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Engineering Delivery Vehicles for Genome Editing

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viral gene therapy, nonviral gene therapy, zinc finger nucleases, TALENs, CRISPR, nanoparticle, virus

Abstract

The field of genome engineering has created new possibilities for gene therapy, including improved animal models of disease, engineered cell therapies, and in vivo gene repair. The most significant challenge for the clinical translation of genome engineering is the development of safe and effective delivery vehicles. A large body of work has applied genome engineering to genetic modification in vitro, and clinical trials have begun using cells modified by genome editing. Now, promising preclinical work is beginning to apply these tools in vivo. This article summarizes the development of genome engineering platforms, including meganucleases, zinc finger nucleases, TALENs, and CRISPR/Cas9, and their flexibility for precise genetic modifications. The prospects for the development of safe and effective viral and nonviral delivery vehicles for genome editing are reviewed, and promising advances in particular therapeutic applications are discussed.

INTRODUCTION

The sequencing of the human genome, the identification of the genetic basis of many diseases, and the exponential increase in genomic sequencing capacity have combined to spark a new era of molecular medicine for precise gene therapies. However, few therapies are currently approved for numerous known genetic diseases. Until recently, the tools to correct the underlying chromosomal mutation did not exist. Gene therapies thus relied on gene replacement strategies, which have made great preclinical and clinical progress to treat various conditions (1–3). However, new techniques for precise chromosomal modification by gene editing enable gene correction in the native chromosomal context and therefore create new possibilities for gene therapy (4). Four major classes of genome engineering tools have been pursued that allow targeted modifications to the host genome. These tools have been rapidly adapted to the study of genetic diseases and as potential therapies for correcting the causal genomic abnormalities (5). The main barrier to clinical translation of these technologies is the efficient and safe delivery of these genome engineering tools to targeted tissues of interest. The creation of improved viral and nonviral gene delivery vectors will drive successful clinical translation of these technologies to an extended variety of diseases and target tissues. This article reviews recent advances in genome engineering with emphasis on the delivery methods and vehicles that are being applied for clinical translation.

GENOME ENGINEERING

Programmable DNA-Binding Proteins

The genome engineering revolution has been fueled by the discovery and application of highly specific DNA-binding proteins (DBPs) and targeted nucleases built from these DBPs. Engineered DBPs permit sequence-specific binding and modification to the genetic and epigenetic code. Four main classes of programmable DBPs have been detailed to date: meganucleases (MNs) (6), zinc finger proteins (ZFPs) (7, 8), transcription activator-like effector proteins (TALEs) (9), and the clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 systems (10). MNs are a class of small endonucleases that recognize DNA sequences of 14–40 bp (11). In addition to the naturally occurring MNs (e.g., I-SceI; **Figure 1*a***) (12), various strategies have been used to modify MNs to recognize desired sequences (13–16), though reengineering of MNs has proven technically challenging. ZFPs are based on the Cys2-His2 zinc finger DNA-binding motif that recognizes 3 bp of DNA and can be assembled into arrays of domains (**Figure 1*b***) (7, 17–19). ZFPs are more amenable to a modular construction strategy than MNs; however, creation of large numbers of ZFPs remains labor intensive. Engineered TALEs are originally derived from the plant pathogen genus *Xanthomonas*, which expresses a repetitive DNA recognition domain in which each repeat recognizes a single DNA nucleotide based on variation in two amino acids, known as the repeat variable diresidue (**Figure 1*c***) (20–23). Engineering TALEs to target new sites is easier than with ZFPs; however, the large size and highly repetitive tracts of TALE proteins made early cloning strategies technically difficult, particularly in the preparation of viral vectors (24, 25). The recent discovery of the RNA-guided endonuclease CRISPR system has provided a platform for modifying genomic and epigenomic sequences with a simplicity and scale that were previously impossible (**Figure 1*d***) (26, 27). CRISPR systems, including the Cas9-based type II CRISPR system, function in bacteria and archaea as adaptive immune systems against invading phage (28–31). The ability of these systems to create double-strand breaks at programmed DNA target locations has led to their reengineering for use as gene editing tools in mammalian cells. Candidate guide RNAs (gRNAs) that target new sites can be assembled and tested with great speed and lower cost and in much higher throughput than other technologies; in fact, libraries of

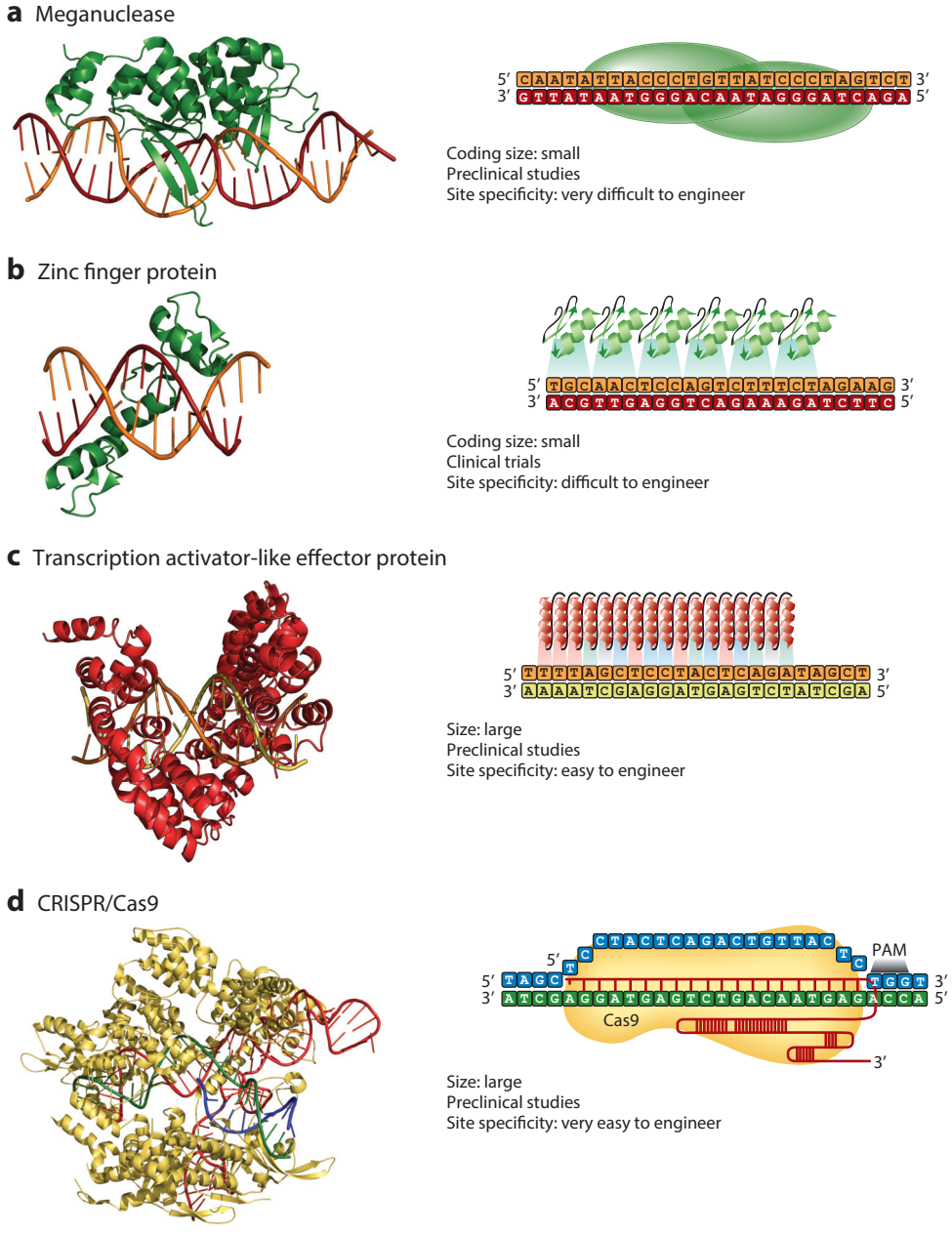


Figure 1

Genome engineering tools. (*a*) Meganuclease family of specific nucleases (pictured I-SceI). (*b*) Zinc finger proteins with 3–base pair (bp) recognition by each domain. (*c*) Transcription activator-like effector proteins with 1-bp recognition by each domain. (*d*) RNA-guided CRISPR/Cas9 endonuclease systems (pictured *Streptococcus pyogenes* Cas9). Crystal structures derived from Protein Data Bank (RDB: 1R7M, 1MEY, 4OSI, 4UN3) and images acquired with open source PyMOL.

gRNAs targeting all genes can be assembled for functional screens (32–35). Furthermore, a Cas9 variant with deactivated nuclease function (dCas9) can be used as a platform for other genome engineering functions (36–40).

Genome Editing and Engineering Methods

Functionalized site-specific DBPs can be used to replace regions of DNA, disrupt genes by shifting the reading frame, enhance or repress transcription, or modify epigenetic states (**Figure 2**). Site-specific nucleases built from DBPs can be used to modify or repair a targeted genomic sequence by enhancing homologous recombination with a donor DNA template by several orders of magnitude (**Figure 2*a***) (41, 42). Homologous recombination or homology directed repair (HDR) can also be used for targeted integration of exogenous genes into safe harbor regions for gene replacement or augmentation (43, 44). In the absence of a donor repair template, the error-prone nonhomologous end-joining (NHEJ) pathway resolves DSBs with small insertions or deletions (indels) that can be used to disrupt the coding region of a gene of interest (**Figure 2*b***). NHEJ can also be used to delete regions of the genome to repair the reading frame of a protein by removing nonessential exons (45, 46) (**Figure 2*c***). Large deletions of up to ~1 Mb have been reported (47), which increases the potential flexibility of genomic deletions.

MNs and CRISPR/Cas9 occur naturally as nucleases; however, ZFPs and TALEs must be modified for nuclease activity. The catalytic domain of the FokI endonuclease fused to either ZFPs [zinc finger nucleases, ZFNs (48)] or TALEs [TALE nucleases, TALENs (49)] has been used extensively. Because FokI functions as a dimer, this requires engineering two opposing DBPs, both of which are fused to a FokI monomer. ZFPs and TALEs have also been modified with recombinase and integrase domains to create targeted recombination and integration events (7, 50–52). These fusion proteins can be used to target the integration or exchange of DNA without requiring the creation of DSBs or endogenous DNA repair mechanisms (4) (**Figure 2*d***). ZFPs, TALEs, and dCas9 can also be fused with effector molecules that allow activation or repression of gene expression and modification of epigenetic states (**Figure 2*e***) (37–40, 53, 54).

DELIVERY

Translational gene therapy and genome engineering are contingent upon the safe and effective delivery of DNA, RNA, or active protein to the target tissue of interest. Carrier-free delivery of DNA and RNA has been used with some success for specific applications; however, use of viral or synthetic gene delivery vectors is required to reach therapeutic levels of activity in most cases. Delivery of genome engineering tools can rely on the decades of research aimed at viral gene therapy (reviewed in References 2 and 55) and nonviral gene therapy (reviewed in Reference 3). Viral- and nonviral-based methods have been developed in parallel, with preclinical and clinical success in both fields. Viral gene therapy has the advantage of efficient transduction and high transgene expression levels. However, viral gene therapy was initially set back owing to safety concerns, including immunogenicity and insertional mutagenesis, which are being addressed with the current generation of vectors (56–59). Nonviral vectors are generally lower in efficiency but have the advantage of diverse available chemistry, capacity for functionalization and targeting, and ease of manufacturing. Both delivery systems have seen success in specific applications.

Nonviral Delivery Vectors

Many classes of nonviral vectors have been considered for gene delivery, including carrier-free delivery, physical membrane disruption, liposomes, polymers, nanoparticles, and cell-penetrating

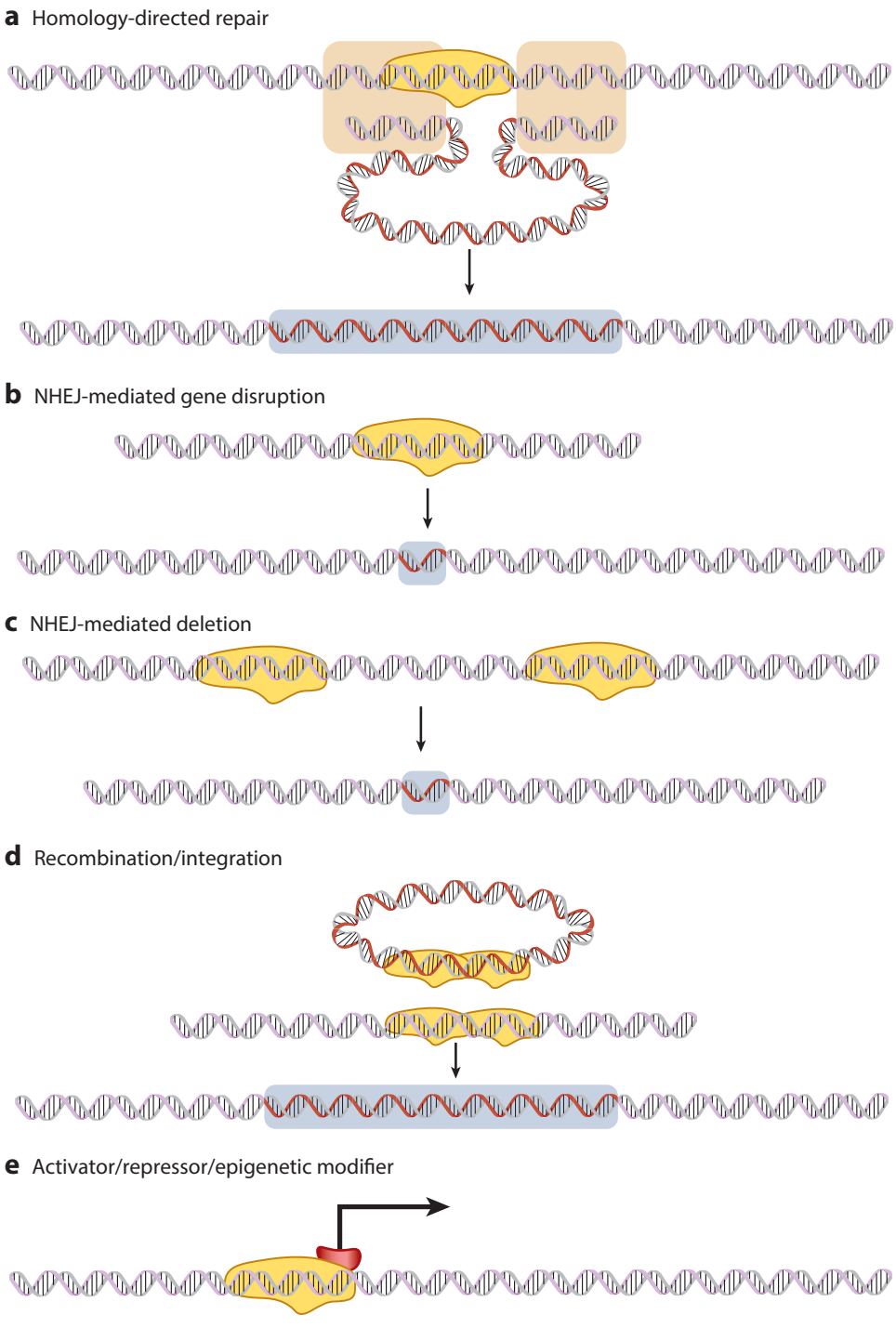


Figure 2

Methods of genome editing. (*a*) Homology-directed repair (HDR): a template with homology arms to the cut site is provided, which mediates HDR. (*b*) Nonhomologous end-joining (NHEJ) repair leads to indel formation to disrupt the gene reading frame. (*c*) NHEJ-mediated deletion of a genomic region by two neighboring nuclease-induced breaks. (*d*) Recombinase/integrase-mediated plasmid insertion without stimulating DNA repair. (*e*) ZFP-, TALE-, or dCas9-based transcription factors including activators, repressors, and epigenetic modifiers.

peptides. Drawbacks of viral vectors that have motivated the development of nonviral gene delivery systems include immunogenicity of viral proteins, insertional mutagenesis (56–59), and cost of viral vector production. A more diverse set of chemical functionalities is available for synthetic nonviral systems than for natural viral systems, thus enabling novel strategies for targeting and stabilization. Several nonviral gene delivery vectors have entered clinical trials, notably for cystic fibrosis and cancer (3). Nonviral vectors are beginning to be applied for the delivery of genome engineering technologies (3), including plasmid DNA (pDNA), messenger RNA (mRNA), and protein delivery.

Nonviral gene vectors: payload. In most published cases for preclinical models of gene therapy and genome engineering, pDNA is the typical payload, as pDNA can encode expression cassettes for both genome engineering enzymes and gRNA for CRISPR-based approaches. pDNA is considered a safe biomolecule for transgene expression, as pDNA remains episomal with very low, albeit measurable, rates of integration. mRNA encoding genome engineering tools is being pursued as an alternative with no risk of insertional mutagenesis (60). Moreover, short bursts of nuclease expression are associated with high activity and low off-target effects (61). Though mRNA delivery can be associated with an innate immune response (62) and RNA is less stable than DNA, chemically modified RNA has addressed some of these concerns (63, 64). For CRISPR/Cas9, chemical modification of gRNA is being pursued to improve stability (65). Both pDNA and mRNA transiently express their cargo, which is well suited for genome engineering tools where persistent expression increases risk of off-target activity (66).

Nonviral gene delivery barriers. Evolutionary processes have generated viral vectors capable of overcoming systemic and local gene delivery barriers. However, synthetic delivery vehicles must be engineered with consideration of these significant delivery hurdles (**Figure 3**). Serum stability is a necessary feature of nucleic acid delivery vehicles owing to rapid degradation of pDNA and mRNA by serum nucleases and clearance by the hepatic or renal system (**Figure 3**, point 1) (67, 68). Nonviral vectors can be designed to shield and protect DNA/RNA from serum nucleases and avoid red blood cell aggregation, in vivo toxicity, and pulmonary emboli associated with many conventional cationic vectors (69–71). Large (>20-nm) nanoparticles were thought to avoid renal clearance through size exclusion. However, polycationic complexes of larger size can be disassembled at the glomerular basement membrane in the renal capillaries, leading to disassociation and clearance of the DNA or RNA (72). Extravasation and cell-specific targeting can be achieved through the use of specific targeting ligands (73) or, in some cases, the enhanced permeation and retention effect (**Figure 3**, point 2) (74, 75). Intracellular trafficking to the cellular compartment of interest is a major barrier (**Figure 3**, points 3–8). For the delivery of RNAs that function in the cytoplasm, disruption of endosomes enhances activity (76). However, for pDNA, nuclear localization is a rate-limiting step in gene delivery (77). This phenomenon explains the higher transfection efficiency noted in dividing cells in which the nuclear membrane breaks down. Use of mRNA-encoding genes, small interfering RNA, antisense oligonucleotides, or miRNA that act in the cytoplasm does not require nuclear localization. Recognition of nucleic acids by the innate immune system by Toll-like receptors has been a setback in the development of delivery of mRNA (78), siRNA (79), and pDNA (80). To address this, removal of unmethylated CpG in plasmid backbones can reduce immune detection (81). mRNA and siRNA sequences should be screened for activation of the innate immune system (82) and can be chemically modified to reduce Toll-like receptor interaction.

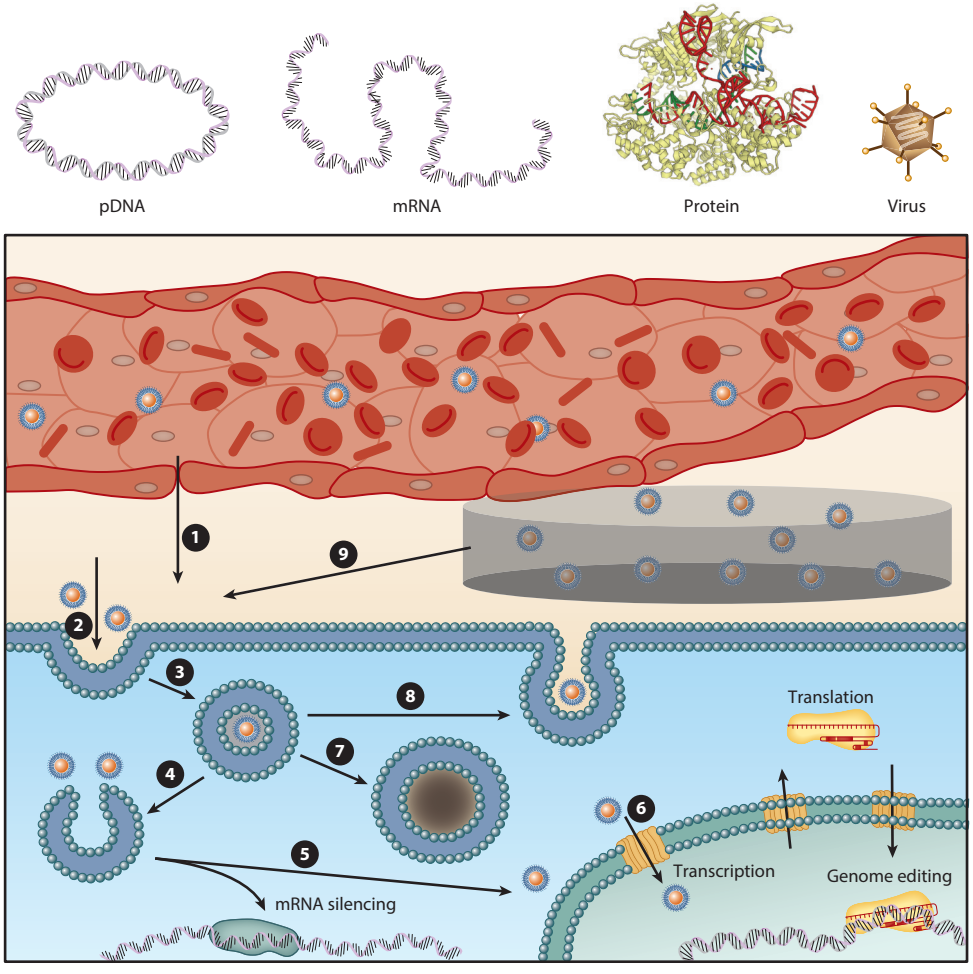


Figure 3

Gene delivery barriers. Systemic administration of genome engineering tools requires ❶ blood/serum stability, ❷ extravasation into the target tissue, ❸ cellular uptake, ❹ endosomal escape, and finally ❺ cytoplasmic or ❻ nuclear availability. ❼ Avoiding lysosomal degradation and ❽ exocytosis improves availability of the delivered genes. ❾ Local delivery to the tissue of interest bypasses many of the systemic barriers but still must solve local delivery barriers, including cellular uptake and proper intracellular localization. Abbreviations: mRNA, messenger RNA; pDNA, plasmid DNA.

Carrier-free delivery. Carrier-free delivery has been pursued through physical disruption of membranes to achieve intracellular delivery or through selective entry into damaged membranes. Electroporation, sonoporation, and magnetofection can be used to temporarily disrupt cell membranes for plasmid delivery. The potential of cell death and the availability of the target tissue make this technique useful only for ex vivo cell modification or in a local in vivo setting. Hydrodynamic injection also can be used to temporarily disrupt the liver and drive efficient delivery in animal models, but safety concerns over the volume required limit this approach clinically to isolated limb delivery in humans (83). This has been used in mouse models to deliver CRISPR/Cas9 to correct hereditary tyrosinemia (84) and to disrupt PTEN and p53 as a model for cancer development

(85). Nucleic acids, including AONs, have been delivered through damaged membranes in skeletal muscle to modify mRNA splicing and correct gene expression for Duchenne muscular dystrophy (DMD) and spinal muscular atrophy, an approach now in phase III clinical trials (86–88).

Liposomes. First adapted for DNA delivery in 1980, liposomes are the most widely used transfection reagent for pDNA and siRNA in cell culture and preclinical models owing to the broad range of cell types that are transfected (89). The cationic head group electrostatically condenses nucleic acid into the liposome, preventing nuclease degradation (**Figure 4*a***). The liposome reaches the target tissue of interest and facilitates cell uptake through the net-positive surface charge or conjugated targeting molecules. Finally, nucleic acid is released from intracellular compartments or endosomes, leading to release and nuclear localization (**Figure 3**). To improve the availability and activity of liposomal formulations, combinatorial libraries have been produced that extend circulation time, improve tissue targeting, and enhance activity (90). Liposomal gene therapy has advanced into clinical trials, notably in cystic fibrosis gene therapy. In this phase IIb trial, monthly application of the liposomal/pDNA gene therapy produced a modest improvement in pulmonary function with no significant difference in adverse events (91). Genome engineering ex vivo has been accomplished through pDNA transfection using several commercially available lipofection products.

Polymers. Owing to abundant chemical diversity, numerous groups are pursuing polymeric materials for gene delivery (**Figure 4*b***). Polymeric gene delivery vehicles use a net-positive charge to form electrostatic complexes with anionic nucleic acid. Intracellular delivery of these polymers is likely driven by the proton sponge effect of amine groups in the acidified endosome and lysosome compartments (92). Early polymeric transfection reagents include poly(L-lysine) (93) and polyethylenimine (PEI) (94), which have high charge densities but poor toxicity profiles and are not suitable for intravenous administration owing to pulmonary accumulation (95). Naturally occurring polymers, including histidine/imidazole-containing copolymers, atelocollagen, and chitosan, have also been used for improved toxicity profiles and acidic protonation for endosomal escape (96, 97). Notably, oral gene delivery has been achieved with naturally occurring chitosan polymers for FVIII gene replacement therapy (98). Dendrimers of cationic polymers, including PEI, poly(propylene imine), and poly(amidoamine), have been used for control of molecular weight and monodispersity of polymers (99). Controlled polymerization techniques, including reversible addition fragmentation chain transfer (RAFT) and atom transfer radical polymerization, allow for controlled, narrowly dispersed polymers with finely tuned environmental response properties (100, 101). More advanced polymer architecture is available with controlled polymerization methods, including block copolymers of poly(dimethylaminoethylmethacrylate) or biodegradable poly(β-amino esters), that rival the better liposome formulations for cell culture delivery (102, 103). These polymers can be designed to form micelles or polymerosomes for more stable polyplexes and are expected to have a more active membrane-disruption function during endocytosis (104). To further improve delivery efficiency, combinatorial panels of polymers of diverse chemistry have been evaluated for enhanced transfection in high throughput (105). External functionalization, including PEG shielding or targeting moieties, can be added to liposomes or polymers to improve tissue specificity (**Figure 4*c***) (71, 106). Several clinical trials have investigated PEI as a gene delivery vector (3). Cyclodextrin was examined in a phase I clinical trial that was terminated early but showed signs of gene silencing in patient tumors (106). A DNA vaccine for cytomegalovirus infection is currently in phase III clinical trials and has shown efficacy in reducing cytomegalovirus infection in patients undergoing allogeneic hematopoietic stem-cell transplantation (107).

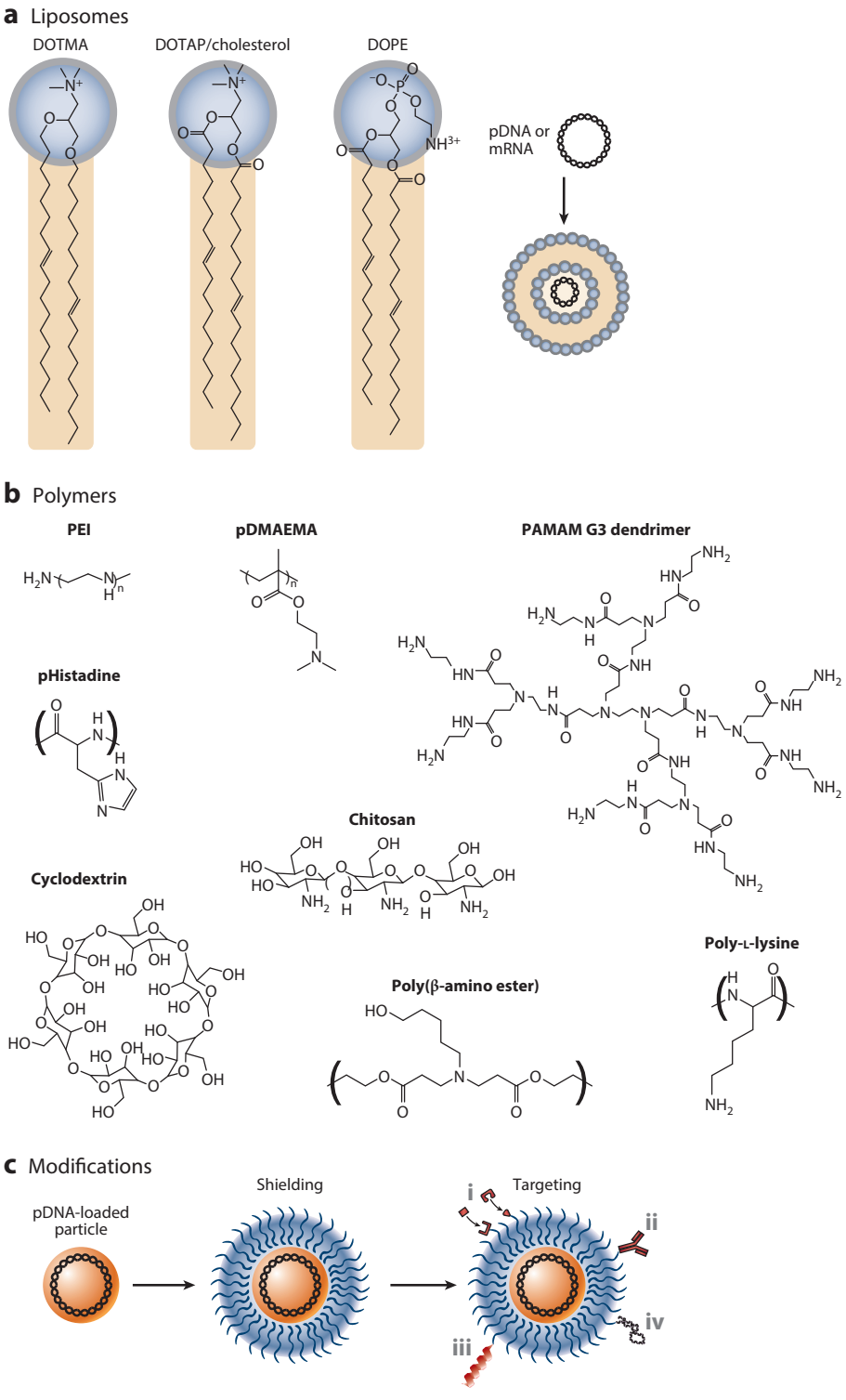


Figure 4

Nonviral delivery vectors for genome engineering. (*a*) Notable liposome-based gene delivery materials that have a hydrophilic cationic head group (DOTMA or DOTAP) or neutral head group (DOPE) shown in blue and a hydrophilic tail shown in yellow. (*b*) Polymeric gene delivery materials include cationic monomers (PEI, pDMAEMA, PAMAM, PBAE, chitosan) or block copolymers of other chemistries. Cyclodextrin, the base unit of the RNA-delivering nanomaterial that entered phase I clinical trials, is also shown. (*c*) Gene carrying polymers can be surface functionalized to improve circulation time (e.g., PEGylation) or conjugated with (i) small molecules, (ii) antibodies, (iii) cell-penetrating peptides, or (iv) aptamers to improve target cell uptake. Abbreviations: mRNA, messenger RNA; pDNA, plasmid DNA.

Protein delivery. In contrast with conventional gene therapy that may require sustained transgene expression, temporary availability of genome engineering proteins can make permanent modifications to the genome. Therefore, protein delivery can replace DNA and RNA delivery and eliminate the possibility of transgene integration or problems with RNA stability and immunogenicity. This approach requires direct delivery of the functional MN, ZFP, TALE, or Cas9/gRNA complex directly to the cytoplasm of target cells. Carrier-free ZFNs were used for genome editing and demonstrated dose-dependent endogenous genome modification in various cell types, which was enhanced by hypothermia treatment and repeat dosing (108). A more recent study used a commercially available cationic lipid (RNAiMAX) to deliver Cre recombinase, TALENs, and Cas9/gRNA, resulting in 80% genome modification. Delivery to the mouse inner ear reached 20% of GFP loss in outer hair cells with Cas9 and GFP-targeted gRNA (109). Ribonucleoprotein-based delivery of the CRISPR/Cas9 system has also been improved by chemical modifications to the ends of the gRNA, leading to enhanced genome editing activity in primary T cells and hematopoietic progenitor cells (65). Ribonucleoprotein-based delivery of Cas9/gRNA complexes has also been used in electroporation of $CD4^+$ T cells, causing 40% reduction in CXCR4 cell surface levels and up to 20% gene knock-in efficiency (66). Together, these studies validate the use of protein delivery in vitro and in local settings in vivo as a potential method to eliminate risks associated with gene delivery.

Viral Delivery Vectors

Viral vectors are idealized gene delivery vehicles, as the natural course of evolution has optimized their delivery behavior to address the formidable nuclear delivery barriers. After initial safety setbacks (56–59), new classes of effective vectors have been designed to address the original safety concerns. Building off of more than 1,000 viral gene therapy clinical trials, viral delivery of genome engineering tools is a natural transition (110). Although numerous viral vectors have been considered for gene therapy, adeno-associated virus (AAV), lentivirus, and adenovirus have been most commonly applied for genome engineering (55).

Adeno-associated virus. The most used viral vector for genome engineering to date is AAV. AAV is the only approved gene therapy product in Europe [Glybera (111)], is the vector of choice for >100 clinical trials (2), and is generally considered a safe and effective delivery vehicle. AAV has a small packaging size of ~4.7 kb (112, 113). The virus exists episomally, has very low rates of insertion (114), has strong expression profiles, and has been engineered for tropism for several target tissues, including eye (115), liver, brain (116), cardiac muscle, and skeletal muscle (117). Rational design, DNA shuffling, and directed evolution have been used to extend the available tropism of AAV and could potentially address immunological barriers (2, 118–123). The AAV capsid is composed of 60 copies of 3 viral proteins (VP1,VP2, and VP3) assembled into an icosahedron. Wild-type AAV is a dependovirus reliant on the activity of adenovirus for assembly, which can be accomplished by plasmid transfection in cell culture.

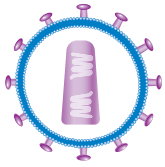
ZFNs are small and compatible with AAV production. TALE monomers have been included within a single AAV (124); however, dual vectors are needed for expression of both monomers of a TALEN. For the CRISPR/Cas9 system, the packaging capacity of AAV [~4.7 kb dependent on the serotype (112, 113)] is a limiting factor. The largest component of the CRISPR system is the Cas9 nuclease. *Streptococcus pyogenes* Cas9 is encoded by a 4.2-kb cDNA, leaving only a small space for packaging and regulatory sequences. *Staphylococcus aureus* Cas9 (SaCas9) is encoded by a 3.2-kb cDNA, which allows for one gRNA expression cassette to fit within the AAV genome (125). By driving guide RNA expression with smaller promoters, multiple gRNAs can be included in

one AAV vector with SaCas9 (126). Still, the addition of most activation and repression domains, epigenetic modifying domains, and homologous repair templates necessitates an additional vector for packaging the gRNA expression cassette and/or donor sequence. For transgenes larger than the packaging capacity, dual AAV vector approaches have shown efficacy in vivo (127, 128), though some efficiency loss is expected with this method. New variants of CRISPR systems are still being characterized, and the potential for smaller variants compatible with AAV is being pursued (129). A major consideration of clinical development of AAV-delivered genome engineering therapies is that large segments of the human population have neutralizing antibodies against AAV capsids (130, 131). Although patients can be screened for neutralizing antibodies prior to treatment, this reduces the treatable patient population. Engineered AAV variants could assist in immune avoidance (2). Another potential approach to reducing immunogenicity is depleting CpG motifs from the AAV genome (132).

Adenovirus. To date, adenovirus is the most common vector used in gene therapy clinical trials owing to its prevalence in antitumor strategies and ex vivo cell modification. Adenovirus is a larger (90–100-nm) icosahedral virus, with ~100 serotypes characterized in primates. The primary advantages of adenovirus are the large genome size (up to 36 kb), which makes the virus amenable to multiplexed editing with multiple ZFNs, TALENs, or gRNAs, and the high titers that can be produced. Also, the large size permits fusion proteins and Cas9 variants that are too large for AAV. Drawbacks of adenovirus include the immunogenicity and high adjuvant properties of adenovirus that may lead to T cell–mediated cytotoxicity in vivo (56, 133), although this may be useful for antitumor gene therapy. Delivery of ZFNs by adenovirus to T cells ex vivo was used for a clinical trial for HIV treatment (NCT00842634) (134). Adenovirus has been used to deliver the highly repetitive TALENs in human cells (24) and more recently CRISPR/Cas9 in vivo (135). Although the adenoviral delivery of Cas9 and gRNA to the liver led to the generation of neutralizing antibodies against Cas9, gene editing was still successfully achieved (135). Therefore, in addition to ex vivo cell modification, a potential utility of adenoviral delivery for genome engineering is in targeted cancer therapy, where immune recognition of targeted cells is desired (136).

Lentivirus. Lentiviral vectors derived from HIV-1 have overcome many of the original limitations of gamma-retroviral vectors, including some safety concerns (44, 137) and the inability to target nondividing cells. Lentivirus is an enveloped virus with ~10-kb transgene packaging capacity. Lentivirus can transduce dividing and nondividing cells, leading to stable integration of transgenes in cultured cells. The lentiviral integrase can be disabled to make integrase-deficient lentivirus (IDLV) for temporary expression of genes that remain episomal (137, 138). The transient activity of IDLV is attractive for genome engineering, where permanent genomic corrections can be made and sustained expression is not desired. The packaging size is sufficiently large for most genome engineering applications, including multiplexed CRISPR involving SpCas9 fusions with four simultaneous gRNAs (139). IDLV can also act as a template for HDR (44). As a unique feature, lentivirus can be used to deliver protein cargo, which has been demonstrated with MNs, ZFNs, and TALENs (140, 141), and could potentially be extended to CRISPR. Tissue tropism can be accomplished through pseudotyping of the lentiviral envelope (142). Challenges associated with lentiviral gene therapy are the high cost of producing sufficient vector and the risks associated with insertional mutagenesis.

Other. A few other viral vectors have received attention for gene therapy in the past and could impact genome engineering in the future. These including vaccinia virus (165 clinical trials), poxvirus (101 clinical trials), herpes simplex virus (73 clinical trials), measles virus, and polio

	Size	Genome size	Applications	Immunogenicity
a Adeno-associated virus 	20 nm	~4.7 kb ssDNA	in vivo: Eye, liver, muscle, central nervous system	Preexisiting antibodies to wild-type virus
b Adenovirus 	90–100 nm	~7.5 kb ~35 kb*	ex vivo in vivo: tumors	High
c Lentivirus 	80–100 nm	~10 kb RNA	ex vivo: T cells HSCs	Low

d Gene therapy clinical trial vectors

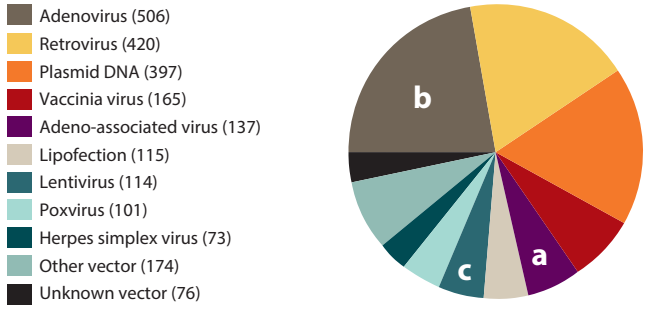


Figure 5

Viral vectors for genome engineering. (*a*) Adeno-associated virus has a 4.7-kb packaging capacity, is replication deficient, and has very low rates of integration. (*b*) Adenovirus has a packaging capacity of 7.5 kb that can be extended to 35 kb in gutted vectors. (*c*) Lentivirus has a packaging capacity of 10 kb and is capable of integration or can be modified to be integration deficient. (*d*) Distribution of historical gene therapy clinical trials through October 2015 by vector type. Information derived from the *Journal of Gene Medicine* Clinical Trial Database. Abbreviation: HSC, hematopoietic stem cells.

virus, although a majority of these clinical trials are for oncolytic viral gene therapy (**Figure 5*d***). Another recently engineered vector is a bacteriophage with an AAV genome (143). Bacteriophage does not efficiently infect human cells; however, replacing the bacteriophage genome with an AAV genome has created a novel vector for transducing multiple cell types by functionalization via peptide display, leading to high expression profiles (143). Bacteriophage can also be harnessed for delivery to bacterial cells as a means for strain-specific bacterial clearance (144, 145).

Local Delivery

Many pathologies are not amenable to local delivery and require a systemic approach. However, for local pathologies, delivery of genome engineering tools to the surrounding environment can circumvent systemic barriers (**Figure 3**, point 9). Local delivery increases the dose in the tissue of interest and reduces the dose at distal tissues, thereby decreasing off-target interactions. Local delivery has been pursued as an initial approach to genome engineering, including intraocular (146), intramuscular, intracranial (116), and topical delivery (109). Material depots of viral particles and polymeric vectors have been shown to have ten- to hundredfold enhanced activity when delivered from a material substrate (147–149). Local delivery also permits combinatorial therapies, including designing material matrices for regenerative medicine or sustained delivery of other active molecules or cells.

THERAPEUTIC APPLICATIONS

Genome engineering applications are broadly divided into gene modification for fundamental studies of disease, ex vivo modification for cell therapy, and in vivo genome editing to treat disease, as outlined in **Table 1**. Genome engineering is already being widely used for basic science. Cell therapy has witnessed extensive preclinical development and has entered clinical trials. In vivo genome engineering still has significant delivery barriers to overcome; nonetheless, preclinical approaches have made substantial progress, notably in the use of AAV to deliver ZFPs/ZFNs and CRISPR/Cas9. Genome engineering approaches with promising preclinical and clinical advances are highlighted in **Figure 6**.

Studying Disease

Genome engineering tools, and CRISPR/Cas9 in particular, are general platforms for the rapid development of engineered cell lines and genetically modified mouse models. Multiplexed CRISPR/Cas9 can be introduced to mouse embryos for the introduction of multiple genetic modifications in a single generation to create more accurate models of human disease (150). Improved models of disease development can also be generated with in vivo genome engineering tools. For example, PTEN and p53 were disrupted by hydrodynamic plasmid injections of CRISPR/Cas9 as a model for cancer development (85). This in situ cancer model could be a substitute for conventional xenograft models that better reflects tumor development and cancer progression. AAV encoding Cas9 and a gRNA has been injected intracranially to study gene function of single or multiple genes in postmitotic neurons in the brain (116). The generation of constitutive and inducible Cas9-expressing mice has simplified the delivery requirements so that only gRNA delivery is required (151, 152). Genome-wide screens for studying tumor growth or regenerative medicine are also being pursued. For example, a CRISPR screen of 67,405 gRNA introduced by lentiviral transduction of non-small-cell lung cancer cells revealed a selective advantage for candidate genes in lung metastasis (153).

Cell Therapy

Genome engineering has seen the most development toward clinical translation in cell therapies, with several ongoing clinical trials of engineered T cells to control HIV infection (e.g., NCT01543152) (134). Manipulation of patient-derived cells is handled in vitro and therefore can use delivery methods that are not feasible in patients (e.g., electroporation and adenoviral transduction). Corrected cells can be screened extensively for off-target DNA integration and nuclease activity before cell transplantation to increase the safety of the approach. In the case of

Table 1 Clinical and preclinical development of genome engineering technologies

Disease	Target gene	Model	Delivery	Platform	Result	Reference
Duchenne muscular dystrophy	Dystrophin	Cell implantation Mdx mouse	Cell AdV AAV	MNs, ZFNs, TALENs, CRISPR/Cas9	Dystrophin restored in patient-derived myoblasts and in the mdx mouse leading to improvements in muscle function	45, 46, 167–171, 173, 174, 175, 176
Hemophilia	Factor IX	Humanized mouse model	AAV	ZFN	23% factor IX levels after 60 days post treatment	164
X-SCID	*IL2RG*	Cell therapy	Cell	ZFN	3–11% gene correction in patient-derived HSCs	158
HIV	*CCR5*	Cell therapy	Cell	ZFN	~3-fold increase in $CD4^+$ T cells in humans	134
Hepatitis B virus (HBV)	Viral genome	Mouse HBV infection	Hydrodynamic plasmid	TALENs or CRISPR	Inhibited viral replication ~70%	180, 181
Hereditary tyrosinemia	*Fah*	*Fah5981SB* mouse	Hydrodynamic plasmid	CRISPR/Cas9	~1/250 cells repaired, repopulation to 33%	84
Cardiovascular protection	*PCSK9*	Mouse	AdV, AAV	CRISPR/Cas9	~50% decrease in plasma levels	125, 127
Bacterial infection	Bacterial genome	Mouse colonization and *Galleria mellonella* larvae	Bacteriophage	CRISPR/Cas9	5-fold decrease in virulent *Staphylococcus aureus* proportion Increased survival of *G. mellonella* larvae	144, 145

Abbreviations: AAV, adeno-associated virus; AdV, adenovirus; HSCs, hematopoietic stem cell; MNs, meganucleases; TALENs, TALE nucleases; ZFN, zinc finger nuclease.

ex vivo transfection, genome engineering tools in the form of mRNA or protein can be delivered to avoid the risk of insertional mutagenesis of pDNA or viral vectors.

HIV. ZFN-mediated editing of T cells is the most clinically advanced therapeutic genome engineering strategy. In 2008, Perez et al. (154) used adenovirus to disrupt the *CCR5* gene in 50% of transduced human $CD4^+$ cells, which provided resistance to infection of these cells in a mouse model of HIV infection. In 2014, the results were reported of an open-label, nonrandomized single-dose infusion of autologous $CD4^+$ T cells with ZFN-mediated *CCR5* disruption. The authors found the treatment to be safe and measured significantly increased median CD4 T-cell counts from 448/mm^3 to 1,517/mm^3, with modified cells having a mean half-life of ~48 weeks (134). By targeting hematopoietic progenitors, Holt et al. (155) demonstrated a more sustained method of producing *CCR5* knockout in all blood cells. ZFNs targeting both HIV receptors *CXCR4* and *CCR5* have also been shown to protect $CD4^+$ T cells from HIV with tropism for either receptor both in vitro and in vivo (156).

X-Linked Severe Combined Immunodeficiency. In 2005, nucleofection of ZFNs and corrective plasmid templates achieved ~5% correction of the *IL2RG* gene and the causative mutation of

X-SCID in $CD4^+$ T cells (157). Lombardo et al. (44) applied this HDR method with IDLV for delivery of ZFNs and a gene template to correct 5% of the *IL2RG* alleles in human embryonic stem cells. Building on this work, in 2014, Genovese et al. (158) reported overcoming barriers to HDR in hematopoietic stem cells to correct the mutation of the *IL2RG* gene with ZFNs. In this study, IDLV carrying the donor DNA and mRNA encoding the ZFNs were electroporated into bone marrow–derived $CD34^+$ cells from a symptomatic patient, achieving 3–11% correction.

Sickle cell anemia. Several strategies have been used to address the mutations that cause sickle cell anemia. Use of ZFNs and either an IDLV or an oligonucleotide donor repair template yielded 15–18% HDR-driven correction of the β-globin gene. Engraftment of corrected $CD34^+$ cells restored functional hemoglobin in an immunodeficient mouse model (159). Another strategy used ZFPs with the self-association domain of LBD1 targeting the fetal globin (γ-globin) promoter to increase γ-globin levels to compensate for β-globin deficiency (160). Removal or disruption of the BCL11a erythroid-specific enhancer region is another approach that can increase fetal hemoglobin levels. BCL11a negatively regulates fetal hemoglobin; however, a BCL11a complete knockout is harmful to nonerythroid cells. By targeting the erythroid-specific enhancer region, fetal hemoglobin levels can be recovered specifically in red blood cells for the treatment of β-hemoglobinopathies (161, 162).

In Vivo Genome Engineering

Many therapeutic approaches require gene correction or disruption directly in tissues, which require more complex delivery methods. The published studies in this area include several genome engineering approaches, including HDR, NHEJ-based gene disruption, NHEJ-based gene deletions, and transcriptional control with both nonviral and viral delivery strategies.

Liver-targeted genome engineering. Genome engineering in the liver is a particularly attractive target for correcting secreted proteins such as clotting factors; therefore, it is the most developed in vivo genome engineering application to date. The liver has a high level of uptake of most viral and nonviral vectors. It also has a significant capacity for regeneration, and thus can be repopulated by corrected hepatocytes with a selective growth advantage. For example, hydrodynamic injection of plasmid encoding CRISPR/Cas9 into the liver of mice carrying a mutation of the fumarylacetoacetate hydrolase gene, which causes tyrosinemia type I, achieved initial low editing rates (~1/250 cells), but a selective advantage was conferred such that 33% of hepatocytes were corrected after 30 days (84). AAV has been used in the majority of these studies for in vivo delivery of ZFPs and Cas9 with gRNA. For example, ZFNs targeted to the factor IX locus have been used for HDR-driven repair in neonates, which improved clotting times in mice (163). In 2013, Anguela et al. (164) reported the same strategy for factor IX correction in the liver of adult mice, reaching 23% of normal levels that was sustained for 60 weeks. A more recent, generalized approach uses a ZFN targeted to the albumin locus in hepatocytes to drive HDR with a repair template containing any secreted protein (165). This approach is a potential general platform for protein replacement therapy because the targeted transgene will be expressed at high levels from the exceptionally strong albumin promoter even if the efficiency of knock-in by gene editing is low. Moreover, slight loss of albumin has no negative effect. Targeted gene knockout in the liver has been demonstrated for proprotein convertase subtilisin/kexin type 9 (PCSK9) because loss of function of this gene is associated with lower risk of heart disease. Both AAV delivering SaCas9 and adenovirus delivering SpCas9 targeting *PCSK9* in the liver led to decreased plasma cholesterol levels (125, 166).

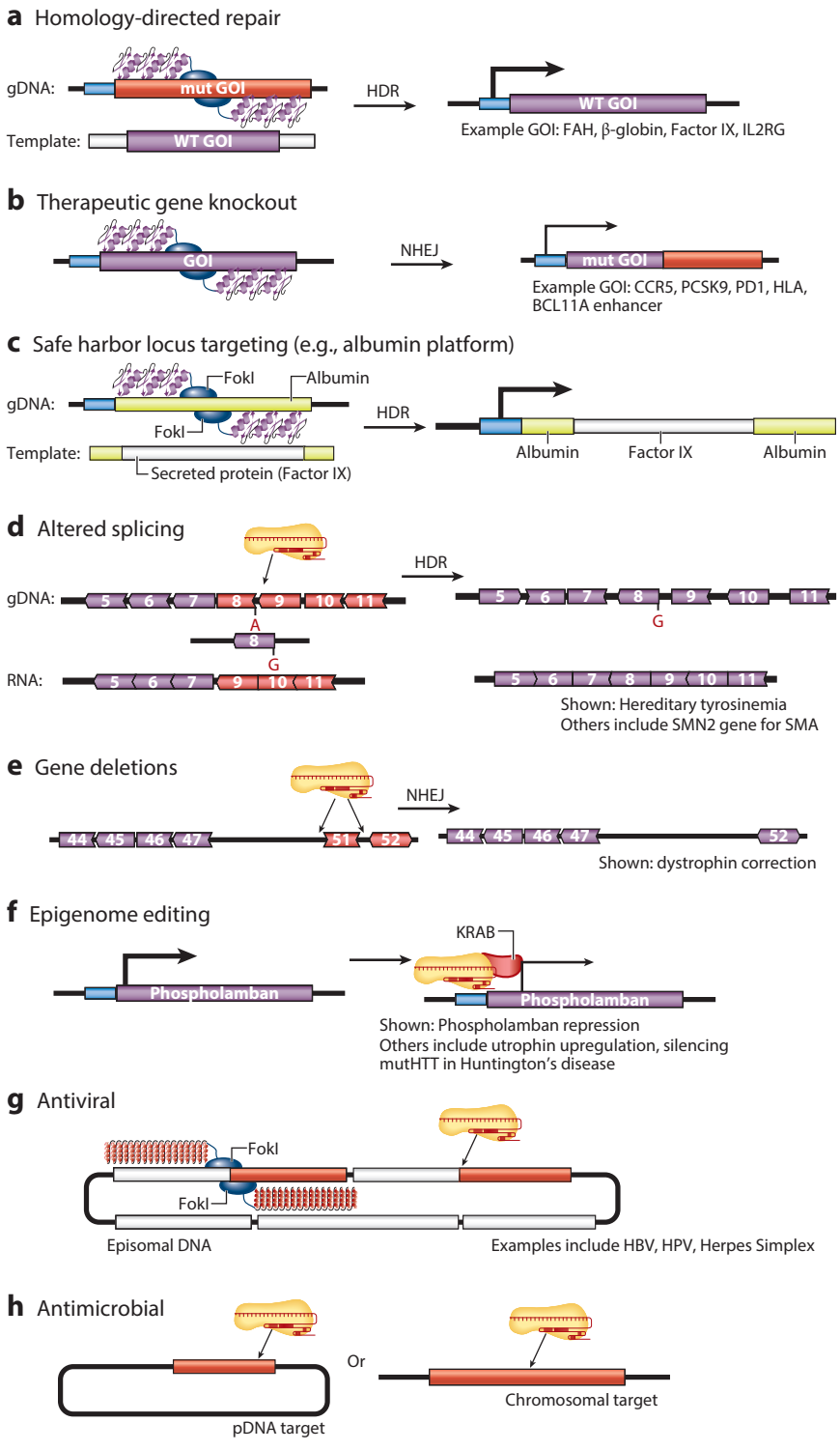
Cardiac and skeletal muscle–targeted genome engineering. Genome engineering approaches are being developed for various neuromuscular and cardiac conditions, including DMD and spinal muscular atrophy. To correct the DMD mutation, missing exons can be replaced (167, 168), or a mini-dystrophin can be inserted into a safe harbor that compensates for the absent gene (169). HDR has also been demonstrated in mouse embryos, with chimeric mice having improved phenotype (170). Targeted NHEJ-mediated frameshifts have also been shown to restore the reading frame in patient-derived cells (167, 171). Building upon years of research using exon-skipping oligonucleotides for DMD (172), genome engineering approaches have been applied to delete nonessential exons from the coding region of the dystrophin to restore protein expression. This has been shown in patient-derived myoblasts with ZFNs and with CRISPR/Cas9 (45, 46, 167). Another report demonstrated AdV-delivered CRISPR/Cas9 excised a 23-kb portion of the dystrophin gene and restored dystrophin expression in the mdx mouse model of DMD (173). Recently, three groups reported AAV-delivered CRISPR/Cas9 repaired the causative mutation in the mdx mouse, showing restoration of dystrophin, improvements in muscle biochemistry and strengthened muscle function (174, 175, 176). An alternative approach uses transcriptional activators to increase levels of utrophin, which can compensate for absent dystrophin. AAV delivery of a ZFP-based activator has shown increases in utrophin and improvement in the phenotype of a mouse model of DMD (177).

Phospholamban plays a role in calcium kinetics in cardiac muscle, and repression of phospholamban has shown improvements in contractility without side effects of activation of the β-adrenergic pathway. An AAV encoding a ZFP-KRAB transcriptional repressor targeted to phospholamban was injected intramyocardially and showed highly specific repression and improved calcium reuptake kinetics in a rat model of heart failure (178).

Antiviral and antimicrobial. Using genome engineering tools as a specific inhibitor of viral or bacterial infection is another promising avenue (179). In 2013, Bloom et al. (180) reported hydrodynamic injection of plasmids encoding TALENs targeting the episomal viral genome of hepatitis B virus, which inhibited viral replication by ~70%. Lin et al. (181) demonstrated a similar

Figure 6

Preclinical or clinical success in genome engineering. (*a*) Homology-directed repair (HDR) in hematopoietic stem cells (HSCs) can correct numerous disorders associated with the hematopoietic cell lineage, including sickle cell disease, thalassemia, and immunodeficiencies. (*b*) Genes with beneficial loss-of-function phenotypes are targeted by nonhomologous end-joining (NHEJ) to confer resistance to HIV infection (CCR5) or reduce cholesterol levels (PCSK9). (*c*) A potential platform for addressing systemic protein deficiencies is through safe harbor integration of transgenes in the liver, notably the AAVS1 and albumin loci. (*d*) Splice site mutations can be corrected by HDR; for example, a splice mutation causing a mouse model of hereditary tyrosinemia was corrected in the liver. (*e*) Gene deletions can be used to treat diseases where removal of nonessential regions of the gene can restore the reading frame, such as Duchenne muscular dystrophy. (*f*) Engineered transcription regulators and epigenome modulators can be used to treat disease or ameliorate symptoms. For example, an engineered transcriptional repressor of phospholamban improved cardiac function in a model of heart failure. (*g*) TALENs or CRISPR/Cas9 targeting latent viral genomes can be used to reduce HBV or HIV viral load. (*h*) Bacteriophage delivery of CRISPR/Cas9 can be used to selectively eliminate strains of bacteria. Abbreviations: BCL11A, B-cell lymphoma/leukemia 11A; CCR5, C-C chemokine receptor type 5; FAH, fumarylacetoacetate hydrolase; GOI, gene of interest; HBV, hepatitis B virus; HLA, human leukocyte antigen; HPV, human papillomavirus; IL2RG, interleukin 2 receptor gamma; mutHTT, mutant Huntington protein; PCSK9, proprotein convertase subtilisin/kexin type 9; PD1, programmed cell death protein 1; SMA, spinal muscular atrophy; SMN2, survival of motor neuron 2; WT, wild type.


a Homology-directed repair
gDNA:
mut GOI
Template:
WT GOI
HDR
WT GOI
Example GOI: FAH, β-globin, Factor IX, IL2RG
b Therapeutic gene knockout
GOI
NHEJ
mut GOI
Example GOI: CCR5, PCSK9, PD1, HLA, BCL11A enhancer
c Safe harbor locus targeting (e.g., albumin platform)
FokI
Albumin
gDNA:
FokI
Template:
Secreted protein (Factor IX)
HDR
Albumin
Factor IX
Albumin
d Altered splicing
HDR
gDNA:
5 6 7 8 9 10 11
A
8
G
RNA:
5 6 7 9 10 11
5 6 7 8 9 10 11
G
5 6 7 8 9 10 11
Shown: Hereditary tyrosinemia
Others include SMN2 gene for SMA
e Gene deletions
NHEJ
44 45 46 47 51 52
44 45 46 47 52
Shown: dystrophin correction
f Epigenome editing
Phospholamban
KRAB
Phospholamban
Shown: Phospholamban repression
Others include utrophin upregulation, silencing mutHTT in Huntington's disease
g Antiviral
FokI
FokI
Episomal DNA
Examples include HBV, HPV, Herpes Simplex
h Antimicrobial
Or
pDNA target
Chromosomal target

result using hydrodynamic injection of plasmids encoding CRISPR/Cas9. Other applications have been demonstrated in vitro, including CRISPR/Cas9 targeted to the LTR region of latent HIV-1 to excise the 9,709-bp genome (182). HPV proteins E6 and E7 are associated with high risk of cervical carcinoma and can be deactivated to restore p53 function and induce cell cycle arrest and apoptosis (183). By using the CRISPR systems targeted to bacterial genomes, a strain-specific method of manipulating or destroying bacteria is feasible. Two groups reported bacteriophage delivery of CRISPR systems targeting specific bacteria populations. Bikard et al. (144) reported a ~5-fold decrease in the proportion of virulent *S. aureus* cells in a mouse skin wound colonization model after phage treatment. Citorik et al. (145) demonstrated significantly improved survival of *Galleria mellonella* larvae with bacteriophage delivery of CRISPR targeting a virulence factor in *Escherichia coli*.

CONCLUSIONS

The primary challenge of translating genome engineering technologies is the development of safe and effective delivery platforms. Nonviral and viral gene delivery methods have had success in clinical trials for gene therapy and can be rapidly adapted for genome engineering. Ongoing challenges for genome engineering include characterizing the prevalence and consequence of off-target gene editing and improving the efficiency of HDR in applications that require this mode of repair. With continued development of gene delivery vectors, the outlook is bright for translation of genome engineering technologies to gene and cell therapy.

FUTURE ISSUES

1. Concerns regarding immunological responses to vector administration for gene therapies have begun to be addressed. However, immune responses to the expression of exogenously delivered meganucleases, ZFPs/ZFNs, TALEs/TALENs, Cas9, or other CRISPR systems, that are not naturally present in humans are the subject of ongoing research. Also, immune responses to previously absent disease-related proteins still need considerable investigation. As the fields of gene therapy and genome engineering progress, strategies to avoid immune recognition and/or induce tolerance to foreign antigens will be critical.
2. Measurable levels of off-target mutations are documented throughout the literature for all of the genome engineering platforms. The biological context and consequence of these off-target effects will need to be better understood for clinical development, and strategies for mitigating these effects continue to be critical contributions to the field (184, 185). Studies in animal models that accurately reflect clinical applications will be important to understand the impact of off-target effects on organismal health, the tolerable level of off-target effects, and the long-term impact of these effects.
3. New CRISPR systems continue to be reported, and only a small fraction of the natural biological diversity of these systems has been sampled. New systems are likely going to continue to be reported with improved sequence specificity, varying targeting restrictions, and novel functionalities. A plethora of distinct CRISPR systems will equip researchers with a diverse tool set for genome engineering.

4. New clinical trials using genome engineering will begin with numerous engineered cell therapies. Several candidate in vivo genome engineering approaches will likely transition into clinical trials. In addition to advancing the field, these trials are likely to uncover new delivery challenges to be addressed.

DISCLOSURE STATEMENT

C.A.G. has filed patent applications related to genome editing and is an advisor to Editas Medicine, a company engaged in development of therapeutic genome editing.

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