we monitor for germ line transduction. We have not observed germline transduction in any dogs treated to date. For insertional mutagenesis, we are primarily concerned with the genomic locus of foamy virus integration. Monitoring these loci of insertion not only tells us whether the foamy virus has proximity to an oncogene, but it also allows us to estimate the number of transduced clones in vivo, which contribute to immune system reconstitution. We have established this clone tracking method in the dogs from the 2014 study and could monitor these parameters in patients enrolled in a clinical trial.

From the standpoint of efficacy, we would want to see reconstitution of a functional immune system. This includes not only development of functional T cells, but also other immune lineages that can be affected, such as NK cells. We observed partial efficacy in the original study published in 2014. Our current research is directed at improving the efficacy in the preclinical canine model.

Any disease treatment or prevention must take into account not just genetic variability between individuals, but also differences in environment and lifestyle. All of these elements together contribute to an individual’s response to therapeutic intervention.
Q What are some of the challenges you’re facing specifically around the use of foamy as a vector?

I would say that we are always challenged to make reproducibly high-titer, concentrated foamy virus vectors. This challenge is not unique to foamy vectors, specifically when compared to lentivirus. There are several groups working on this, including our group here and the laboratory of Grant Trobridge at Washington State University. In general, we are able to produce titers in the range of 10^6 vector particles per milliliter. However, for in vivo delivery you want to infuse as many particles as possible in a very small volume, and you need to clean up the concentrated vector preparation before you would infuse it. Centrifugal concentration in our hands results in loss of vector particles. We’re currently working on other methods of concentration like tangential flow filtration, which also increases our purity of intact viral particles, and removes cellular debris. Thus, concentration by this method also reduces toxicity and cleans up the preparation for infusion.

Codon optimization of the helper genes to increase expression during vector production could be another option for improving the titer. The foamy virus envelope can also be further modified. This envelope glycoprotein has already been modified to increase titer, and we think further modifications in the envelope could be possible.

Scale up of viral vector production such that one preparation could treat many patients is another issue for not just foamy viruses, but lentiviral vectors as well. Creation of a stably producing cell line to make foamy virus particles would also be advantageous. An engineered producer cell line that does not express known restriction factors for foamy virus particle production, such as Trim5α or APOBEC3, could also improve the upper limit of titers and reliability of foamy virus production.

One challenge particular to our experience with foamy viral vectors is a limited stability at room temperature. To address this issue we have developed a rapid freezing protocol and optimized the cryogenic media, specifically with regard to the content of dimethylsulfoxide (DMSO), which stabilized foamy virus preparations. This was particularly important for in vivo administration of foamy virus vectors. Clinically, patients receive DMSO in cellular products, which are infused after thawing, thus the administration of thawed foamy virus particles formulated in DMSO at or below the concentrations received by patients infused with thawed cell products was critical for us.

Q Your group recently developed a prototype semi-automated closed system for point-of-care delivery of lenti-mediated HSC gene therapy. Could you tell us a little about this approach and the impact you think it could have on increasing the accessibility of gene therapies?

First, let me declare that accessibility to early phase clinical trials is something we really need to think about, and act to improve, in
the field of cell and gene therapy. Any disease treatment or prevention must take into account not just genetic variability between individuals, but also differences in environment and lifestyle. All of these elements together contribute to an individual’s response to therapeutic intervention. In the USA, this is supported by the National Institute’s of Health Precision Medicine Initiative (All of Us®).

We started this research in improving accessibility because my work here at Fred Hutchison is translating hematopoietic stem cell gene therapies for a variety of diseases. We’re fortunate to have the multimillion dollar Good Manufacturing Practices (GMP) infrastructure here in Seattle to do these kinds of studies. In contrast to our current preclinical in vivo work with foamy virus vectors for the treatment of X-SCID, all of our current Phase I gene therapy trials require blood or bone marrow to be collected from patients, then manipulated to parse out the stem and progenitor blood cells from the more mature blood cells, then these stem and progenitor cells have to be transduced outside of the body and finally cleaned up and prepared for infusion back into the patient. This requires clean rooms and sterile, heavily monitored equipment, reagents and materials. Thus, patients enrolled on our clinical trials have to come to Seattle for treatment.

Many private industry groups as well as academic institutions are attempting to centralize manufacturing by developing ways to ship cells back and forth from the clinic to the manufacturing site and back to the clinic for administration. However, this is expensive and introduces risks such as cell products being compromised or lost during shipment.

Personally, the issue of accessibility really resonated with me about 4 years ago, when we received a grant to translate a clinical trial of gene therapy to treat human immunodeficiency virus (HIV) infection. I was thinking about the environment and lifestyle of patients in the USA where the treatment is being developed and the prevalence of HIV worldwide. I really felt we needed a better way to distribute this sophisticated type of cell therapy to heavily HIV-infected countries, such as Africa.

For us this research was about creating a mobile gene therapy lab that could be applied to lots of different cell therapies in the local clinic where the patient is being seen or treated. Our proof-of-concept was in hematopoietic stem cells, but all the same components could be applied to T-cell gene transfer or any other cell type using that system.

I also wanted to show the field that you don’t have to have GMP facility infrastructure in place to work on meaningful solutions to translating accessible cell and gene therapy. If more labs have access to genetic modification of purified cells in a simplified system, we can overcome the barriers to widespread use much more quickly and efficiently.

Q Could similar systems be developed for alternative viral vectors such as foamy?

The way we developed the process, the user can define which vector is added to the cells in the system. We have shown it works
with the SIN lentivirus vector backbone currently in clinical use, but we could have just as easily added a foamy virus vector and shown similar results. It’s amenable to any viral vector.

Therefore, I think if we can come up with a viral vector that requires a very low multiplicity of infection to get the same transduction efficiency in the target cells, that’s certainly something that will be immediately applicable to the field.

**AFFILIATION**

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**REFERENCES**


Dr Axel Schambach is Acting Director of the Institute of Experimental Hematology at Hannover Medical School. He completed his medical training at the University of Hamburg, with international electives carried out at UCSD, UCSF, Baylor University and the Children’s Hospital Zurich. In 2005 Axel gained his PhD from the Institute of Experimental Hematology, Hannover Medical School, focusing on novel methods for gene therapy of inherited and acquired diseases. In 2013 he became Professor for Gene Modification of Somatic Cells at Hannover Medical School and Acting Director of the Institute of Experimental Hematology. Axel is also a Visiting Scientist and Lecturer at Boston Children’s Hospital. He is lead author on a number of scientific papers, has received multiple awards including the ESGCT Young Investigator Award in 2010 and is lead on a patent for alpharetroviral SIN vector system.

**Q** Your research has predominantly focused on the development of retrovirus-based vectors, as gene transfer systems for gene therapy. What do you see as the advantages of using these vectors for gene therapy compared to adenoviral vectors?

**R** Retrovirus-based vectors are vector tools that integrate preferentially into actively transcribed regions of the genome, from where the introduced genetic information can be permanently expressed. In that regard, these vectors are very interesting tools to treat
diseases, in which we would like to achieve a long lasting treatment effect. For example, retrovirus-based vectors, including gammaretroviral and lentiviral vectors, have a proven track record in the clinical arena. Gene therapy strategies using retrovirus-based vectors have been successfully used to treat immunodeficiencies, such as forms of severe combined immunodeficiency, globinopathies, such as sickle cell disease and beta thalassemia, and metabolic diseases, such as metachromatic leukodystrophy and adrenoleukodystrophy. In these diseases, it is possible that a one-time treatment could lead to a lifelong therapeutic effect. Evidence has shown that coupling hematopoietic stem cell transplantation and gene therapy of hematopoietic stem cells can result in a remarkable and long-lasting therapeutic effect for the aforementioned diseases.

In contrast, adenoviral vectors, which also represent interesting and clinically useful tools, have a natural tropism towards different tissues, such as the liver and endothelial cells. Thus, adenoviral vectors are also suitable for in vivo delivery. The biggest difference to retrovirus-based vectors is that adenoviral vectors are episomal vectors, which means that they are almost exclusively maintained in an extra-chromosomal, non-integrated fashion. Thus, the therapeutic adenoviral vectors may become diluted over time in rapidly dividing cell populations.

A potential limitation of adenoviral vectors is that immunological responses have been reported in patients, which is likely attributed to the fact that adenoviruses are frequent causes of respiratory tract infections in humans. In summary, retrovirus-based vectors and adenoviral vectors are both very interesting tools in the arena of gene therapy. However, the decision of which vector type is best suited should be decided on a case-by-case evaluation as needed for the specific gene therapy intervention.

Q What are some of the challenges associated with using retrovirus vectors?

In general, genetic modification with viral vectors, and in particular the stable integration of a gene therapy vector into the host cell genome, is considered to be a potential risk. In that regard, the greatest concern is genotoxicity, which is a form of insertional mutagenesis facilitated by either enhancer-mediated dysregulation of neighboring genes or by initiation of aberrant splicing.

To address this issue, stringent deletions of the promoter/enhancer regions from long terminal repeats (LTR) were accomplished and resulted in development of improved retrovirus-based vectors, which are called self-inactivating (SIN) vectors. These SIN vectors have a reduced likelihood of cis-activation of genes due to viral integration and represent an important milestone toward reduction of genotoxicity, especially when lineage-specific or more physiologic internal promoters are used to drive expression of the therapeutic transgene.
In this context, it’s also worth mentioning the need to further develop meaningful genotoxicity assay systems for improved and simplified assessment of genotoxic risk as well as to predict safer gene therapy vectors at an early stage in (pre)clinical development.

Further potential risk factors, including the immunogenicity of vector particles, the phenotoxicity of the transgene – for example a toxic gene product – as well as potential vertical or horizontal transmission by so-called replication-competent retroviruses, RCR, should also be considered and excluded to ensure an acceptable safety profile.

One additional challenge is the current bottleneck for clinical-grade lentiviral vector production, from which one GMP-grade vector production is only sufficient to treat 2–3 patients.

**Q** You recently published an update on your exciting work in developing an alpha retrovirus self-inactivating vector platform, could you share the science behind the creation of these vectors?

**A** Alpharetroviral vectors are a very promising new addition to the retroviral vector toolbox. These vectors are derived from the Rous sarcoma virus, a retrovirus identified in 1911 by Peyton Rous and co-workers. Since it was already identified more than 100 years ago, the Rous sarcoma virus is currently one of the best studied retroviruses.

In contrast to gammaretroviral and lentiviral vectors, this avian retrovirus exhibits an intrinsically more random integration pattern into the host genome. This was especially interesting for us as this means that the alpharetroviral vectors have a reduced tendency to integrate close to transcriptional start sites or transcription units, and is thus less likely to interfere with these genomic features.

The initial generation of the alpharetroviral vector was challenging, because it was only known to be producible in avian cells. We re-designed this system, used codon-optimization to improve its performance in human cells and adapted it for production in human packaging cells, which is desirable for use in a clinical setting.

Furthermore, we developed a SIN (self-inactivating) vector design and removed cryptic splice sites to decrease the risks of genotoxicity. As the final step, we generated a so-called split packaging system for our vector, in which structural proteins, replication enzymes as well as envelope glycoproteins were encoded by separate plasmids, thereby decreasing the risk of replication competent retrovirus (RCR) formation.

**Q** You specifically looked at using these vectors in T-cell and NK cell engineering. What were the key outcomes of this study?

**T** Lymphocytes, natural killer (NK) cells and NKT cells are very interesting cell populations for immunotherapeutic approaches. For example, the application of T-cell receptor genes, chimeric antigen
receptors and suicide genes to prevent graft versus host disease (GvHD) are a few of the clinical strategies currently being developed.

We found that alpharetroviral vectors are very well suited to transduce human T-cells and human NK cells. For NK cells, it is often very difficult to reach high transduction efficiency using low or moderate vector doses. Use of the alpharetroviral vector system allowed us to achieve very high transduction rates using a low vector dose.

We tested different clinically relevant transgenes, such as chimeric antigen receptors and suicides genes (for GvHD prophylaxis), and obtained proof-of-principle that these transgenes performed as expected in human T and NK cells. Moreover, we established a stable packaging cell line technology, which robustly produced alpharetroviral SIN vectors with high titers for more than 6 months, which is quite remarkable. This is interesting from a manufacturer standpoint, as this allows generation of high titer stable producer clones.

Taken together, these vectors are very interesting as – using this improved and cost-effective stable production system - we envision that vector scale-up would be simplified and more patients could be treated. Furthermore, in the ideal setting, one would only need to create one large batch of alpharetroviral vector and, thus, only have to test it once in terms of preclinical vector characterization to meet the requirements instructed by regulatory agencies.

Q How could these vectors be manipulated to further enhance their therapeutic potential?

These vectors are already fairly well advanced retroviral vector tools, due to the several optimization steps and synthetic biology approaches that were applied to improve their clinical behaviour. Previous work from us and others have resulted in vectors that have an improved and partially synthetic design, with no cryptic splice sites and no overlap of gag/pol (encoding structural proteins and replication enzymes) or env (envelope) sequences.

We also incorporated further features such as codon optimization and improvements in post-transcriptional processing, to ensure efficient expression of the therapeutic gene.

Certainly, further fine tuning of vector expression to mimic physiologic expression is required for each new clinical application strategy. To accomplish this, one could incorporate more physiologically relevant promoter sequences and miRNA sponges to drive transgene expression and to restrict expression in more physiological terms.

Additionally, one could also consider use of improved vector pseudotyping and equip these vector tools with new envelope variants, e.g. the recently described antibody- or darpin-guided Measles or Nipah Virus systems developed by Christian Buchholz at the Paul Ehrlich Institute in Langen. One could envision using these tools to achieve cell specific targeting in vivo and potentially even more applications.
Q What do you see as the major challenges in moving this and other new vectors towards clinical translation for future human gene therapies?

F irst of all, we have obtained proof-of-principle that we can generate a stable human packaging cell line and validated the vector system in murine and human hematopoietic stem cells as well as human T cells after transplantation in suitable mouse models. That’s very interesting.

As a next step, we are now in the process of generating a fully traceable stable packaging line and, in collaboration with David A. Williams (Chief Scientific Officer, Boston Children’s Hospital), we are designing additional strategies to approach several clinically interesting diseases.

We do not foresee major bottlenecks, and have already developed a plan to move this promising technology into the clinical arena.

As for the field in general, it would be desirable to have further improvements in the preclinical pipelines and the efforts to bring an interesting therapeutic and safe approach closer to clinical use to benefit the patients. Therefore, it is important that we as a scientific community develop better tools for the risk-benefit assessment of our vector tools, and also to streamline the procedures required to move interesting research from bench to bedside, i.e. into a clinical trial.

It will certainly be necessary to form teams consisting of experienced clinicians and knowledgeable scientists in order to translate these strategies more efficiently into clinical reality in settings where there is a high unmet clinical need.

One final and important point that certainly deserves more attention is how to form productive general alliances with industrial partners and to identify win–win settings to streamline development of clinical gene therapy procedures into promising and safe drugs for the benefit of patients.

AFFILIATION

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