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REVIEW

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Adeno-associated Virus (AAV) Dual Vector Strategies for Gene Therapy Encoding Large Transgenes

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The use of adeno-associated viral (AAV†) vectors for gene therapy treatments of inherited disorders has accelerated over the past decade with multiple clinical trials ongoing in varying tissue types and new ones initiating every year. These vectors are exhibiting low-immunogenicity across the clinical trials in addition to showing evidence of efficacy, making it clear they are the current standard vector for any potential gene therapy treatment. However, AAV vectors do have a limitation in their packaging capacity, being capable of holding no more than ~5kb of DNA and in a therapeutic transgene scenario, this length of DNA would need to include genetic control elements in addition to the gene coding sequence (CDS) of interest. Given that numerous diseases are caused by mutations in genes with a CDS exceeding 3.5kb, this makes packaging into a single AAV capsid not possible for larger genes. Due to this problem, yet with the desire to use AAV vectors, research groups have adapted the standard AAV gene therapy approach to enable delivery of such large genes to target cells using dual AAV vector systems. Here we review the AAV dual vector strategies currently employed and highlight the virtues and drawbacks of each method plus the likelihood of success with such approaches.

INTRODUCTION

Recombinant adeno-associated viral (AAV) vectors are now well described and established in the field of gene therapy and being developed for treatments of numerous disease states [1]. Briefly, they originate from the wild-type AAV, which is a member of the *Parvoviridae* family of viruses. It is a non-enveloped virus with an icosahedral capsid structure generated from three capsid proteins (VP1, VP2, and VP3) [2]. The capsids contain a single-stranded DNA (ssDNA) genome of 4.7kb that carries two genes, *rep* and *cap*, flanked by palindromic inverted terminal repeat sequences (ITRs). Both *rep* and *cap* have multiple open reading frames (ORFs) that express proteins necessary for genome replication and packaging [3]. AAV is a dependovirus, meaning that it cannot replicate

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†Abbreviations: AAV, adeno-associated virus; ABCA4, ATP-binding cassette transporter family member 4; AP, alkaline phosphatase; CDS, coding sequence; HR, homologous recombination; ITR, inverted terminal repeat; MYO7A, myosin VIIA; NHEJ, non-homologous end joining; OAGR, oligo-assisted /AAV genome recombination; ORF, open reading frame; polyA, poly-adenylation signal; RPE, retinal pigment epithelium; SSA, single-strand annealing; ssDNA, single-stranded DNA; VP, viral protein; WT, wild-type.

Keywords: Dual vector, AAV, large transgenes, gene therapy

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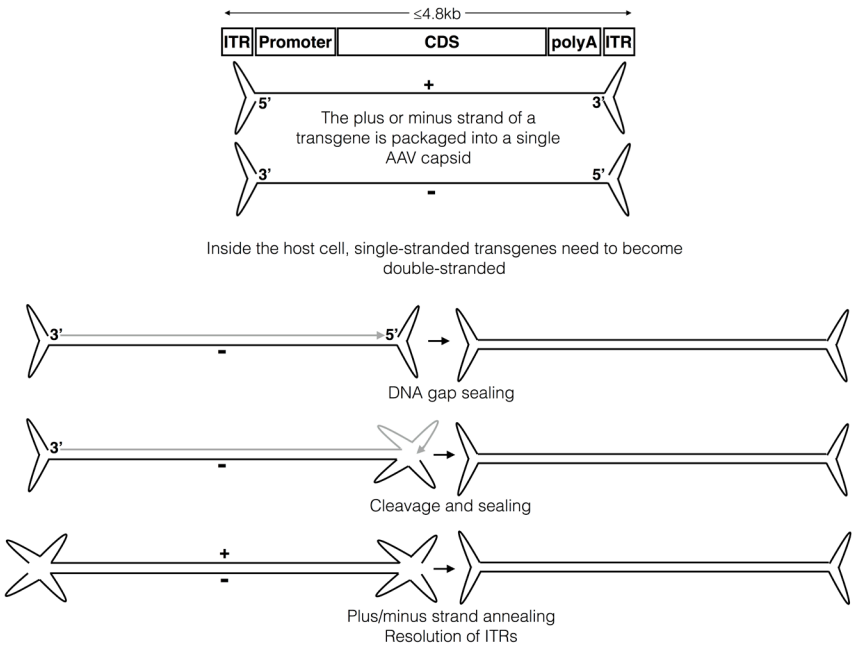

Figure 1. **Transgene structure for a traditional AAV gene therapy**. Each AAV capsid carries either a plus or minus strand version of the transgene, which is delivered to the host cell nucleus. Inside the nucleus the single-strand transgene is transformed into a double-stranded episomal structure either by second-strand synthesis or by annealing of complementary plus and minus transgene strands. Mechanisms of sealing and resolution of ITRs are based on previous work [77]. ITR = inverted terminal repeat; CDS = coding sequence; polyA = polyadenylation signal; AAV = adeno-associated virus.

or infect without the aid of another virus, for example, adenovirus or herpesvirus [4]. For generation of the recombinant AAV vectors used for gene therapy purposes, the native genome of AAV has the *rep* and *cap* genes removed and replaced with the genetic elements required for gene therapy. These elements must be flanked by the ITRs, the only required *cis*-elements of the original AAV genome, and this structure is known as the transgene. For vector production purposes, the *rep* and *cap* genes are provided in *trans* along with "helper" sequences derived from the adenovirus genome [5]. Packaging of the transgene into the assembled capsids occurs from the 3' end of both the plus and minus strand of the template transgene, which is double-stranded DNA, and the resulting AAV population is expected to comprise of a 50:50 mix of capsids containing either the plus or the minus strand version of the transgene [6,7]. Each AAV vector can then deliver a ssDNA version of the transgene to the target cell, which needs to become double-stranded before it can express the desired therapeutic protein (Figure 1). This can occur either by annealing of the plus strand of a transgene to a minus strand delivered to the same cell [8] or from native nuclear mechanisms initiating second-strand synthesis from a single-stranded transgene [9].

The wild-type AAV genome is 4.7kb in size and the packaging capacity of recombinant AAV vectors is limited to therapeutic transgenes up to this size though they can be encouraged to package larger genomes, albeit not efficiently [10]. As the length of a transgene increases, the packaging efficiency into the capsids diminishes [11] therefore the ideal transgene size is considered to be anything up to 4.7kb. Given that the structure of a therapeutic transgene requires, as a minimum, inclusion of a promoter, gene coding sequence (CDS), and poly-adenylyation signal (polyA) flanked by ITRs, this means the treatment of disorders caused by mutations in genes over 3.5kb in size is currently not achievable as the transgene would not fit into a single AAV capsid [11–13]. There are multiple inherited diseases that have a relatively large patient population that would benefit from a gene therapy treatment but result from mutations in large genes, including: Duchenne muscular dystrophy, hemophilia A, and the retinal degeneration disorders Stargardt disease and Usher syndrome. Development of a gene therapy treatment for these disorders is currently a great challenge as there is no larger vector available that has the equivalent safety and efficacy profile as AAV. While other vectors, such as retroviruses, are able to package larger transgenes, their use brings greater potential risks. Retroviruses integrate the desired transgene into host DNA, which creates the opportunity for insertional mutagenesis or oncogene activation [14]. Lentiviral vectors share features of the

Table 1. A summary of the different AAV dual vector strategies.

Dual vector system	Advantages	Disadvantages
Fragmented	• successful transgene expression observed in multiple studies in various models of disease	• poor vector production quality • lack of transgene packaging control • strong potential for unwanted transgene products
Overlapping	• successful transgene expression observed in multiple studies in various models of disease • no additional genetic sequences required	• pre-clinical testing required to determine the optimal overlap sequence of a given coding sequence • potential for unwanted transgene products
Trans-splicing	• successful transgene expression observed in multiple studies in various models of disease	• pre-clinical testing required to determine the optimal splice sequence • requires additional genetic elements • requires efficient transcript processing (removal of the unwanted splice/ITR junction) • potential for unwanted transgene products • relies on an inefficient concatemerization process
Hybrid	• successful transgene expression observed in multiple studies in various models of disease • offers two opportunities for transgene reformation • once optimized, universal dual transgene structures can be applied to generate other treatment vectors	• pre-clinical testing required to determine the optimal splice and recombinogenic sequences • requires additional genetic elements • requires efficient transcript processing (removal of the unwanted splice/ recombinogenic region) • potential for unwanted transgene products

retroviral systems but have undergone modifications over the past decade to generate safer non-integrating vectors though they are still more complex and currently display less cell-specific targeting abilities than AAV [15,16]. While showing some success in cancer immunotherapy [17] for the treatment of other diseases such as retinal disorders, lentiviral vectors are exhibiting less ability than AAV at transducing the non-dividing cells of the central nervous system [18]. Their larger size, while good for transgene packaging, also creates problems such as making it difficult to diffuse through the multi-layered cell structure of the retina. Whereas good transduction has been achieved in very young mouse models (post-natal days 1 to 4) [19], in adult mice reporter gene expression is restricted to the site of injection and to the retinal pigment epithelium (RPE) [18,20]. Given the more complicated nature of lentiviruses, their increased potential to cause unwanted immune responses and current restricted transduction abilities (relative to AAV), developing dual vector AAV systems for the delivery of larger transgenes is considered worthwhile.

The generation, assessments, and use of such dual vectors is relatively simple [21] and there is good evidence to show that co-transducing a cell population with two different AAV vectors can be efficient [22]; the next challenge is to then encourage two transgenes delivered to the same cell to recombine and form a single larger transgene. There are different approaches to this problem being attempted [23] but they generally begin with the generation of two transgenes: the first can be referred to as an upstream transgene as it carries the promoter element and 5' upstream portion of a given CDS flanked by ITRs. The second can be referred to as the downstream transgene as it carries the downstream portion of a given CDS and polyA signal, also flanked by ITRs. The two transgenes are packaged separately and provided as a dual vector mix to the target cell population. A target cell would need to receive a copy of both the upstream and the downstream transgene and, based on the specific dual vector design, these transgenes combined would lead to generation of an mRNA transcript containing the complete large gene that could not be carried and packaged in AAV on a single sequence of DNA. The different strategies for achieving this are discussed in detail below with the advantages and disadvantages of each summarized in Table 1.

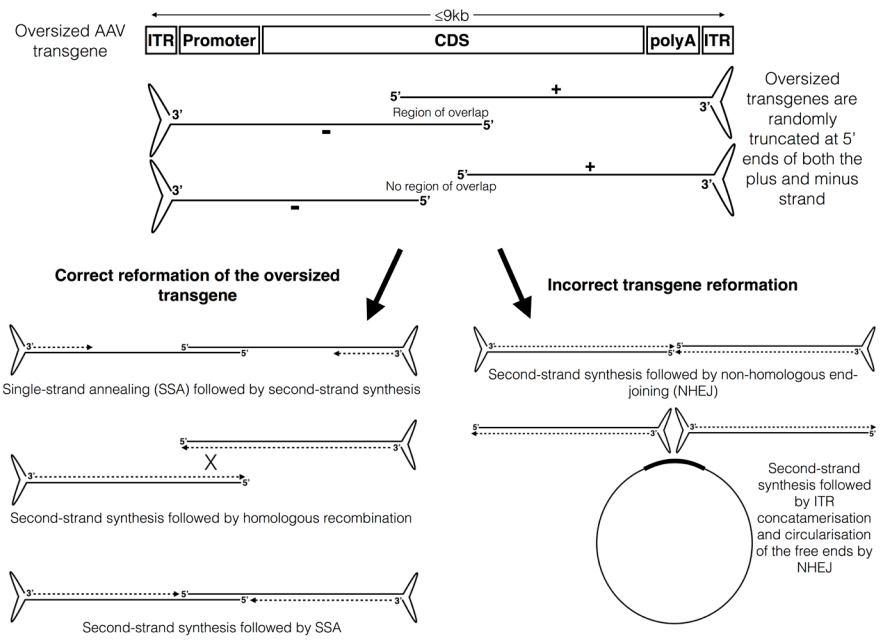

Figure 2. **Fragmented packaging of oversized transgenes can lead to different outcomes**. In the therapeutic scenario, there is correct reformation of the oversized transgene via a region of homology. This could occur due to single-strand annealing of plus and minus strands at the region of homology or by homologous recombination (HR) following second-strand synthesis of the truncated transgenes. Alternative outcomes involve non-homologous end joining (NHEJ) of transgenes following second-strand synthesis, which may also occur in combination with ITR concatemerization. These outcomes were presented in our previous publication [32]. ITR = inverted terminal repeat; CDS = coding sequence; polyA = polyadenylation signal; AAV = adeno-associated virus; NHEJ = non-homologous end-joining.

AAV DUAL VECTOR STRATEGIES

Fragmented AAV Dual Vectors

The potential of AAV to deliver large genes to a target cell population was investigated in 2008 by Alloca *et al*. and their data surprisingly indicated that AAV vectors could package large transgenes of nearly 9kb in size [24]. However, investigations published soon after revealed that this was not the case [11]. Despite the unknown reason at the time, Alloca *et al*., did show successful expression of their desired proteins following transduction with AAV vectors in which they had attempted to package oversized transgenes. This success was later elucidated to have resulted from the packaging of fragmented transgenes [11–13]. When a transgene is large, packaging that begins from the 3' ITRs of both the plus and minus strands and becomes truncated at an undefined point, therefore each capsid carries an incomplete fragment of transgene. This results in a mixed population of AAV vectors carrying different truncated lengths of the transgene plus and minus strands (Figure 2). The successful generation of target product despite this heterogeneous vector population was deduced to result from the plus and minus strands carrying overlapping regions of the original therapeutic transgene that could undergo homologous recombination (HR) or annealing at the complementary regions prior to second-synthesis [25,26].

Following on from the success shown by Alloca *et al*. in 2008, other research groups have attempted this fragmented strategy and exhibited variable success. Assessments of the transduction success of three fragmented AAV vector preparations in HEK293 cells revealed they were clearly less effective than other dual vector strategies (considered in the sections below) [27]. In contrast, an *in vivo* investigation showed evidence of the fragmented approach working better at delivering large transgenes to the retina and skeletal muscle than the *trans*-splicing approach (see section: Trans-*splicing AAV Dual Vectors*), as measured by levels of luciferase activity post-injection [28]. This fragmented dual vector success appeared to be supported in another study comparing it with an overlapping dual vector system (see section: *Overlapping AAV Dual Vectors*), in which both vector types were assessed in the retina of *Myo7a*$^{-/-}$ mice and were attempting to provide MYO7A expression [29]. The data from Lopes *et al*. indicated the fragmented approach led to greater expression levels of MYO7A in treated eyes and also provided indications of therapeutic outcomes in the mouse model.

However, it may be that the overlapping dual vector strategy that was used as a comparison dual vector system was not optimal as it relied on a large region of overlap sequence (1,365 bases) that had not been optimized and therefore may have been recombining inefficiently (see section: *Overlapping AAV Dual Vectors* for further discussion on this).

Despite the success shown by research groups utilizing this fragmented approach, it is apparent that AAV capsids predominantly package shorter than expected transgenes, which then limits the chance of successful regeneration in this dual vector strategy [11,13,28,30]. Enriching the AAV preparation by fractionation and collection of capsids containing larger transgenes may aid the success of this approach [28,31]. However, these enriched fractions will still contain heterogeneous transgenes, which are capable of joining without a region of overlap, forming hybrid transgenes that then express hybrid, mutant forms of the therapeutic gene [32]. From a potential treatment perspective, this then becomes a concern for the safety of any treatment arising from the fragmented dual vector AAV strategy, making further progress to clinical trial with this strategy unlikely.

Overlapping AAV Dual Vectors

An advancement on the fragmented dual vector approach is the overlapping approach. In this strategy, there are two defined transgenes that each carry a demarcated fragment of the therapeutic gene CDS that includes a portion of specified sequence overlap in each transgene. This can be a region of the CDS contained in both transgenes [27,29,31,33–38] or a designated recombinogenic sequence [31,39–41], although in this latter scenario this becomes a hybrid dual vector approach (discussed in section: *Hybrid AAV Dual Vectors*). The overlapping strategy relies on the same premise that enables the fragmented approach, whereby a region of sequence overlap initiates joining of two separate transgenes into a single larger one. This was originally shown to be achieved when comparing the expression of alkaline phosphatase (AP) dual vectors that carried AP overlap zones of 440 or 1,000 bases. Successful expression of AP was achieved but at low efficiencies (50- to 100-fold less) compared to a single vector control *in vitro.* However, when tested *in vivo*, the best performing overlapping dual vector performed similarly to a single gene comparison following delivery to the airways of WT mice [36].

Numerous studies have employed the overlapping approach *in vitro* and *in vivo* in numerous tissue types assessing for potential dual AAV vector treatments for retinal degeneration, dysferlinopathy, hemophilia, and muscular dystrophy. For this latter disorder, overlapping vectors carrying a 372-base region of dystrophin CDS overlap were intravenously delivered into the striated muscle of the *mdx* mouse model with a subsequent improvement in limb muscle performance observed despite expression levels of the desired mini-dystrophin gene not reaching WT levels [37]. When compared to expression levels achieved from a hybrid dual vector, the overlapping dual vector gave 3-fold higher expression levels when delivered locally to the muscle of dystrophin-null mice [33]. The same research group have since shown similar success with their overlapping dual vectors when providing the vectors systemically [42]. Investigations into the treatment of dysferlinopathy mouse models have used a larger 1kb CDS overlap region to achieve successful expression of the dysferlin gene following muscle transduction [35]. Comparison of a shorter 859 bases of dysferlin overlap to a fragmented dual vector system determined the overlapping vectors offered up to 10-fold higher expression levels than the fragmented vectors following intra-muscular injection [31]. Whether expression levels may be further improved by assessing different overlap regions of a given CDS has yet to be presented.

Dual vectors have also been assessed in different models of retinal degeneration, namely for the delivery of myosin VIIA (MYO7A, mutations in which cause Usher syndrome) and ATP-binding cassette transporter protein 4 (ABCA4, mutations in which cause Stargardt disease). Trapani *et al*. performed an extensive study comparing all the dual AAV vector strategies considered in this review for delivering both MYO7A and ABCA4 *in vitro* and *in vivo* [27]. Their data indicated that *in vitro*, the overlapping approach was more successful than both the fragmented and a hybrid approach using an alkaline phosphatase (AP) derived recombinogenic region. However, when using an F1-phage derived recombinogenic sequence in their hybrid vector system, they achieved much greater expression levels. Despite this being a different dual vector design, it is evidence indicating that the region of overlap is critical to the success of a strategy relying on recombination via regions of homology. Interestingly, despite the success achieved with their overlapping vectors *in vitro*, this did not translate to their *in vivo* experiments when targeting the photoreceptor cells of the retina. Attempts at delivering ABCA4 following sub-retinal injection in WT mice were unsuccessful yet RPE expression was achieved. This poor success when attempting the overlapping strategy to deliver a big gene to the retina was also shown elsewhere [29]. These data highlight the point that particular dual vector strategies may be more or less likely to succeed depending on the cell type being targeted as, interestingly, the delivery of overlapping vectors to muscle appears to have been more consistently successful than photoreceptor targeting to date. However, other groups investigating dual vector strategies for the delivery of MYO7A to the retina have

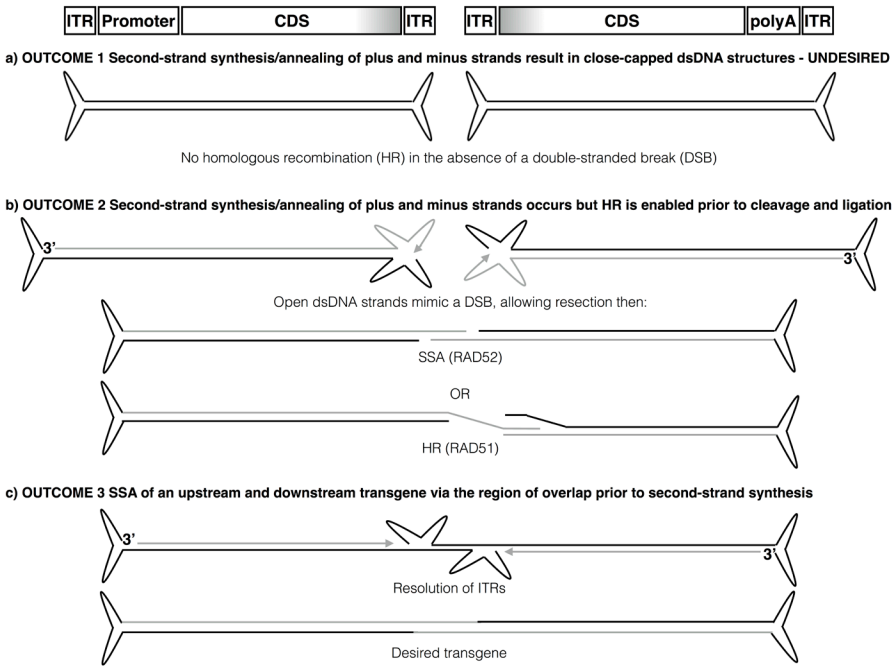

Figure 3. **Potential outcomes of the overlapping dual vector strategy**. In the undesired scenario, the two transgenes may be delivered to the same host cell yet not recombine and exist as independent forms (**a**). Alternatively, the two transgenes may undergo homologous recombination (**b**) or single-strand annealing (**c**) via their shared regions of homology to create the desired transgene. ITR = inverted terminal repeat; CDS = coding sequence; polyA = polyadenylation signal; AAV = adeno-associated virus; HR = homologous recombination; DSB = double-stranded break; SSA = single-strand annealing. Shaded areas indicate regions of homology.

shown contrasting results with one study indicating better expression in photoreceptors was achieved from an overlapping approach compared to one that is fragmented [38]. Furthermore, the authors commented that changing their vector serotype to AAV8 Y733F enabled better observation of expression in the photoreceptor cells of the retina, which indicates more general (not dual vector-specific) optimizations of the AAV strategy could enhance the success of the overlapping approach.

Studies have indicated the success of the overlapping approach relies on homologous recombination (HR) but in the case of many gene therapies, the target cells types will be terminally differentiated, non-dividing cells. Despite the variability in the data presented from research groups employing the overlapping approach, the positive results achieved indicate the target cells do employ some form of molecular mechanism to recombine opposing transgenes. The effectiveness of these mechanisms may be tissue-dependent therefore the success of a dual vector system may rely on the cell types being targeted. HR is typically associated with dividing cells and occurs between sister chromatids but there are other forms that are used in DNA repair [43], and through one of these sub-pathways the overlapping dual vector transgenes may be recombined. Non-homologous end joining (NHEJ) is another mechanism of DNA repair and both NHEJ and HR have been shown to be active in mouse rod photoreceptor cells [44], indicating dual vector strategies for retinal degenerations should be viable. Importantly, NHEJ is prone to error and joining by this mechanism would generate mistakes in the subsequent CDS [32]. If consistently correct reformation of the larger transgene occurs following dual vector transduction (as has been indicated, [38]), that is a strong indicator of a HR pathway being preferred.

There is evidence to suggest that in a normal recombinant AAV gene therapy scenario, stable double-stranded transgenes are formed preferentially by the recruitment of the corresponding plus and minus ssDNA transgene forms rather than by second-strand synthesis of complementary strands [8]. However, second-strand synthesis is an alternative mechanism by which stable transgenes are formed [9]. Following either route, the subsequent double-stranded structures will be closed ITR-capped elements (Figure 1). Without inducing a double-stranded break, these would likely stay as stable, unrecombined structures, which is undesirable in an overlapping strategy (Figure 3a). In this form as stable closed structures in non-dividing cells, it would seem unlikely that HR mechanisms would be able to join these overlapping transgenes. This would then lead to the hypothesis that the most likely mechanism for success of the overlapping approach would be single-strand an-

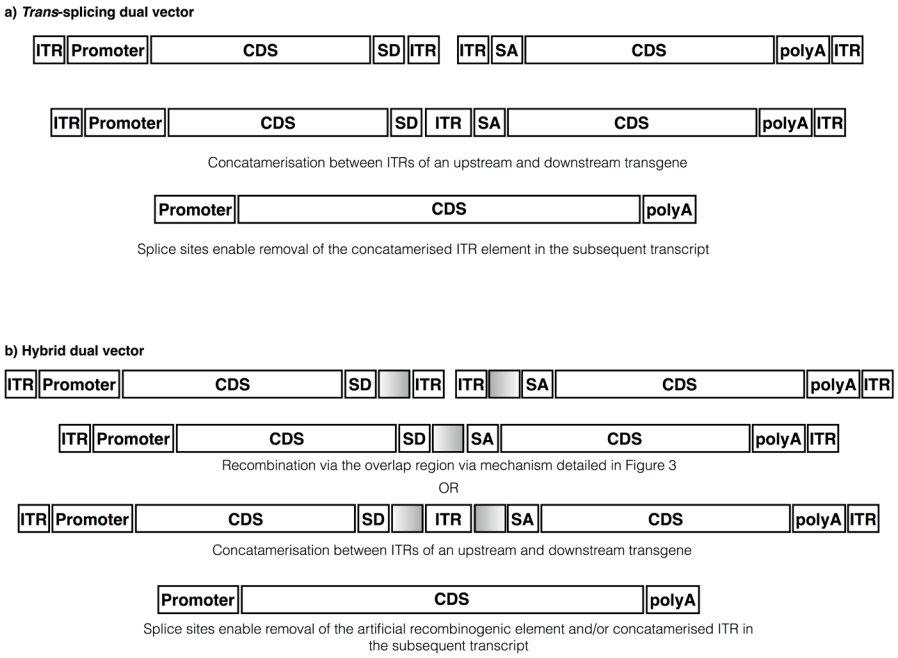

Figure 4. **Principles of the *trans*-splicing** (**a**) **and hybrid dual vector strategies** (**b**). Two transgenes are delivered to the same host cell and in the *trans*-splicing mechanism, success relies on the concatemerization of the ITR structures of an upstream and downstream transgene with the splice donor (SD) and splice acceptor (SA) elements allowing for subsequent removal from the transcript form (**a**). The same mechanism may occur in the hybrid approach but a more likely scenario is that recombination of the transgenes will occur via the artificial region of overlap, which then gets removed by splicing from the resulting transcript (**b**). ITR = inverted terminal repeat; CDS = coding sequence; polyA = polyadenylation signal; AAV = adeno-associated virus; SD = splice donor element; SA = splice acceptor element. Shaded boxes indicate regions of homology.

nealing (SSA) of complementary regions from opposing transgenes (Figure 3c). Intriguingly, the success of the fragmented dual vector AAV approach has been shown to be reliant on RAD51C [19], yet SSA has been shown to be a RAD51-independent process as it does not require strand invasion [45]. This suggests the fragmented AAV dual vector strategy recombines following second-strand synthesis. Given the single-ITR nature of the transgenes, this would generate two double-stranded transgenes that mimic a double-stranded break, in which case RAD51-dependent repair is to be anticipated. A similar mechanism is plausible with the overlapping approach if it were to predominantly occur after second-strand synthesis but before complete annealing of the double-stranded transgene (Figure 3b) but the SSA homology-directed repair pathway independent of RAD51 seems the most likely mechanism for recombination success with the overlapping approach [36,46].

The efficiency of this mechanism may be strongly reliant on achieving high numbers of upstream and downstream transgenes within each target cell to increase the chance of subsequent intermolecular interaction. Improvements in transduction success and transgene survival will therefore be critical to the overlapping dual vector outcome. In addition, the overlap region will also be important. One could argue that too short and the interaction would not be viable to form an attachment strong enough for DNA polymerase to recognize and bind. If the region were too long, it may be more likely to form a secondary structure that would prevent complementary annealing with the opposing transgene. Therefore, optimization of the overlap region used in this strategy is highly likely to be critical to its success [41].

The overlapping dual AAV vector approach is the simplest in design and the transgenes require less foreign or artificial DNA elements. However, one of the potential downsides of this approach is that with each new gene therapy treatment to be made, much work will need to be done to determine the optimal region of CDS overlap to be used. If a universal region of recombinogenic sequence could be used, this would be transferrable to multiple dual vector treatments (see section: *Hybrid AAV Dual Vectors*).

Trans-*splicing AAV Dual Vectors*

This strategy has no region of sequence overlap and therefore the two transgenes are completely distinct and

contain two different fragments of the therapeutic CDS. The approach relies on the tendency of ITRs to concatemerize (form linked circular genomes) as it has been shown that following transduction and second-strand synthesis, AAV transgenes form stable episomal structures through joining of their ITR structures, a process known as concatemerization [47–50]. The *trans*-splicing approach piggy-backs on this process and so with appropriate dual vector design, following joining of the ITRs from the dual vectors, the concatemerized ITR structure that would lie in the middle of the therapeutic CDS can be removed by native cellular mechanisms during transcription due to the inclusion of a splice donor site following the 3' end of the CDS contained in the upstream transgene and a splice acceptor site prior to the 5' end of the CDS contained in the downstream vector (Figure 4a). This approach was the first AAV dual vector system utilized and has been successfully employed by numerous research groups in different cell lines and tissue types albeit to varying degrees [27,28,34,38,51–57]. The first success with this strategy was shown in 2000 [54,57–59] with a comparison to an equivalent overlapping dual vector system published in 2001 [34]. The *trans*-splicing approach was indicated in these early studies to perform better *in vitro* and *in vivo* in skeletal muscle than an overlapping dual vector although as discussed in section *Overlapping AAV Dual Vectors*, there may be overlapping transgene design reasons for this. These data were supported by later investigations that included an additional comparison of a hybrid vector system, which appeared to lead to better expression levels than both the *trans*-splicing and the overlapping approaches *in vitro* and *in vivo* [52]. But, as will appear as a common theme, there are varying results from different research groups and other studies have revealed less expression from *trans*-splicing vectors than a fragmented system [28] and overlapping and hybrid approaches [38] *in vitro* and *in vivo.*

There have been studies that indicate transgenes favor self-circularization [49,60,61] and transgenes will concatemerize in both the correct orientation (upstream:downstream) and incorrect (*e.g.* downstream:upstream/upstream:upstream/downstream:downstream) [62–64]. Investigations have been conducted attempting to improve and encourage concatemerization of ITRs in the correct orientations through the use of heterologous ITRs, which have shown improvements in the success of *trans*-splicing dual vectors in skeletal muscle studies [65,66]. By generating an upstream transgene with a 5' AAV2 ITR and a 3' AAV5 ITR and a downstream transgene with a 5' AAV5 ITR and 3' AAV2 ITR, a 3- to 6-fold increase in expression following intramuscular injection was achieved when compared with typical AAV5:AAV5 and AAV2:AAV2 *trans*-splicing dual vectors. However, use of heterologous ITRs for orientation-directed concatemerization has not been shown by other investigations to lead to any improvements and in fact led to difficulties in achieving high titer vector preparations [67]. Other attempts to improve orientation-specific concatemerization of these dual vectors have involved oligo-assisted AAV genome recombination (OAGR) [61].

In addition to the problem of ensuring concatemerization between appropriate transgenes, a further issue for consideration involves the splice sites selected for the subsequent removal of unwanted sequence in transcripts. Different splice elements will undergo splicing to different efficiencies and may need to be optimized to ensure their removal [51]. Utilizing natural splice junctions may be better suited to therapeutic transgenes than synthetic sequences but further investigations and optimizations may prove otherwise, particularly as splicing efficiencies differ between natural splice junctions from the same gene [68,69]. Another factor that may influence the success of splicing this junction is the concatemerized ITR structure, which may enhance or inhibit the splicing process.

Evidently there are potentially big issues to overcome with the *trans*-splicing approach to enable it to be less problematic and more efficient. Adapting the design and combining the strategy with the overlapping approach may be the solution, as has occurred with development of the hybrid dual vector strategy.

Hybrid AAV Dual Vectors

With the *trans*-splicing approach, there is a concern that the dual vector transgenes will join in an undesirable way or indeed not concatemerize at all. With the overlapping approach, a concern is that concatemerization would occur at all as there would be no feature to remove an unwanted ITR structure present in the middle of a CDS. The hybrid strategy counters both these concerns by combining the two approaches and was first described by Ghosh *et al*. 2008 [52]. This hybrid dual vector strategy incorporates both an overlap region and splice donor/splice acceptor sites in the dual vector transgenes (Figure 4b) [27,31,38,52,67,70]. Recent studies suggest this hybrid approach is the most effective of the dual vector methods, which is perhaps not unexpected as it offers two opportunities for large transgene regeneration. The initial experiments by Ghosh *et al*. compared LacZ expression *in vitro* and found comparable levels from hybrid dual vectors versus a traditional single vector design. These vectors outperformed both the overlapping and *trans*-splicing dual vectors compared in the same study. All vectors were then compared *in vivo* in mouse muscle with very similar results achieved [52]. The downside of this initial hybrid vector system was that the overlap region used was 872 bases of alkaline phosphatase sequence (AP), which would be too large to use in therapeutic dual vector transgenes. This recombinogenic region had been

previously characterized [40] and the research group later assessed shorter versions and found all variants of the AP overlap fragment in hybrid vectors led to improvements in expression compared to a *trans*-splicing vector [39]. This enabled a defined 270 base recombinogenic region of AP to be used by other research groups and was shown to work successfully in the delivery of MYO7A to the retina of mice [38]. However, in a separate study, hybrid vectors with this short AP recombinogenic region were not able to achieve good expression of ABCA4 in mouse photoreceptor cells whereas inclusion of a 77bp sequence from filamentous phage F1 homology region (referred to as AK) in hybrid dual vectors led to much greater ABCA4 expression *in vivo* [67].

Given that recombination between transgenes is likely to occur via SSA of the overlap region prior to any concatemerization, the overlap zone used is likely to be critical to the success of the hybrid approach just as it is for the overlapping approach [41]. The region of overlap has been shown to strongly influence the success of transgene reformation and it may be that including the *trans*-splicing elements only enhances results when the overlapping region is inefficient [31]. Indeed, from the studies published so far, the presence of the splice elements seems not to enhance the strategy when the overlapping region is highly recombinogenic, indicating the overlap sequence is the critical feature of a successful dual vector strategy.

Issues with AAV Dual Vector Strategies

Currently, all these dual vector strategies face similar issues: variable success and potential for unwanted expression products. Both transgenes used in a given dual vector system appear to be capable of generating undesired expression products in their individual forms. A successfully delivered upstream transgene that does not recombine with a downstream transgene but is transformed into a double-stranded episomal structure, will be identified as a viable transcriptional start point and high levels of truncated transcripts could potentially be generated. Given the lack of a polyA signal in the upstream transgene design, it would be expected that any such transcript population would not exist for long as they would not resemble stable mRNA transcript structures in the absence of a polyA tail. Furthermore, without a stop codon present in the mRNA transcript, there is a likelihood that any subsequent peptide would not survive to become a stable protein [71]. However, despite the absence of these genetic features, some research groups are identifying protein products when testing their upstream vector not in combination with the downstream vector [38,67]. This indicates the transcripts are stable and survive for translation, suggesting there must be existence of stop codons and cryptic polyA sites within the upstream transgene structure. This would be something to consider in future dual vector strategy development as assessment of the sequence designs could be critical to prevent such products forming. Interestingly, Dyka *et al*. identified a truncated product from their *trans*-splicing and hybrid upstream vectors but not from their overlapping upstream vector so it may be a problem more likely to arise in particular dual vector strategies/designs. Inclusion of an in-frame CL1 degradation sequence after the splice donor site has been shown to prevent accumulation of the unwanted protein products [67]. However, in attempting to overcome this unwanted expression, yet another genetic element needs to be included in the upstream transgene which will limit space for the gene CDS.

Expression from the downstream transgene is less reported but has been presented [53,67]. While there are no designated promoter elements included in the downstream transgene designs, expression is believed to initiate from the 5'ITR, which has been shown to have promoter activity [72,73]. With the polyA signal included in the downstream transgene, transcription initiated from the 5'ITR would create stable transcript forms. The likelihood of these transcripts generating protein products would then depend on the existence of a cryptic translational start sequences within an appropriate distance from the start of the transcript. Were this to arise and to then provide an in-frame open-reading frame, a truncated form of the therapeutic protein would be generated. Such products may or may not be a problem but would need to be assessed for safety/toxicity prior to any clinical trial application. Potentially they would be present at very low levels and be non-functional, recognized as unnecessary forms and degraded but there is a possibility they may elicit toxic dominant-negative effects. An alternative outcome would be that a cryptic translational start site would arise out of frame and then lead to generation of short foreign peptides. These would likely be degraded quickly due to their size [74,75], but clearly these issues indicate that for any dual vector strategy, the design and specific nucleotide sequence of the transgenes is critical and requires multiple considerations and adaptations, which may include codon-optimizations to remove cryptic genetic signals.

CONCLUSIONS AND OUTLOOK

With AAV vectors offering such hope to patients suffering from inherited disorders that currently have no treatments available, the possibility of expanding the use of these safe vectors to the treatment of disorders caused by mutations in large genes is very exciting. Research over the past decade indicates this is a real possibility but given the complexities of such treatment strategies, there are many considerations to be made. Currently one

of the main factors to contemplate are the inconsistencies of success shown when assessing the different dual vector strategies between independent research groups. Where one approach might show great success in one study, another achieves greater success with another strategy in head-to-head comparisons. This will likely be due to multiple factors including cell types used, culture conditions, AAV preparation purity and titer, variations in transgene designs, and transgene delivery. Despite this, what is encouraging is that the dual vector strategies are showing success despite the variations in the data but clearly there are improvements to be made both universally for all strategies and within each specific approach itself.

Despite some success shown when utilizing the fragmented AAV approach [24,27–29], the lack of control in transgene packaging and subsequent transgene reformation makes this approach inappropriate for further consideration as a dual vector treatment without significant improvements to these safety aspects. Similarly, the lack of control of ITR concatemerization in the *trans*-splicing strategy and the poor efficiency of intermolecular concatemerization versus intramolecular concatemerization may make it an undesirable strategy moving forward to clinical use.

The overlapping approach using the CDS as the overlap region is the simplest and most elegant dual vector design but requires extensive pre-clinical optimization steps to determine the most efficient overlap sequence. The suspected DNA repair molecular mechanisms involved in the success of this approach should be active in most target cell types therefore making the overlapping approach a good strategy for therapeutic success. A similar approach using an artificial region of overlap could be equally successful and indeed more universal as the dual vector transgene designs could be applied to all large genes once optimized. However, the use of an additional recombinogenic sequence would then require its removal from resultant transcripts and therefore further genetic sequences would be required in the dual vector transgene designs. The more additional sequences required, the less space there becomes for the actual CDS, which may then limit the use of the hybrid dual vector system. Indeed, some investigations have already begun on multi-vector systems for delivering genes that do not fit even in two AAV transgenes [55]. Furthermore, the splice sites used for the removal of unwanted sequence in transcripts may need to be optimized to ensure the efficient removal of undesired genetic sequence in the recombined gene CDS.

A common question posed when it comes to AAV dual vector gene therapy strategies is: will they be successful enough to generate therapeutic levels of the target protein? It is clearly possible to provide two different vectors in a single mix and successfully transduce a target cell population with both vectors. When these vectors then require transgene interactions and specific molecular mechanisms to occur in an appropriate and efficient order to achieve therapeutic success, there are further considerations to be made in terms of enhancing the process of intermolecular interaction to achieve the single desired therapeutic transgene. Even traditional AAV gene therapy strategies are undergoing changes and optimizations to improve the chances of a given vector successfully transducing a target cell population and surviving to the point of delivering intact ssDNA transgenes into the target nucleus. Improvements in the stages that make up this transduction process are universally required for all gene therapy treatments but may be particularly critical in aiding the success of a dual vector strategy where the number of transgenes delivered and maintained in the target cell may be fundamental to the chance of intermolecular interactions occurring. We have discussed key features of each dual vector strategy and the efficacy of each approach may depend on the severity of the disease to be treated.

Taking the example of retinal degenerations, the dual vector strategies may be very likely to achieve success given the isolated nature of the eye, safety profile of AAV following sub-retinal delivery in clinical trials and the progression of disease. For Stargardt disease, it is known that carriers do not show any disease phenotype [76] therefore providing 50 percent of the levels of native ABCA4 should be sufficient to treat the disorder. Understanding the nature of the disorder, biochemistry, and physiology of progression is critical when considering the chances of dual vector success. Being able to provide even a sub-population of the photoreceptor cells of the retina with a correct copy of the *ABCA4* gene would likely be good enough to prevent further visual loss and at a minimum slow the disease progression. Given that vision is lost from a young age and gets progressively worse over the course of a lifetime, any delay from further degeneration of the retina would provide a significant improvement to the quality of life to individuals that currently have no treatment opportunities. For the condition Usher syndrome, patients suffer both retinal degeneration and hearing loss and as yet there is no efficient way of delivering gene therapy to treat the hearing loss aspect of the condition. Being able to provide some relief to the blinding aspect of the condition would be considered highly significant for these individuals that will become both deaf and blind in the absence of any treatment. While it is not known whether a dual vector gene therapy strategy will provide enough therapeutic protein for complete rescue of the disorder, being able to offer some level of vision rescue would be considered a major achievement for these patients.

Currently there are very encouraging signs from the field of AAV dual vector research. If the dual vector

strategies can be shown to be safe and not have negative outcomes, for example from unwanted expression products, then a successful dual vector treatment might be applicable for a variety of diseases in which a medium-sized gene can be replaced.

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