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REVIEW

Synthetic Biology Approaches for Engineering Next-Generation Adenoviral Gene Therapies

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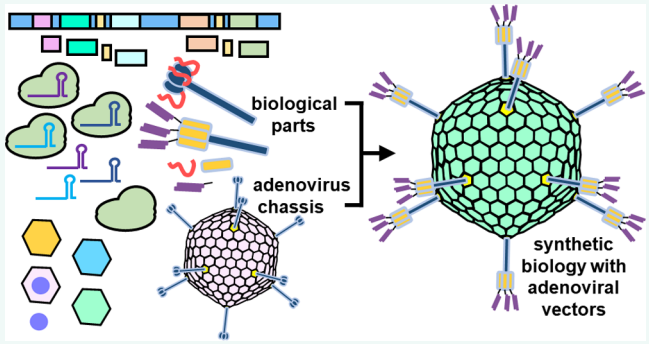
ABSTRACT: Synthetic biology centers on the design and modular assembly of biological parts so as to construct artificial biological systems. Over the past decade, synthetic biology has blossomed into a highly productive field, yielding advances in diverse areas such as neuroscience, cell-based therapies, and chemical manufacturing. Similarly, the field of gene therapy has made enormous strides both in proof-of-concept studies and in the clinical setting. One viral vector of increasing interest for gene therapy is the adenovirus (Ad). A major part of the Ad's increasing momentum comes from synthetic biology approaches to Ad engineering. Convergence of gene therapy and synthetic biology has enhanced Ad vectors by mitigating Ad toxicity *in vivo*, providing precise Ad tropisms, and incorporating genetic circuits to make smart therapies which adapt to environmental stimuli. Synthetic biology engineering of Ad vectors may lead to superior gene delivery and editing platforms which could find applications in a wide range of therapeutic contexts.



KEYWORDS: *adenovirus, CRISPR, gene therapy, genetic circuits, protein engineering, synthetic biology, viral capsids, viral tropisms*

The advent of key technologies such as cheap DNA sequencing and synthesis, cloning tools, CRISPR-Cas systems, and computer-aided biomolecular design software has facilitated the maturation of synthetic biology as an engineering field.[1] While synthetic biology started in bacteria and yeast,[2] it has greatly expanded over the past decade. Synthetic biology has brought neuroscience the toolbox of optogenetics,[3] has treated cancers *via* cell-based immunotherapies,[4] and has aided manufacturing of synthetically challenging drugs.[5] Gene therapy represents another key area upon which synthetic biology has already exerted a major influence. Synthetic biology has spurred advances in experimental and software tools which have facilitated more extensive and widespread engineering of viral vectors.[6,7] Much of contemporary gene therapy has started to incorporate approaches which are often associated with synthetic biology as a discipline. With the increasing synergy between synthetic biology and gene therapy, the next decade is poised to bring another wave of exciting technological changes.

Adenovirus (Ad) vectors are a prime target for applying synthetic biology strategies to gene therapy. One advantage of Ad vectors for synthetic biology approaches comes from their large packaging capacities. Even first-generation Ad vectors, which feature only E1 and E3 deletions, possess packaging capacities of about 8.5 kb.[8,9] Second-generation and third-generation (gutless) Ad vectors have more complex modifications which, respectively, facilitate packaging of around 10 kb and 35 kb. These large capacities provide fertile space for the extensive genetic engineering involved in synthetic biology. Furthermore, Ads have historically shown themselves as highly amenable to protein engineering modifications.[10] Numerous rationally designed modifications to the Ad fiber have granted tissue-specific tropisms and modifications to the hexon proteins have mitigated immunotoxicity.[11,12] In addition, foundations for Ad genetic circuit engineering have been laid by studies on conditionally replicative Ads (CRAds) for cancer therapy which leverage tumor-selective promoters.[13] While many of these CRAds have used simple switch-based logic, recent work has begun to incorporate more complex operations into oncolytic Ad vectors.[14,15] The future is bright since, as synthetic biology and Ad gene therapy experience ever more cross pollination,

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possibilities for environmentally responsive and programmable therapies will continue to grow.

Though Ad vectors experienced a setback after the immunotoxicity-related death of Jesse Gelsinger during an adenoviral gene therapy clinical trial in 1999, a great deal of work has since been done to ensure the safety of Ad therapies.[16] More recent clinical data have shown that, with appropriate dosing and administration, properly developed Ad therapies are safe for patients.[17,18] That said, it is still crucial to consider the immunogenicity levels of Ad vectors when designing treatments. Importantly, synthetic biology provides a variety of powerful approaches by which to create Ad vectors with lower immunogenicity levels.

This review introduces the influences of synthetic biology on Ad gene therapy and considers where these converging fields might go in the future. First, the types of thinking which underlie synthetic biology are elucidated. Synthetic biology techniques for mitigating Ad immunogenicity, for engineering tissue-specific Ad tropisms and for developing Ad-based genetic circuits which perform complex functions are discussed. Future possibilities of employing computational protein engineering methods to enhance Ad gene therapies, utilizing deep learning to optimize Ad design, and leveraging Ads to deliver CRISPR-Cas systems that treat polygenic disorders are explored. The overlap between synthetic biology and Ad vectorology is emphasized throughout.

WHAT DO SYNTHETIC BIOLOGY APPROACHES ENTAIL?

Synthetic biology exhibits a number of distinguishing qualities which have contributed to its success as a discipline. One fundamental idea in synthetic biology is the modular decomposition of biological systems into biological parts (Figure 1).[2,19] To facilitate engineering, synthetic biologists often partition biological systems into subcomponents which retain key functional activities outside of their native contexts. Biological parts vary widely in their granularity. They may come in the forms of single nucleotides, single amino acids, individual protein domains, functional nucleic acid sequences such as promoters and enhancers, proteins and protein complexes, entire plasmids, and gene regulatory circuits which perform functional operations. Biological parts fit neatly into block diagrams, streamlining the engineering process of synthetic biology. Distinguishing biological parts can also facilitate predictive modeling of biological systems and thereby enable computer-aided design of complex biology.[20] It is of interest to note that much of this thinking parallels ideas from electrical engineering due to synthetic biology's historical ties with electrical engineering fields.[2] The biological part concept turns biology into a vast LEGO set, ready to be taken apart and put back together in nigh-infinite configurations.

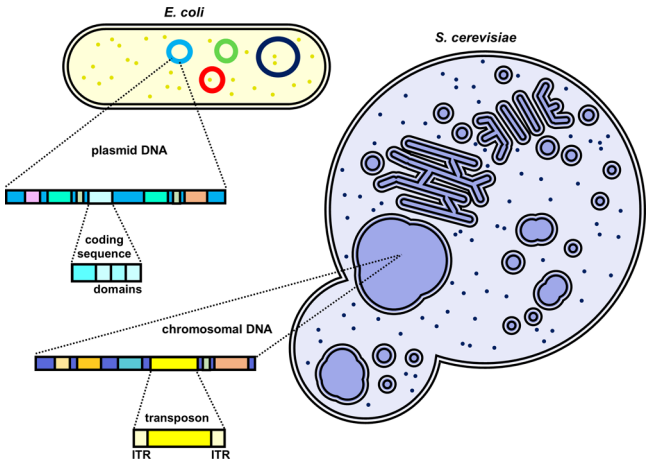

Figure 1. Philosophy of synthetic biology. From the perspective of a synthetic biologist, all biology is decomposable into modular biological parts which can be synthesized, characterized, and rearranged like LEGO bricks to create functional systems.[2,19] A few examples of biological parts are plasmids, chromosomes, coding sequences, transposons, protein domains, and inverted terminal repeats. Synthetic biologists also employ chassis, which are existing biological systems that incorporate edits and parts.[21] Two canonical examples of chassis are *E. coli* and *S. cerevisiae*. The Ad represents another important chassis system.

Another important aspect of synthetic biology is to understand and mitigate the imperfections associated with biological parts. Though distinguishing separate functional parts can be useful, the practice represents a model rather than a reality. Biological systems are often noisy, and synthetic designs can exhibit unexpected crosstalk with endogenous biology.[21] Stated more simply, parts are not perfectly modular. Ameliorating this challenge typically may involve characterization experiments, combinatorial screening, computational optimization, rational design, and directed evolution.[21,22] That said, many biological parts have already been thoroughly characterized,[23,24] making them much more suitable for plug-and-play approaches. The biological parts concept is most powerful when its limitations are understood and addressed using appropriate methods for the situation at hand.

For most synthetic biology applications, biological parts are added into a chassis. The chassis is a preexisting biological system which incorporates synthetic biological circuits and modifications (Figure 1).[21] It typically takes the form of some type of a host cell such as *Escherichia coli* or *Saccharomyces cerevisiae*, though cell-free systems and vesicular systems are also sometimes considered chassis.[25] Cellular chassis allow synthetic biology to take advantage of the given cell's stable self-maintenance, replication, and optimal operation under appropriate environmental conditions. One might argue that a virus coupled with a production cell line can serve as a powerful chassis system as well. The production cell line contributes the stable self-maintenance and replication, while the viral particles themselves act as synthetic biological machines which carry out the effector function of gene delivery to target cells. As described earlier, the Ad represents a key example of a viral chassis which can be modified using biological parts. Applying this "chassis and parts" style of thinking to Ad gene therapy has enabled versatile next-generation designs.

SYNTHETIC BIOLOGY FOR MITIGATING AD IMMUNOGENICITY

One of the most prominent challenges of Ad vectors is immunogenicity, which can prevent gene delivery and exert systemic toxicity, yet synthetic biology design of Ad vectors is providing ways to overcome such issues. One successful strategy has been to swap out Ad capsid proteins for rationally designed versions of themselves which can hide from neutralizing antibodies.[12] This may soon take advantage of machine learning techniques which could help guide capsid protein design.[26] In the context of Ad-based COVID-19

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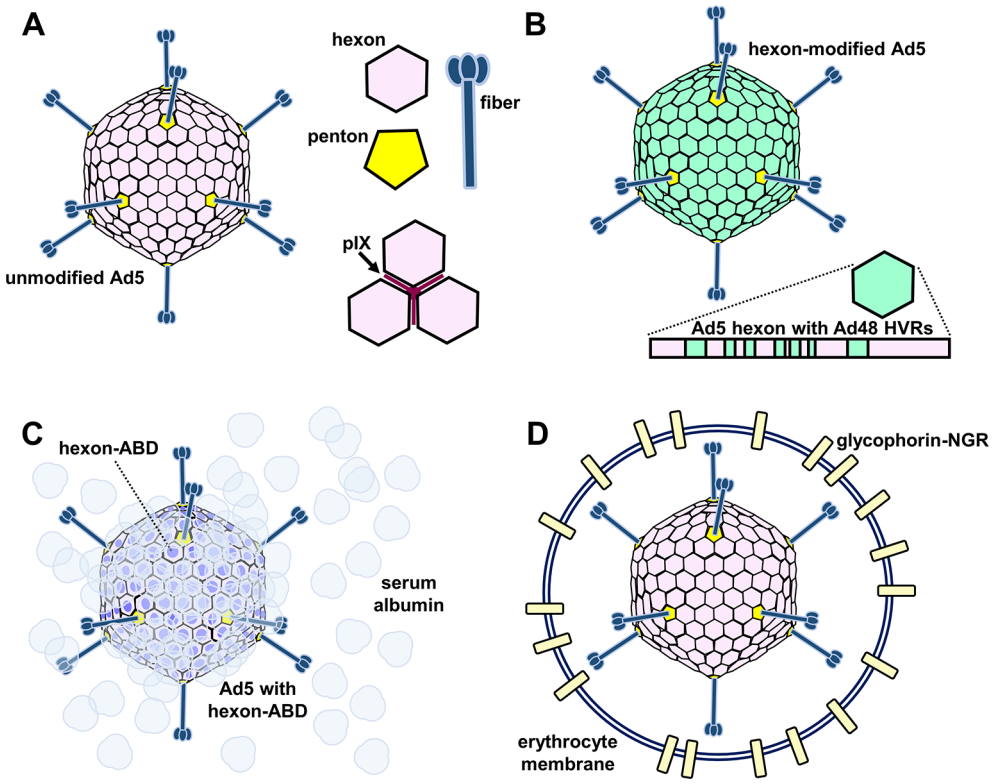

Figure 2. Synthetic biology strategies to help mitigate the immunogenicity of Ads. (A) Structure of the Ad capsid and its external components. Three more structural proteins (not shown) can be found on the inner surface of the Ad capsid: pIIIa, pVI, and pVIII. (B) The Ad hexon includes seven highly immunogenic HVR sequences. Replacing these sequences with versions from rare Ad serotypes such as Ad48[12] can mitigate immunogenicity since the human body has fewer antibodies against rare Ad serotypes. (C) Insertion of an albumin binding domain (purple) into the hexon protein has sterically shielded the Ad from neutralizing antibodies by sequestering serum albumin upon injection.[38] (D) Encapsulation of the entire Ad inside of erythrocyte-derived membrane has protected the Ad from immunological assaults.[39] To facilitate tissue targeting, glycophorin proteins with an NGR tripeptide have been included in the encapsulating membrane.

vaccines, the usage of alternative Ad serotypes as chassis for expression of SARS-CoV-2 antigens has played a key role.[27] Another approach has been to shield the Ad surface by adding biological parts onto the outside of the capsid. Synthetic biology has made an array of contributions to helping decrease immune responses to Ad vectors.

Upon injection into the human body, Ads encounter innate, cellular, and humoral immune responses.[28] Innate immunity against Ads starts with recognition of the virus by pattern recognition receptors and the complement system, leading to early (<24 h after injection) toxicity in the forms of thrombocytopenia and massive cytokine production. After around 3−7 days, the cellular response comes into play, mainly through cytotoxic T lymphocytes lysing infected cells. Humoral immune responses take effect after a few weeks, with antigen presenting cells activating $CD4^+$ T lymphocytes *via* MHC-II and the $CD4^+$ cells subsequently activating B cells. The B cells manufacture anti-Ad neutralizing antibodies and develop immunological memory, blocking any further therapeutic effects from Ads. The tendency for wild-type (WT) Ads to accumulate in the liver exacerbates these immune responses and can lead to hepatotoxicity.[29] These events can harm patients as well as preclude the beneficial effects of Ad therapies.

Synthetic biology methods have started to ameliorate these challenges. Ad capsids consist of seven proteins: three major proteins including the hexon, penton, and fiber proteins as well as four minor proteins including pIIIa, pVI, pVIII, and pIX (Figure 2A).[30] From a synthetic biology standpoint, engineered versions of these proteins may serve as modular biological parts which can decrease immunogenicity. Various capsid engineering strategies have indeed been investigated in pursuit of this goal.[10] Many investigators have also appended nanomaterials and biomaterials onto the surface of the capsid as ways of shielding the immunogenic components of the Ad.[31] In many cases, the nanomaterials and biomaterials in question can be thought of as exogenous biological parts. These approaches borrow principles from synthetic biology to reduce Ad immunogenicity.

Modifications of the existing capsid proteins have aided construction of Ads which exhibit less immunotoxicity. One helpful strategy for circumventing antibodies has been to swap out hexon hypervariable region (HVR) sequences. Most of the preexisting antibodies against the Ad5 serotype target these regions.[11] In an example of this approach, Roberts *et al.* replaced the HVRs of Ad5 with those of the rare serotype Ad48, creating a chimeric vector which avoided antibody responses in mice (Figure 2B).[12] That is, modification of the Ad5 chassis with Ad48-derived biological parts retained Ad5's high infectivity and decreased the effects of neutralizing antibodies. Cutting-edge strategies which have been used for decreasing immunogenicity of adeno-associated virus (AAV) capsids easily might apply to Ad as well. Bryant *et al.* used a high-throughput viability assay to determine the impact of various combinations of mutations on successful virus production and subsequently leveraged their results as training

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data for deep learning models.[26] The deep learning models found a vast sequence space consisting of sets of mutations which were predicted to maintain viability in AAV capsids while varying from the WT. This sequence space constrained the design of capsids with multiple mutations to only those which were predicted to be viable, greatly accelerating the downstream process of finding mutant AAVs which evade immune responses. Since Ads typically exhibit even more immunogenicity than AAVs, similar deep learning platforms may show strong applicability to the field of Ad gene therapy. Capsid engineering in Ad is helping the field to move toward next-generation therapies which circumvent the effects of Ad immunogenicity.

Several of the most widespread COVID-19 vaccines have utilized Ad chassis as delivery vehicles for DNA encoding SARS-CoV-2 spike protein antigens.[32,33] In the tradition of synthetic biology, these vaccines have combined rational chassis selection criteria (*i.e.*, stability and minimal immunogenicity) with a payload of functional genetic parts that encode spike protein antigens. The COVID-19 vaccines developed by AstraZeneca and by Johnson & Johnson, respectively, use the simian Ad known as ChAdOx1 and the human Ad26 vector.[32] As biological chassis, both of these Ads provide a key advantage in that they are stable 4 °C, unlike the mRNA vaccines of Moderna and Pfizer. Furthermore, they both exhibit low seroprevalence in humans, so patients are less likely to carry preexisting antibodies against these vectors.[33,34] It should be noted that, while some immunotoxic side effects have occurred with some Ad vaccines, the prevalence of serious manifestations of these side effects has been extremely low.[35] One might further envision using directed evolution, rational design, or machine learning (as described earlier)[26] on Ad capsid proteins to develop stable vaccines with even less immunotoxicity. Synthetic biology principles have been successfully applied to Ad vaccine vectors for COVID-19.

Constructing Ads with extra parts on their surfaces can shield their most immunogenic capsid regions. One classic approach in this category has been to chemically link polyethylene glycol chains onto the exposed lysine residues of the Ad capsid.[36] This work has spawned a wide array of polymer-based methods for protecting Ad capsids.[37] While these polymer approaches are beyond the scope of this review since they fall under the umbrella of nanomaterials engineering rather than synthetic biology, some synthetic biology methods have utilized the principles of Ad-shielding nanomaterials. Rojas *et al.* inserted an albumin-binding domain (ABD) into the middle of the Ad hexon protein, resulting in sequestration of protective serum albumin onto Ad particles (Figure 2C).[38] One drawback of this approach was the decreased infectivity of the ABD-modified Ads, likely due to bulky albumin interfering with intracellular transport processes. Nonetheless, the immune evasion benefits of ABD-modified Ads quantitatively outweighed the infectivity detriment relative to controls. In another synthetic biology approach, Lv *et al.* used CRISPR-Cas9 to create a transgenic mouse strain which expressed a Asn-Gly-Arg (NGR) tripeptide on the erythrocyte membranes *via* a glycophorin-NGR fusion protein.[39] These erythrocyte membranes were collected and used to encapsulate oncolytic Ads (Figure 2D). The erythrocyte-derived lipid membranes shielded the Ads from antibodies, while the NGR peptide targeted the Ads for uptake by tumor cells. Surface modification of Ad chassis with biological parts is making strides toward less immunogenic Ad vectors.

Synthetic biology has begun to infiltrate the area of Ad vector design toward minimal immunogenicity. Replacing capsid protein domains with sequences from alternative Ad serotypes has helped decrease immunogenicity.[12] Machine learning methods which have mitigated AAV immunogenicity by identifying optimal capsid mutations may also find utility with Ads.[26] Rational chassis selection has been employed to help develop minimally immunogenic Ad-based vaccines for SARS-CoV-2 which show high stability at 4 °C.[33,34] Decoration of the immunogenic Ad capsid surface with occluding biological parts has also helped to prevent immune factors from binding Ads. As the field of synthetic biology continues to proliferate, it will further aid engineering of Ad gene therapy vectors to circumvent immune responses.

SYNTHETIC BIOLOGY FOR ENGINEERING TISSUE-SPECIFIC AD TROPISMS

Making Ads which can target specific cells and tissues represents an important component of Ad vectorology. As mentioned earlier, most of the commonly used unmodified Ads (*e.g.*, Ad5) tend to accumulate in the liver.[29] This can result in hepatotoxicity and prevent the Ads from reaching their intended therapeutic targets if those targets are outside of the liver. In addition, unmodified Ads cannot transduce cells which lack the coxsackievirus and Ad receptor (CAR), limiting their tropisms to just a few cell types.[31] Synthetic biology approaches have been applied to help overcome these issues and increase therapeutic efficacies in general. Engineering of the Ad fiber protein has taken center stage as a way to induce altered tropisms.[10] This has involved modifying the fiber with peptide fusions, with replacements from other Ad serotypes, and with antibodies. Through incorporating exogenous biological parts into and onto the Ad, desired tropisms have been achieved.

Peptide fusions in the Ad fiber have shown success in redirecting the virus to CAR-deficient tissues (Figure 3A). In an early example of this method, Wickham *et al.* made vectors with fusions including an Ad with an RGD motif on the fiber's C-terminus and an Ad with a polylysine motif on the fiber's C-terminus.[40] These vectors displayed improved transduction in a variety of nonhepatic cell types such as macrophage, endothelium, and smooth muscle. Some further examples include fusions of an epitope from the vesicular stomatitis virus glycoprotein[41] and of a cell penetrating domain from the HIV Tat protein.[42] Such peptide fusions exemplify the synthetic biology practices of borrowing parts from the diverse biological world (*e.g.*, the RGD, VSVG, and Tat peptides) and of employing rationally designed *de novo* parts (*e.g.*, the polylysine peptide). These early examples of peptide fusions to the fiber have laid the groundwork for further synthetic biology toward programmable Ad tropisms.

Fiber pseudotyping and xenotyping have also acted to expand the range of tissues that Ads can transduce beyond tissues expressing CAR (Figure 3B).[43] Pseudotyping involves genetically swapping fibers or fiber domains with alternative human serotypes. Xenotyping involves doing the same using fibers or fiber domains from nonhuman Ad serotypes. These methods typically allow Ads to target a wider array of tissues, but do not enable highly specific targeting of desired cell types.[9] Fiber pseudotyping and xenotyping capitalize upon the modularity of biological parts from various Ad serotypes, a key aspect of synthetic biology. So, these methods also have

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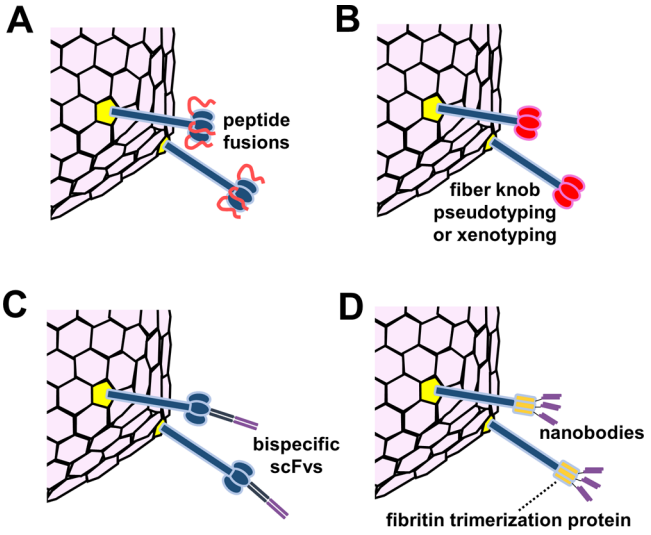

Figure 3. Synthetic biology approaches to engineer tissue-specific tropisms in Ads. (A) Peptide fusions on the C-terminus of the Ad fiber knob can expand tropisms to nonhepatic tissues which do not express CAR.[40,42] (B) Replacing the fiber knob of a commonly used Ad serotype like Ad5 with knob domains from less frequently occurring Ad serotypes can also alter the vector's tropisms, allowing targeting of CAR-deficient tissues.[43] When using knobs from other human Ad serotypes, this is referred to as pseudotyping. If using knobs from nonhuman Ad serotypes, this is referred to as xenotyping. (C) Bispecific scFvs can facilitate tissue-specific tropisms.[44] For this approach, two scFvs are linked together. One scFv binds the fiber knob, while a second scFv binds a tissue-specific marker. (D) Nanobodies can also enable tissue-specific tropisms.[46] Unlike bispecific scFvs, nanobodies can be genetically fused to the Ad fiber. To ensure good efficacy of binding to target antigens, the nanobodies must be oriented away from the Ad capsid. However, C-terminal knob domain fusions would orient the nanobodies toward the Ad capsid. Swapping out the knob domain for a fibritin trimerization protein can solve this problem since the fibritin C-terminus points outward.

contributed to the foundation of synthetic biology which underlies Ad tropism engineering.

Antibody-based strategies have been employed to target desired cell types more precisely. In a precursor to later antibody approaches, Haisma *et al.* fused two single-chain variable fragments (scFvs), one of which was directed against the Ad fiber knob and the other of which was directed against the EGF receptor (Figure 3C).[44] This bispecific scFv attached to the fiber knob and gave the Ads tropism for cell lines expressing the EGF receptor such as those derived from epidermal carcinomas. Since the Coughlan *et al.* study, many similar adaptor methods of attaching antibodies (or antibody fragments) to the fiber knob have been explored.[45] Unfortunately, the noncovalent associations of these adaptors with the knob may undergo some level of disruption upon *in vivo* delivery.[10] To circumvent this problem, van Erp *et al.* made Ads with camelid nanobodies genetically fused onto the fiber (Figure 3D).[46] Because the C-terminus of the knob orients back toward the Ad capsid, the knob was replaced by a T4 fibritin trimerization protein which instead allowed the nanobodies to point outward. This clever protein engineering approach incorporated the fibritin and nanobody biological parts directly into the Ad chassis, avoiding the complications of the adaptor strategies. Ways of combining Ads with immunoglobulins provide versatile platforms for programming precise tropisms into Ad vectors. Such molecular precision and programmability represent key attributes sought after in synthetic biology design.

Synthetic biology has demonstrated a growing influence over Ad tropism engineering. Peptide fusions, pseudotyping and xenotyping, and antibody-based modifications have all involved adding biological parts to Ad fibers or replacing existing components of the fibers. While many of the earlier examples of these approaches made relatively simple adjustments to the Ad chassis, the more recent trend of incorporating multiple biological parts from distinct sources[46] illustrates how the design-centered ethos of synthetic biology has gained more traction in Ad vectorology. The continued acceleration of the field of synthetic biology will likely provide tools, parts, and ideas which may facilitate further improvements in Ad tropism design.

SYNTHETIC BIOLOGY FOR ENGINEERING AD GENETIC CIRCUITS

Ad genetic circuits represent an emerging frontier in synthetic biology. The high packaging capacities of Ads make them ideally suited to carry complex genetic circuits consisting of multiple interacting elements.[8,9] Sets of modular biological parts have been used to construct genetic circuits, creating Ad vectors which dynamically respond to external events.[14,15] Sets of modular biological parts have also been employed to create Ad vectors which carry complex CRISPR-Cas systems.[47] With the increasing availability of tools for genetic circuit design and characterization, Ad genetic circuits have great potential for propelling next-generation gene therapies.

Ad vectors which respond to external signals may grant immense benefits. Though this area is still in its infancy, proof-of-concept studies have shown the promise of dynamically responsive Ad vectors. Huang *et al.* constructed an oncolytic Ad vector with a complex genetic switch to facilitate conditional Ad replication and expression of immune effector molecules in tumor cells (Figure 4A).[14] Expression of the Ad replication factor E1A and an immune effector was regulated by a tumor-specific promoter and host microRNAs miR-199a-3p and miR-21, which are, respectively, found in normal liver cells and hepatocellular carcinoma cells. Huang *et al.* designed the circuit to only induce expression of E1A and immune effector when the tumor-specific promoter is activated, miR-21 is high, and miR-199a-3p is low. This was accomplished by compiling modular promoters, coding regions, and regulatory sequence elements into a logic gate configuration which yielded the desired output. In another example of an externally responsive Ad vector, Takayama and Mizuguchi used a pair of Ads to deliver optogenetic gene activation machinery and thereby to spatiotemporally control gene expression (Figure 4B).[15] One of the Ads carried a gene encoding a catalytically inactive dCas9 with fusions to the CIBN domain. The other Ad carried a gene encoding a protein called CRY2 fused to a VP64 domain as well as a gene encoding a gRNA. CIBN-dCas9-CIBN bound to its target gene *via* the gRNA. During exposure to blue light, CIBN sequestered CRY2-VP64, and the VP64 domain activated the gene. The system enabled optically programmable gene expression in mice infected by the Ads, laying the foundation for future targeted therapies. These studies illustrate how synthetic biology techniques can empower Ad gene therapy to exhibit smart responsiveness to outside signals and to thus gain superior precision and efficacy.

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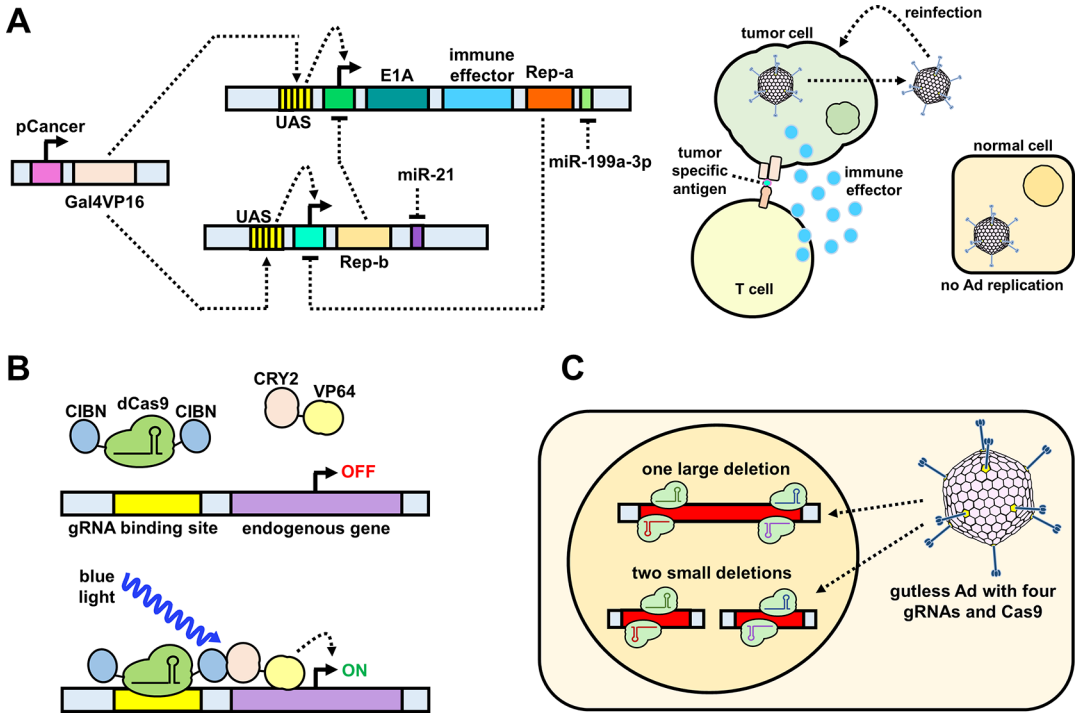

Figure 4. Ways of using synthetic biology to engineer genetic circuits in Ad systems. (A) Genetic circuit to ensure that Ad replication and immune effector expression only occurs in tumor cells.[14] The tumor-specific promoter pCancer encodes an activator (GalVP16) which binds to the upstream activating sites (UAS), turning on two genes. These genes mutually inhibit each other through the Rep-a and Rep-b repressors. The genes also feature microRNA binding sites which regulate their activity at the RNA level. Because of this circuit, the Ad replication initiator E1A and the immune effector are only expressed when pCancer is active, host cell miR-21 is high, and host cell miR-199a-3p is low. (B) Optogenetic circuit delivered *via* two Ads.[15] By leveraging dCas9 and its gRNA, the VP64 activator domain, and protein domains which associate upon exposure to blue light, this circuit can facilitate spatiotemporal control of endogenous genes. (C) Multiplex CRISPR-Cas system delivered *via* gutless Ad.[47] The Ad encodes a Cas9 nickase and four gRNAs. In this way, two small deletions can be made by double nicking at separate genomic loci, or a single large deletion of a desired region can be made by double nicking around that region.

Another important area of Ad genetic circuit design involves CRISPR-Cas machinery. Though there is currently a strong push for CRISPR therapeutics delivered by AAVs in the translational sector, Ad vectors have much higher packaging capacities and lower manufacturing costs, so they could soon gain significant traction as well.[9] Furthermore, proof-of-concept studies have illustrated the utility of bringing together Ads and synthetic biology principles for CRISPR-Cas delivery. As an illustrative recent example, Nakanishi *et al.* engineered a gutless Ad vector to simultaneously express four different gRNAs alongside a Cas9 nickase (Figure 4C).[47] In mice, this allowed disruption of two target regions using double nicking for each as well as deletion of a large target region by applying double nicking to a pair of flanking sites. The study demonstrates how CRISPR-based synthetic biology and Ad gene therapy are coming together to lay the foundation for future gene therapies. The Cas9 nickase and four gRNAs acted as modular biological parts installed on the gutless Ad chassis, facilitating complex *in vivo* gene editing. It should be further noted that the Nakanishi *et al.* investigation employed Golden-Gate Assembly,[48] which is a cloning method that falls squarely under the umbrella of synthetic biology. Bringing together CRISPR and Ad vectors represents a prime area in which synthetic biology approaches could be exploited to create superior gene therapies.

Engineering the genetic circuits of Ads has enabled the creation of biological functions, both in making Ads respond dynamically to outside stimuli and in giving Ads the ability to perform more complex gene editing in target cells. MicroRNA-based logic gate circuits have been leveraged to enhance tumor specificity in oncolytic CRAds.[14] Spatiotemporal control of gene expression has been achieved through Ad-mediated delivery of optogenetic gene regulatory circuitry.[15] Multiplexed gene editing has been performed in mice using Ad vectors encoding four gRNAs and a Cas9 nickase.[47] Though the synthetic biology of Ad genetic circuits is still in the early stages of technology development, the work done so far has shown great potential for propelling the field toward future clinical applications.

OUTLOOK ON THE FUTURE

As synthetic biology continues to expand, it may further enhance Ad gene therapy. Computational design of proteins may begin to play a more central role in rationally specifying the structure and function of Ad vectors.[49] As has already been seen in the field of AAV gene therapy, high-throughput experimental techniques could provide training data for machine learning models which might extract superior Ad vector designs.[26] Ad delivery of multiplexed CRISPR-Cas systems may allow genetic treatment of polygenic conditions.[47,50] Synthetic biology may help construct superior Ad vectors and enable polygenic Ad therapies.

Emerging computational approaches involving protein design software may support greatly improved Ad vector design (Figure 5A). Computational protein engineering has made immense strides, and it is now feasible to create complex

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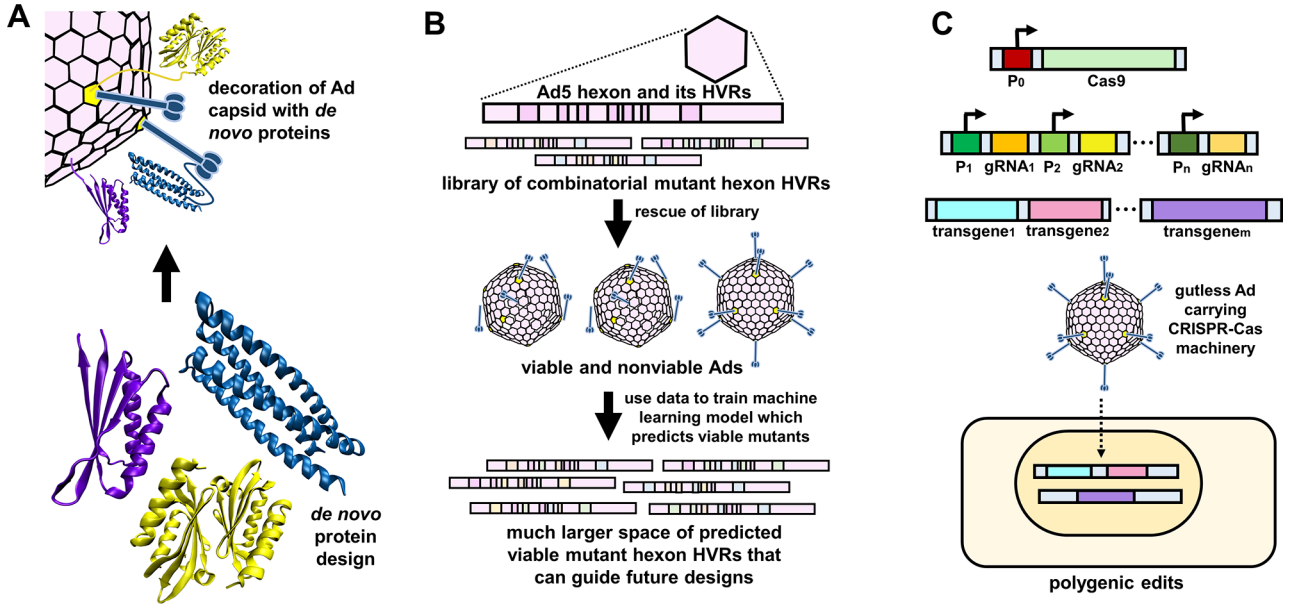

Figure 5. Promising directions for the future of synthetic biology in adenoviral gene therapy. (A) Computational protein engineering approaches[51−53] may facilitate the design of *de novo* structures which could be incorporated into Ad vectors. Decorating the Ad capsid with such precisely designed proteins may enable programmable responses to various environmental conditions. PDB structures 5J2L (blue), 6WI5 (purple), and 5TPH (yellow) are pictured here as illustrative examples of *de novo* protein designs.[64−66] (B) Machine learning may help guide Ad design.[26] For instance, machine learning could predict a large space of mutated (and therefore potentially less immunogenic) hexon HVR regions which do not interfere with Ad viability. This could accelerate downstream experiments by ruling out a vast number of likely nonrescuable designs. (C) Gutless Ads have large packaging capacities of up to 35 kb.[8] Because of this, they can carry enough CRISPR-Cas machinery to implement multiple edits in target cells.[47] In combination with technologies that improve understanding of the effects of multiple genetic perturbations on host organisms, these Ad vectors may help enable polygenic gene therapies.

de novo structures using software.[51,52] One important software suite for protein engineering is Rosetta; this software has been applied to the design of antibodies, the design of interfaces between proteins and their interaction partners, the design of symmetric protein assemblies, the design of membrane proteins, the design of proteins with completely synthetically created functions, and more.[53] As an example, Divine *et al.* recently used Rosetta to develop modular protein nanocages that incorporate antibodies into diverse multivalent nanocage architectures.[54] Rosetta-based or similar methodologies might be employed to design Ad hexon, penton, or fiber proteins with improved properties or with synthetically created functions. Related computational techniques could also be used to design protein complexes which decorate or encapsulate the Ad capsid at physiological pH and disassemble upon exposure to acidic conditions such as in the endosome or tumor microenvironment.[55] This could shield the Ad capsid from circulating antibodies while minimizing interference with transduction and intracellular trafficking. Engineered proteins might also modify Ad tropisms or act as scaffolds which bind and transport many copies of useful biomolecules (DNA, RNA, enzymes, Cas proteins, photocleavable molecules, *etc.*) on the outside of the capsid. Though directly appending bulky proteins to the Ad capsid is often difficult since they can interfere with viral assembly, there are ways to add small adaptors which can sequester larger designed proteins. For example, the SpyTag peptide reacts in a highly specific fashion to form a covalent isopeptide bond with the SpyCatcher protein.[56−58] One could feasibly attach large *de novo* protein designs to the Ad capsid by fusing SpyTag onto a chosen capsid protein and then mixing the Ad-SpyTag particles with a *de novo* protein that has been fused to SpyCatcher. Improved computational tools and user interfaces for protein engineering may enable numerous rationally designed Ad vector enhancements.

High-throughput experiments coupled with machine learning may take on an increasingly important role in Ad vector engineering (Figure 5B). The principles seen in the Bryant *et al.* study on AAVs could easily be applied to Ads as well.[26] High-throughput experiments might characterize sets of mutations in the Ad hexon HVRs which retain viability. These data could subsequently act to train artificial neural networks which might infer a larger sequence space of possible variants. This sequence space could greatly narrow the number of potential designs, making it unnecessary to test every possible combination of mutations in the HVR domains. Similar strategies might be implemented to facilitate tropism engineering. This might start with high-throughput experiments to characterize sets of mutations in the fiber knob that increase binding efficacy and specificity onto a desired receptor. One could also apply rational design prior to the mutational characterization experiments, shifting the space of possibilities to be explored through machine learning. The combination of high-throughput experimental methods with machine learning may guide and accelerate Ad vector design.

Ad delivery of multiplexed CRISPR-Cas systems could help usher in an age of polygenic gene therapies (Figure 5C). The extremely large packaging capacities of gutless Ad chassis make them ideally suited to carry Cas proteins, multiple gRNAs, and one or more transgene sequences. Platform technologies such as organoids,[59] multiorgan-on-a-chip platforms,[60] and spatial transcriptomics[61] are granting a better understanding of how polygenic changes can influence cell, tissue, organ, and whole-organism physiology. While the experiments necessary to

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understand the multiscale effects of polygenic gene editing can produce massive amounts of data, artificial intelligence (AI) methodologies are well-poised to extract useful insights from such large data sets.[62,63] By putting together gutless Ad vectors equipped with multiplexed CRISPR-Cas systems with platforms which decipher physiological responses to polygenic changes, polygenic gene therapy may gain feasibility in the near future.

Ad synthetic biology represents a diverse and exciting field which will likely expand significantly in the coming decade. The properties of Ad vectors have made them well-suited as chassis for a broad range of useful biological parts. Synthetic biology has historically made use of computational techniques as well.[2] Though computational methods have so far received minimal attention in Ad vectorology, the proliferation of synthetic biology into Ad design may soon expand the computational aspects of Ad gene therapy. In particular, *de novo* protein design software[52] and AI-guided sequence engineering[26] could gain prominence. Multiplexed CRISPR-Cas parts in gutless Ad chassis could give Ad synthetic biology a strong foothold as a player in the emerging field of polygenic gene therapy.[50] These possibilities in Ad synthetic biology represent an opportunity for the field to grow and to potentially transform the future landscape of gene therapy.

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Notes

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VOCABULARY

Adenovirus, a type of nonenveloped virus which carries double-stranded DNA and can be used as a therapeutic gene delivery vehicle; **camelid nanobody**, a monomeric antibody fragment derived from the heavy-chain antibodies of camels. At around 15 kDa, camelid nanobodies are very small compared to other types of antibody fragments; ***de novo* protein engineering**, the practice of designing entirely artificial proteins from scratch rather than borrowing domains from preexisting proteins. This often involves starting with a desired structure and utilizing biophysical principles to identify an amino acid sequence which will reliably fold into that structure; **immunogenicity**, the degree to which a substance elicits an immune response upon introduction to a host organism; **oncolytic virus**, a viral vector which has been engineered to induce tumor shrinkage or slow tumor proliferation

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