Small But Increasingly Mighty: Latest Advances in AAV Vector Research, Design, and Evolution

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Recombinant gene delivery vectors derived from naturally occurring or genetically engineered adeno-associated viruses (AAV) have taken center stage in human gene therapy, fueled by rapidly accumulating and highly encouraging clinical data. Nonetheless, it has also become evident that the current generation of AAV vectors will require improvements in transduction potency, antibody evasion, and cell specificity in order to realize their full potential and to widen applicability in larger patient cohorts. Fortunately, in the recent past, the field has seen a flurry of exciting new developments that enhance our understanding of AAV vector biology, including virus–host interactions, and/or that expand our arsenal of technologies for AAV capsid design and evolution. This review highlights a collection of latest advances in these areas, which, in the authors’ opinion, hold particular promise to propel the AAV vector field forward in the near future, especially when applied in combination. These include fundamental novel insights into the AAV life cycle, from an unexpected role of autophagy and interactions with other viruses to the (re-)discovery of a universal AAV receptor and the function of AAV-AAP for capsid assembly. Concurrently, recent successes in the rational design of next-generation synthetic AAV capsids are pointed out, exemplified by the structure-guided derivation of AAV mutants displaying robust in vivo immune evasion. Finally, a variety of new and innovative strategies for high-throughput generation and screening of AAV capsid libraries are briefly reviewed, including Cre recombinase-based selection, ancestral AAV capsid reconstruction, and DNA barcoding of AAV genomes. All of these examples showcase the present momentum in the AAV field and, together with work by many other academic or industrial entities, raise substantial optimism that the remaining hurdles for human gene therapy with AAV vectors will (soon) be overcome.

Keywords: AAV, adeno-associated virus, molecular evolution, virus engineering, immune escape, virus–host interaction

AAV: A PROMISING BUT (STILL) ENIGMATIC VECTOR FOR HUMAN GENE THERAPY

More than 50 years after its discovery,1 adeno-associated viruses (AAV) have become the basis of one of the most auspicious and most versatile gene delivery systems available to date, whose power has been well documented in a wealth of preclinical and clinical studies.2–5 A major reason for this overwhelming success is the minimalistic genome and capsid organization of AAV,6 which facilitates genetic modification and thus enables customization of all essential vector properties, including cellular tropism, efficiency of transduction, and reactivity with anti-AAV antibodies.7–11 Curiously, though, despite the relatively simple virus structure, the fundamental biology of AAV vectors, including their interaction with the host cell—from binding and uptake, to trafficking and nuclear uncoating—remains largely enigmatic. Fortunately, over the last few years, the AAV field has seen a

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flurry of activities in both basic and applied virus/vector research that have started to shed more light on the cellular and viral mechanisms of transduction, and that have drastically expanded our arsenal of technologies for AAV vector development and optimization.

Below, a selection of recent studies and exciting accomplishments will be highlighted that in the authors’ opinion showcase the current trends and advances in AAV research. Because of the enormous complexity of the field, and in view of numerous previous reviews that have already covered different aspects of AAV viruses and vectors, the overview will be limited to studies on AAV capsid biology and modification. Moreover, the reports that have been selected merely exemplify the breadth and ingenuity of the various concepts that are being pursued by many groups, but are by no means intended to provide a comprehensive synopsis of the entire field.

FROM ADVANCES IN BASIC AAV RESEARCH...

In light of the rapidly accumulating successes with AAV vectors in early- to late-stage clinical trials for treatment of numerous diseases, and of the fact that AAV has been studied extensively for more than five decades, it is staggering that most of the cellular events and host factors that govern AAV-mediated gene delivery have remained unknown to date. Obviously, a more thorough understanding of the infectious pathway of AAV vectors, of the cellular key players, and of cell type- and/or serotype-specific barriers would be highly instrumental and informative for efforts in the field to improve the AAV capsid through rational design or molecular evolution. Therefore, in the following, a variety of recent, exciting discoveries that have emerged from basic research on AAV biology and that are of strong interest for the AAV vector community as well will be briefly highlighted.

Impact of the cellular “virome” and identification of additional AAV helper virus factors

For many decades, it has been known that AAV requires co-infection with a second unrelated, so-called helper virus for productive infection or for exit from the latent state, respectively. Accordingly, production of recombinant AAV vectors strictly depends on the co-expression of all relevant functions—proteins and sometimes RNAs—of these helper viruses, from expression cassettes that are either stably integrated in, or transiently delivered to, the AAV producer cells. Traditional and well-studied helper viruses are the nongiving adenovirus as well as herpes simplex virus, which both fully support the many steps of the AAV life cycle and of AAV vector production, albeit through different mechanisms.

In an ironic twist, Hösel et al. recently assigned a helper function to another human virus that, as a clinically highly relevant pathogen, actually belongs to the major targets for AAV vector-based gene therapies (i.e., hepatitis B virus [HBV]). To this end, the group first studied reporter gene expression from AAV vectors using different promoters and serotypes in distinct cell culture models of HBV infection. Strikingly, in HBV-replicating cell lines as well as in HBV-infected HepaRG cells or primary human hepatocytes, the authors observed a significant increase in AAV transduction efficiency across all vectors. These unexpected results were further corroborated in HBV-transgenic mice where strongest reporter gene expression from AAV vectors was again observed in hepatocytes that actively produced HBV, showing that HBV supports AAV transduction in vivo too. Interestingly, this effect may not be due to enhanced vector internalization, but rather to improved AAV trafficking into the nucleus and slightly increased transcription. This is an unusual combination of interactions and helper functions that distinguishes HBV from the previously characterized helper viruses. Further noteworthy is that by using a variety of complementary methods and tools, including cells lines inducibly expressing individual HBV proteins, the authors succeeded at identifying HBV protein x (HBx, a pleiotropic protein essential for HBV replication and infection) as the predominant factor exerting the helper function. While a contribution of other HBV products was not ruled out, the conclusion that a single factor is sufficient to enhance AAV transduction is again unique among the known helper viruses and thus further illustrates the novelty of these findings.

Finally, and most important in the context of the present article, the authors were able to exploit the cis helper effect of HBV for AAV-mediated anti-HBV therapeutic strategies. Accordingly, they demonstrated an enhanced expression and efficacy of two therapeutic molecules, interferon gamma and anti-HBV shRNA, in cells or mice that co-expressed HBV, respectively. Thereby, this work not only improves our understanding of the host–AAV interaction in the context of (chronic) infection with pathogenic viruses, but concurrently paves the way for the development of novel antiviral therapies that harness this peculiar virus–host interaction.
A second, equally striking example for newly discovered aspects of the AAV life cycle with respect to helper virus interaction has been provided most recently by Wang et al. In this case, the authors discovered that AAV can also benefit from co-infection with another member of the Parvoviridae family, namely, the human bocavirus 1 (HBoV1). This is a very unique and remarkable finding, considering that all other helper viruses described thus far, including the aforementioned HBV, belong to distinct virus families and are not related to AAV. It should be noted that in this latest study, the helper effect of HBoV1 on AAV was found to be modest and subpar compared to a traditional adenoviral helper. Nonetheless, the authors demonstrated that HBoV1 co-infection supported AAV wild-type gene expression and genome replication in multiple cell culture systems, including primary human airway epithelia, which are very amenable to HBoV1 infection and may represent the natural viral reservoir. Notably, the effect was observed for both production of new AAV and infection of cells with pre-made AAV, evidencing that HBoV1 can indeed support multiple steps of the AAV life cycle. However, the HBoV1 products mediating these effects seem to differ between the two stages, since AAV production required HBoV1 NP1 and NS4 proteins as well as the small non-coding RNA BocaSR, whereas during infection NS2 replaced NS4. Finally notable is that, vice versa, AAV infection had no detrimental impact on HBoV1 infection, implying that AAV hijacks HBoV1 functions without blocking this particular helper virus in return (the latter phenomenon is indeed observed for adenovirus and herpes simplex virus).

With respect to the topic of this article, this latest work is highly intriguing, as it expands both our fundamental knowledge of AAV helper viruses and—related to this—our options for AAV vector production and for capsid optimization. For instance, use of HBoV1 as helper will enable AAV vector manufacturing in cells lacking the adenoviral E1 gene, whose presence is normally a prerequisite for all systems that use adenoviral helper constructs (which are devoid of E1 and thus need trans-complementation in the producer cells). Moreover, it was shown that adenovirus and HBoV1 act differently on AAV gene expression and genome replication, which includes findings that, for instance, BocaSR potentiates expression of AAV Rep proteins but not of VP proteins. Hence, it is tempting to speculate that a combination of traditional adenoviral and newly discovered bocaviral helper functions may result in entirely novel AAV vector production systems, in which the two heterologous helpers synergize to boost AAV particle yields and/or infectivity. Further support for the promise of such a concept is indeed provided by earlier reports, including the finding that AAV virus and vector yields can be improved by supplementing traditional adenoviral helper functions with E1, E2, and E6 from human papillomavirus. Together, these data substantiate the idea that the optimal set of helper functions for AAV production may not have been identified yet, and may eventually be composed of proteins and (small) RNAs from different heterologous sources.

As a final example for the relevance of this latest report, one can readily anticipate novel selection schemes for AAV capsid libraries in which the latter are specifically amplified in cells that are co-infected with HBoV1, which will stimulate AAV progeny production and thus simplify rescue of enriched capsid sequences. In view of the increasing evidence that HBoV1 may be a human pathogen in the lung and the gastrointestinal tract, the ability to exploit this virus as a helper to foster and accelerate the evolution of AAV capsids that infect the same target cells is reminiscent of the abovementioned situation with HBV, and thus also clinically utmost relevant and promising.

**AAVR: a universal (?) AAV receptor**

In 2016, Pillay et al. published a widely recognized study that reported, for the first time, a presumably universal cellular receptor, called AAVR (AAV receptor; KIAA0319L), that impacts infection of different AAV serotypes. Evidence for the seminal role of this receptor included findings that AAVR knockout cells and mice were largely refractory to AAV infection, and that this effect could be rescued in cultured cells through AAVR over-expression. Concomitantly, this study raised a large number of questions into the underlying mechanisms, including whether AAVR directly or indirectly interact with AAV capsids, which AAVR protein domains are involved, and which steps of the infection pathway are affected, such as cell binding and uptake, or trafficking to and/or escape from the trans-Golgi network. Furthermore, the question remained whether the involvement of AAVR in these steps is AAV serotype-dependent, considering the known differences in serotype tropism. Clearly, a better understanding of all these aspects has great potential to result in improvements in AAV vector design and should particularly benefit attempts to enhance capsid specificity.
In this respect, a most recent follow-up study by the same group is noteworthy, as it has started to shed more light on the aforementioned questions about the role of AAVR. In this latest work, Pillay et al. expanded on their previous finding that among the five polycystic kidney disease or PKD domains within the AAVR ectodomain, PKD1–3, comprise a binding domain for AAV2. Using a set of elegant and complementary mutation, over-expression, and domain-swapping experiments, the new study concludes that it is predominantly PKD2—next to a minor contribution by PKD1—that mediates AAV2 binding and transduction. Surprisingly, results were different for the evolutionarily distinct AAV5, which rather relies on the most membrane-distal PKD1 domain instead of PKD2. Mixed phenotypes were found for AAV1 and AAV8, which required PKD2 akin to AAV2, but more strongly depended on PKD1. Besides, this study also confirmed that AAVR is heavily N- and O-linked glycosylated. Yet, these modifications only facilitate AAV transduction but are not strictly required.

As a whole, the discovery that multiple serotypes share the dependence on a single receptor yet differ in their distinctive molecular interactions is intriguing not only from a general standpoint, but also with respect to vector development. Possibly, once the exact binding sites have been delineated further in the future, evolution efforts may harness this knowledge in attempts to breed AAV capsids that more specifically and/or more efficiently interact with an individual AAVR domain. This could be highly relevant considering that AAVR is expressed differently in multiple tissues, and in alternative splice variants and post-translationally modified isoforms. Hence, it is tempting to speculate that continued research into the structure and function of AAVR could provide the AAV vector field with an entirely new treasure box full of options to fine-tune cellular specificity and transduction potency of recombinant AAV particles.

As a side note, this latest report from the Carette group is moreover remarkable, as it confirms previous work by Kevin Brown’s laboratory who, unknown at the time, had already identified the AAVR receptor in a publication more than two decades ago. As there was no direct follow-up to the 1996 study, the independent re-discovery of the same receptor and the enthusiasm it now causes in the field should be very gratifying for the original authors.

Assembly-Activating Protein: AAV’s own little helper

Similar to the (re-)discovery of a “new” AAV receptor after decades of AAV research, many in the AAV field were probably stunned when, in 2010, the Kleinschmidt laboratory reported the identification of a previously unknown AAV protein encoded in the second open reading frame of the capsid gene. Quickly thereafter, this protein—dubbed assembly-activating protein or short AAP—has attracted significant attention for at least two reasons, the first being its unusual and enigmatic biology. As the name implies, and as has been experimentally confirmed in the original study and in five subsequent reports, AAP is critical for assembly of AAV particles. Yet, the underlying mechanisms remain incompletely understood. A straightforward hypothesis is that AAP acts as a scaffold or chaperone that fosters assembly of individual capsid proteins, either directly or through interaction with additional cellular proteins. Indirect evidence against the latter mechanism may be that AAPs of different AAV serotypes exhibit distinct intracellular localization patterns, despite their functional interchangeability. Moreover, members of the Grimm and Ayuso laboratories have recently jointly reported the strong dependency of AAV2 on AAP for baculovirus-mediated vector production also in insect cells. The fact that the same relationship has now been observed in two heterologous systems, mammalian and insect cells, can be interpreted as further evidence for a direct role of AAP in AAV capsid assembly, albeit the existence of orthologous cellular factors cannot be ruled out.

The second reason for the attractiveness and importance of AAP as a research object is the aforementioned fact that its open reading frame overlaps with that of the capsid proteins, more precisely, the N termini of VP2 and VP3. On the one hand, this provokes questions as to how these two proteins or open reading frames, respectively, managed to co-evolve and retain or develop their individual functions, part of which were answered in an intriguing study by the Nakai laboratory. On the other hand, it has raised concerns in the original 2010 study that modulation of the capsid gene, for example via DNA family shuffling or targeted mutagenesis, may inadvertently disrupt the sequence and hence the function of AAP as well. Aggravating these concerns were subsequent and consistent findings by the Kleinschmidt, Nakai, and Grimm laboratories that at least 10 different AAV serotypes all require AAP for production of capsids composed of VP3 only, or of all three capsid proteins. Notably, the extent of this dependency varies and is less pronounced for AAV4 and AAV5, which can form assembled particles even in the complete absence of functional
However, the efficiency of this alternative, AAP-independent assembly pathway is about 10-fold lower, and it is unclear whether these particles represent genuine capsids.

Nonetheless, the possibility remains that one could molecularly evolve or rationally design capsids that result in efficient particle assembly, even without a need for AAP. This would, in turn, instantly resolve concerns about the overlap of the capsid and AAP reading frames, and about the unintended perturbation of the latter during capsid modification. Of note, unpublished data from the laboratory of one of the authors (D.G.) show that the vast majority of chimeric AAPs that result from shuffling of AAV capsid genes retain their functionality, implying that AAP is not a limiting factor, at least during the production of synthetic AAV capsid/vector libraries. Vice versa, it could also be rewarding to try and deliberately evolve chimeric AAPs that exhibit a gain-of-function phenotype and thus promote faster and/or more efficient capsid assembly, which may benefit AAV vector production. Either way, one can readily predict that AAP will remain in the center of attention of basic and applied AAV research for many years to come.

Autophagy: a "biomarker" that determines the outcome of liver-directed gene therapy

As compared to adenovirus, another powerful and widely used gene therapy vector, AAV can be considered as a “stealth virus,” but even AAV infection or vector transduction does not go unnoticed. Classical immune cells such as plasmacytoid dendritic cells (pDC) or Kupffer cells (KC), or sentinel cells such as liver sinusoidal endothelial cells (LSEC) or activated macrovascular endothelial cells, can sense the presence of AAV vector genomes or capsids, respectively, through distinct Toll-like receptors mounting a transient innate immune response.28,29 Of note, innate immune responses are not restricted to conventional immune cells. Thus, it came as a surprise when it was found that hepatocytes, AAV’s main target cells in the liver, did not respond to a challenge with AAV vectors by upregulation of inflammatory cytokines,28 despite the ability of these cells to sense pathogens.

Intrigued by this observation and convinced that a deeper understanding of the AAV–hepatocyte interaction is required to improve the efficacy of liver transduction with AAV, the Büning group characterized the impact of autophagy, a key response pathway to environmental stress.30 Autophagy is of particular importance for the liver with its quiescent hepatocytes, where it contributes to the removal and recycling of protein aggregates and damaged organelles. In addition, autophagy exhibits critical functions in the regulation of hepatic metabolism and supports hepatic innate immune responses. Hepatocytes—like most other cells—exert a basic level of autophagy in the steady state. However, in response to stress such as starvation, injury, toxins, or infection, the level of autophagy increases with the aim of quickly re-establishing liver homeostasis. Strikingly, challenge of hepatoma cell lines as well as primary human hepatocytes with AAV vectors of different serotypes or by wild-type AAV also increased autophagy and fostered de novo autophagosome formation. In contrast, this was not observed in highly AAV-permissive HeLa cells.30–32 Interestingly, AAV not only induced hepatic autophagy, but actually required this cell response for efficient transduction of hepatocytes. Further underscoring the newly discovered importance of autophagy as a critical parameter or “biomarker” determining the outcome of liver-directed gene therapy with AAV are the marked difference in hepatocyte transduction that were observed in preclinical animal models simulating conditions of impaired autophagy.

These results have two important implications for the future use of AAV vectors in the liver. First, they argue for screening of patients beforehand and for taking this dependency into account when designing liver-directed gene therapy approaches exploring AAV as a delivery system. Second, the ease by which the level of autophagy can be manipulated offers a cornucopia of strategies to improve AAV vector efficacy at least in the liver. This was impressively demonstrated in preclinical animal models, including nonhuman primates, in which drug-induced autophagy resulted in elevated AAV vector efficiency. One particularly interesting candidate drug considered worthy of further evaluation in this context is rapamycin, which, besides inducing autophagy by targeting mTOR, the pathway’s gatekeeper, is known for its advantages in modulating anti-AAV immune responses.30,33 Accordingly, it is very tempting to postulate that use of rapamycin as an adjuvant might provide a dual benefit for liver-directed AAV gene therapies by (i) boosting the efficiency of gene transfer and (ii) concurrently alleviating adverse immune responses. In this respect, it would represent a prototype of a scenario where fundamental research into AAV biology in cells and animals not only enhances our understanding of virus–host interactions, but also has direct and pivotal implications for therapeutic use of AAV in humans.
TO THE NEXT STEPS IN AAV CAPSID EVOLUTION

Typically, molecular AAV evolution strategies follow a forward genetics principle, that is, capsid sequences are mutated or shuffled in a high-throughput fashion, and the resulting libraries are selected under positive and/or negative pressure to foster enrichment of desired candidates.\(^8,9,11\) While the power and breadth of these approaches is undisputed and well documented in the literature, it is also clear that the current strategies have inherent limitations that have to be overcome in order to realize the potential of these technologies fully. Below, notable examples of recently reported amendments of existing AAV evolution technology are described that are considered particularly powerful and innovative, and that should help to reach this goal.

Match what belongs together: the challenge of genotype/phenotype coupling

Irrespective of the particular molecular AAV evolution and selection strategies that are applied, the likelihood of success directly correlates with the quality of the initial library that is used in the screen. This statement—although intriguingly easy to acknowledge—conceals the challenge of producing libraries of a high complexity ("diversity"), with a high biological score ("vitality"), and a high fidelity ("coupled geno-phenotype").\(^8\) Two completely different protocols have already been developed in the early days of molecular AAV evolution to produce a library in cells that consists of particles with uniform capsids (all 60 subunits display the same mutation) from a plasmid pool that encodes numerous different capsid variants, while ensuring that each capsid packages a viral genome encoding the cognate engineered capsid sequence. Both protocols start with the plasmid pool that is transfected into HEK293 packaging cells but differ in essential details. In the one-step-protocol, a strictly limited amount of plasmids is transfected per cell to achieve a library with high fidelity, while a large number of cells is employed to cope with the issue of diversity.\(^34–37\) In contrast, the two-step-protocol first produces mosaic AAV particles consisting of engineered and wild-type capsid subunits, and then uses this shuttle library to infect packaging cells with a multiplicity of infection \(<1\) through the wild-type capsid subunits, resulting in generation of the final library.\(^38,39\) The underlying rationale is that controlled infection ideally results in delivery of a single capsid-encoding genome to each cell, thus ensuring production of, and packaging in, the corresponding capsid. Intriguingly, the need to perform such a phenotype/genotype coupling step at all has been challenged by Nonnenmacher et al. who recently postulated the existence of an intrinsic mechanism that packages the "correct" genome into a capsid that assembles in a non-random fashion.\(^40\) This was particularly observed for replication-competent AAV genomes, such as those in an AAV capsid library, but not for AAV vectors produced from replication-incompetent helper constructs, implying a coupling of packaging and genome replication.

While this report and hypothesis await independent confirmation, Körbelin et al. have recently compared the two abovementioned protocols side-by-side with AAV peptide libraries displaying 6, 7, or 12mer peptides in position 588 of the AAV2 capsid by next-generation sequencing (NGS).\(^41\) These comprehensive analyses revealed that the two-step-protocol results in a characteristic bias for an asparagine in the first position of the random peptide insertion. This bias was observed for all three libraries and, interestingly, correlated with a gain in heparin binding. This indicates that in libraries produced by shuttle libraries, there is enrichment during the coupling step of capsid variants that bind to AAV2’s natural attachment receptor, heparan sulfate proteoglycan. As discussed by the authors, this bias may also account for the striking accumulation of AAV capsid variants displaying asparagine in the first position of the peptide insertion that had been selected with these libraries in the past.\(^36,38\) As this bias impacts the diversity of the library and may change the course of the selection procedure, the one-step-protocol appears as method of choice at least for the moment. Thus, akin to the aforementioned autophagy data (see section “Autophagy: a ‘biomarker’ that determines the outcome of liver-directed gene therapy”),\(^36,39\) this may be another example where AAV vector technology could largely benefit from novel insights into fundamental AAV biology, in this case, the processes underlying genome encapsidation.

CREATE: Cre-dependent AAV capsid selection in predefined target cells

Next to library quality, the success of any AAV capsid evolution scheme also largely hinges on a stringent selection pressure that forces the enrichment of particles with desired properties and that concurrently weeds out all others that do not meet the selection criteria. Thus, in the case of strategies that aim at breeding new capsids with unique cell specificities in a multicellular context (e.g., a complex organ within a living organism), success will primarily depend on the ability to
isolate the desired target cells and to rescue the AAV DNA exclusively from this cell type.

An intriguing solution to this challenge was proposed by Deverman et al. with a system they called "CREATE" and which is based on selective in vivo amplification of AAV genomes in cells that express Cre recombinase. Therefore, the authors applied a clever trick, which was to flank the polyadenylation signal in their AAV capsid library with loxP recognition sites, so that it would become inverted in Cre-expressing target cells. Importantly, the polyadenylation signal contained a binding site for a PCR primer which, upon Cre-mediated inversion, enabled PCR amplification of the associated AAV capsid gene. Hence, this ensures rescue and subcloning only of those capsid genes that have entered the cells and were effectively converted to double-stranded DNA, thus rendering them a target for Cre recombination. To prove the power of this novel strategy, the authors demonstrated the selection of an AAV9 peptide display variant, called AAV-HP.B, in the brain of transgenic mice expressing Cre exclusively in astrocytes. Indeed, individual validation of this capsid following intravenous delivery confirmed a superior efficiency and specificity in various regions of the murine central nervous system compared to the parental wild-type AAV9.

Curiously, though, the capsid not only performed well in astrocytes, that is, the cell type it was supposedly selected in, but it also broadly transduced many other neuronal and glial cell populations including neurons, oligodendrocytes, endothelial cells, and Purkinje cells. Possibly, this is an indirect consequence of the in vivo selection of capsids that have not only acquired the ability to transduce astrocytes but also to cross the blood–brain barrier as a prerequisite to reach these cells from in intravenous injection. While this may explain the broader-than-expected tropism of the enriched candidates, it concurrently highlights the need to include negative selection pressure in future iterations of this technology. In this regard, the possibility is noted of employing NGS-based scoring algorithms to rank candidates not only by efficacy of on-target cell infection but also by off-target activity. In fact, the latter strategy has successfully been applied in a recent in vivo AAV peptide display screening for AAV capsid variants with tropism for the endothelium of the pulmonary vasculature. In addition, CREATE would be even more beneficial if one could overcome the need for Cre-transgenic animals, for example via transient and targeted Cre delivery by another viral or non-viral vectors, as this would significantly expand the cellular and organismal target range for AAV selection. Notwithstanding these necessary improvements, CREATE technology is an original and powerful expansion of the list of current selection methods for which one can readily envision a plethora of in vitro and in vivo applications.

(Semi-)rational engineering of immune escape variants

Next to potency and specificity, another critical parameter that governs the efficacy of AAV gene therapies and that can even lead to patient exclusion from clinical trials is inactivation by neutralizing anti-AAV antibodies that pre-exist because of earlier exposure to wild-type AAV or that have been induced by prior vector treatment. Previously, a variety of measures have thus been devised and tested to tackle this shortfall, including repeated rounds of plasmapheresis, coating of AAV capsids with surfactants, use of immunosuppressive agents that diminish antibody generation, or association of AAV with extracellular vesicles (vexosomes).

In a notable study published earlier this year, Tse et al. have taken a different approach to dealing with prevailing anti-AAV antibodies, namely, a structure-guided evolution scheme that can yield highly divergent antigenic footprints that do not exist in nature. In essence, this novel approach combines (i) structural information from cryo-electron microscopy analysis of a given AAV capsid complexed with antibodies, (ii) saturation mutagenesis of the identified clusters of capsid antigenic footprints, and (iii) iterative selection of the resulting capsid libraries in target cells that are amenable to transduction with the parental wild-type virus, in the absence of antibody pressure. For proof-of-concept, the authors chose to improve AAV serotype 1 and, accordingly, first identified a prominent clustering of antigenic footprints located at the threefold symmetry axis of the AAV1 capsids and spanning parts of the variable capsid regions IV, V, and VIII. Comprehensive mutagenesis of the latter and selection of the ensuing libraries in vascular endothelial cells resulted in enrichment of specific capsid antigenic motifs (called CAM) in each region. Importantly, analysis of selected CAM variants confirmed that mutagenesis and selection had not impaired or altered the key physical properties of the underlying AAV1 particle, including yield, morphology, and ratio of full-to-empty capsids. Even more noteworthy, all studied variants indeed exhibited some degree of resistance to anti-AAV1 antibodies in cultured cells and in mice (following injection of pre-mixed antibody–CAM complexes).
Finally, it was shown that not all combinations of individual antigenic footprints are viable when grafted onto each other, but that these defects can be overcome by additional iterative selection rounds. Impressively, the most antigenically distinct lead candidate emerging from this extended strategy, CAM130, not only evaded most neutralizing antisera from mice, nonhuman primates, and humans more efficiently than wild-type AAV1, but also maintained very similar transduction and biodistribution profiles in mice.

As a whole, this new approach is a welcome, promising, and needed addition to the arsenal of current technologies to overcome the problem of anti-AAV immunity in humans. Still, much more work is required to be able to conclude whether mutagenizing multiple regions of AAV capsids is widely tolerated among other serotypes and mutants. Likewise, it will be important to study thoroughly whether improvements in antibody resistance measured in this study truly bear clinical implications, especially considering that antibody escape was incomplete and that not all tested primate or human antisera were evaded. However, there is reason to hope that these concerns may be resolved through the juxtaposition of this new combinatorial approach with previously validated strategies for evasion or reduction of anti-AAV antibodies. This would, in turn, help to relieve further the current restrictions on enrollment in AAV vector-based clinical trials and largely expand the cohorts of prospective gene therapy patients.

Finally, beyond the specific problem of antigenicity, it is a generally encouraging outcome of this and similar other studies to see how targeted and rational engineering of exterior capsid moieties can amend or resolve intrinsic limitations of AAV variants. In combination with the ever-improving modalities for high-throughput molecular evolution and selection of AAV capsid mutants (see above and also the next two paragraphs), this fuels hopes that the field will soon be in possession of a new generation of designer viral vectors that are tailored to a given application, target, and antibody profile, and hence an optimal tool for clinical gene therapy.

Ancestral capsid reconstruction: one step back, many steps forward in evolution

As noted, conventional AAV evolution strategies follow a forward principle because novel capsids are generated that do not exist in nature and are selected from a pool of variants based on specific criteria. Therefore, the original strategy called “ancestral reconstruction” that was reported in recent publications from the Vandenberghe and Schaffer laboratories represents a radical departure from this paradigm. This is because rather than going forward in evolution, the two groups have taken a step back in time and have thus inverted the traditional concept of molecular AAV evolution. As the details of this technology have recently been covered elsewhere, it should suffice to recapitulate briefly the essential working steps: (i) a group of extant AAV capsid sequences are integrated through phylogenetic analysis, (ii) ancestral nodes and putative parents to the contemporary AAV variants are identified computationally, (iii) a probabilistic sequence space is provided by creating a library covering all possible permutations at uncertain residues, and (iv) this library is screened under stringent selection pressure and under low- or high-throughput conditions, depending on library size.

Best exemplifying the enormous power of this “back to the future” approach is the work by Zinn et al., who first identified a single node (Anc80) from which most of their 75 preselected AAV capsids had likely evolved, and who then managed to isolate a single lead candidate (Anc80L65) from an ensuing screening of nearly 800 capsid variants that were derived from Anc80. Impressively, this ancestral capsid not only performed very well in multiple tissues in adult mice, but even surpassed AAV8—a capsid that is currently in clinical trials—in the liver of nonhuman primates. Furthermore, recently published work illustrates the great potency of this particular capsid for gene therapy in the inner ear, where Anc80L65 mediated reporter gene transfer throughout the entire cochlea and the vestibular sensory organs, without causing any adversity. In addition, Pan et al. successfully used Anc80L65 to improve symptoms of Usher syndrome in a murine model of this hearing disorder, including partial restoration of auditory function, startle response, and balance function, all of which are clinically highly relevant phenotypes.

Notwithstanding these encouraging advances and data, ancestral sequence reconstruction ultimately faces the same challenges like any other AAV capsid evolution technology, that is, a dependency on stringent selection schemes and a need for further fine-tuning of lead candidates. Exemplifying the latter are findings that Anc80L65, despite representing an extinct capsid that probably no longer exists in nature (or never has), partially cross-reacts with antibodies against contemporary serotypes. Fortunately, though, ancestral reconstruction technology is fully compatible with the other advances noted above, including the engi-
neering of antigenic footprints. Consequently, it must primarily be viewed as a novel and creative approach to expand the repertoire of AAV capsid variants and to thus increase chances to evolve superior candidates, in particular when applied in a combinatorial fashion.

DNA barcoding for accelerated high-throughput AAV evolution

The advances highlighted above—Cre-based selection, epitope engineering, and ancestral reconstruction—all hold great potential to propel the molecular AAV evolution field forward, but they still share a particular limitation with the wealth of previously reported screening technologies. This is due to the fact that even under the most stringent selection conditions, these strategems will typically result in a collection of interesting capsids rather than a single lead candidate (note that also Anc80L65 was picked from a large set of possible candidates and turned out to be a lucky choice). Moreover, it is challenging to devise and apply intricate schemes that concurrently select for or against multiple relevant features in a capsid, such as high activity in a desired on-target cell, low activity in all possible off-targets, and no/low reactivity with pre-existing immunity. Instead, these properties are usually tested and then optimized sequentially, which can rapidly pose significant logistical, financial, temporal, and ethical hurdles.

A very elegant solution to this problem was introduced into the AAV field by the Nakai laboratory in 2014, when they first applied DNA barcoding technology as a means to interrogate AAV capsid libraries in vivo rapidly and comprehensively.\(^6\) The underlying concept is to tag each capsid—wild-type or mutant—with a short, 12 nucleotide-long DNA barcode that is inserted into the cognate, capsid-encoding genome. Following packaging of each barcoded genome into its corresponding capsids, all particles are pooled, purified, and then delivered to mice in order to study, for example, capsid biodistribution, blood clearance, or antibody reactivity. In all these cases, interesting capsids are identified through NGS analysis of the library before and after infusion into animals. In striking contrast to conventional selection schemes that take multiple rounds of iterative selection and secondary library preparation, this will instantly reveal the lead candidates in a single round of experimentation, and thus save substantial work, time, and, last but not least, animals.

These benefits were further corroborated in more recent work from the Zolotukhin laboratory, who expanded the DNA barcoding strategy to AAV vector genomes rather than capsid-encoding genomes as in the original study by Adachi et al.\(^6\) The principle remained the same, however, that is, interesting AAV mutants were identified among a pool of variants by NGS analysis of barcode and hence capsid biodistribution. An important improvement was that Marsic et al. exploited a second barcode that was introduced during the preparation of the NGS libraries and that allowed them to identify concurrently the tissue in which the DNA capsid(s) performed best. This further drastically reduces the required workload, costs, and animal numbers, since all tissues of a mouse (or any other experimental animal) can be studied simultaneously in a single sequencing run.

In the future, it should be most rewarding also to include RNA-Seq analysis in this strategy in order to track and quantify AAV capsid mutants concurrently on the DNA and RNA level. This will be important, since many AAV variants may be capable of delivering their encoded genomes into a target cell, but only a fraction of them may also reach the nucleus and result in transgene expression. Indeed, this is what we observed in recent screening of two different comprehensive capsid libraries using AAV vector genomes that carried a DNA barcode in their 3′ untranslated region (Weinmann and Grimm, manuscript in preparation). Moreover, it will be highly beneficial to combine DNA/RNA AAV barcoding technology with the other advances highlighted above in order to accelerate the identification of successfully engineered immune-evasion mutants or the screening of ancestral capsid libraries.

CONCLUDING REMARKS

As noted initially, the purpose of this article was to provide an overview of some of the most recent and, in the authors’ personal opinion, most exhilarating and most influential developments in the areas of basic and applied AAV research. As also noted, this overview was restricted to selected examples that represent emerging concepts and avenues but which clearly only reflect the tip of the massive iceberg that is the current research in the AAV virus and vector community. As members of this community who have both been involved in, and actively contributing to, this research for more than two decades, the authors are thrilled to witness the continued level of creativity, thrive, and ingenuity in the field, and the extremely rapid pace at which it is presently advancing, fueled by the clinical successes with early-generation AAV vectors. It is particularly delightful and intriguing to
see that even after >50 years of intensive research, AAV still holds a large variety of secrets that we are only now beginning to uncover, as perhaps best exemplified by the recent discoveries of the roles of AAP, autophagy, and AAVR. Likewise, it is astounding and highly motivating how the field manages to improve the technologies for molecular AAV capsid evolution consistently, from advanced methodologies for library creation that go back or forth in evolution, to ingenious novel in vivo selection strategies that are becoming increasingly stringent, fast, and powerful. In this context, the authors also briefly mention further work by others that they could not cover in detail but that perfectly complements the examples in this article and that comprises, for instance, the identification of small molecules or secreted host factors such as albumin that boost AAV transduction.\textsuperscript{63,64} Another relevant example is the discovery of an expanded set of host cells that may represent natural AAV reservoirs and thus, vice versa, can be added to the list of therapeutic AAV targets, such as CD34-positive hematopoietic stem cells and T cells.\textsuperscript{65,66}

Last but not least, all of these inspiring and important developments are of course accompanied and complemented by equally stimulating research on the other seminal AAV component (i.e., the genome). While details of these studies and results could unfortunately not be covered here, some of most remarkable achievements and findings should be noted, such as the bioinformatical engineering of cis-regulatory modules (CRMs) for tissue-specific AAV transgene expression,\textsuperscript{67,68} or the finding that strong secondary structures, such as shRNAs or gRNAs, can impact AAV vector integrity, stability, and expression.\textsuperscript{69} Notably, also in this research area, we are still acquiring and benefitting from new knowledge of basic cellular mechanisms that govern the functionality of AAV virus/vector genomes, as exemplified by latest findings on a role of nucleophosmin or the U2 snRNP spliceosome as critical host factors for AAV.\textsuperscript{70–72} Finally, the ongoing discussion on the potential impact of elements in the 3′ untranslated region of the wild-type AAV2 genome that may exhibit enhancer-promoter-like activity and may thus be involved in tumorigenesis in the liver is also noted.\textsuperscript{73–75} However, it important to underscore that the actual relevance of these peculiar results— that were all obtained exclusively in the wild-type AAV context and with partial viral genomes—for recombinant AAV vectors remains largely unclear and highly controversial at this point.\textsuperscript{75–77}

In summary, one can readily (fore-)see how our ever-increasing knowledge of basic AAV biology and the concomitantly growing toolbox for applied AAV technology will continue to stimulate and mutually benefit each other for many years to come. Importantly, one can also clearly anticipate that focused research on these aspects will have numerous indirect and positive implications for additional facets of AAV virus and vector biology. For example, a better understanding of AAV–host interactions combined with deliberate measures to increase transduction efficiency will allow clinical vector doses to be reduced, which will, in turn, help to limit immune responses, including a reduction of cytotoxic T cell activities against the capsid. Accordingly, the AAV vector and gene therapy communities have every reason to believe that we have probably still only seen a glimpse of the fascinating biology and applications of this presumably simple virus, and that the future holds many more stirring and encouraging surprises for researchers, doctors, and patients alike.

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AUTHOR DISCLOSURE
The authors declare no conflict of interest.


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