Delivery technologies for genome editing

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Abstract | With the recent development of CRISPR technology, it is becoming increasingly easy to engineer the genome. Genome-editing systems based on CRISPR, as well as transcription activator-like effector nucleases (TALENs) and zinc-finger nucleases (ZFNs), are becoming valuable tools for biomedical research, drug discovery and development, and even gene therapy. However, for each of these systems to effectively enter cells of interest and perform their function, efficient and safe delivery technologies are needed. This Review discusses the principles of biomacromolecule delivery and gene editing, examines recent advances and challenges in non-viral and viral delivery methods, and highlights the status of related clinical trials.

RNA interference
(RNAi). Process by which one strand of double-stranded RNA binds to complementary mRNA and degrades or regulates the mRNA via an enzymatic process, usually resulting in a decrease in the expression of a desired protein.

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More than 3,000 human genes have been identified that are associated with Mendelian diseases, and ~500 genes are associated with susceptibility to complex diseases or infections (see Orphanet and OMIM Gene Map Statistics websites). These numbers are rapidly increasing, and it is predicted that ~4,000–7,000 additional disease-associated genes will be uncovered in the next decade[1]. However, despite tremendous advances in genomic sciences and substantial progress in drug development, effective therapies are still needed; for example, <5% of rare diseases have an effective treatment[2].

Cystic fibrosis is an example of a genetic disease with effective treatments for a large portion of patients, with the successful development of small-molecule drugs to target defective transmembrane conductance regulator (CFTR) protein[3]. Small molecules now can treat about 40% of patients with cystic fibrosis and eventually could hopefully benefit >90% of patients. However, small molecules cannot provide a cure for cystic fibrosis, and it is generally difficult to use small-molecule therapies, bone marrow transplantation or surgical approaches to treat most genetic diseases[4]. Protein therapeutics, including protein replacement (or augmentation) therapies and antibodies, have been investigated for treating certain genetic disorders, and some have received regulatory approval. Because administered purified proteins generally do not enter cells, protein augmentation therapies have been applied to treat disorders for which a deficient protein functions at least partially in the extracellular milieu[5]. Examples of regulatory-approved protein therapeutics include recombinant acid α-glucosidase, recombinant factors VIII and IX, and a humanized vascular endothelial growth factor A (VEGFA)-specific antibody. Recombinant acid α-glucosidase effectively treats Pompe disease, a type of lysosomal storage disorder, resulting in improved function of multiple organs in patients[6]. Recombinant factors VIII and IX are used to treat haemophilia A and haemophilia B, respectively, to correct bleeding episodes[7], whereas the VEGFA-specific antibody is used to treat age-related macular degeneration[8]. However, most of these protein therapeutics target a limited number of biomolecules that are involved in a small proportion of genetic diseases[9].

Meanwhile, viral and non-viral delivery strategies of functional copies of mutated genes have been developed to treat loss-of-function genetic diseases[10,11], and many are currently in clinical trials (for examples, see ClinicalTrials.gov). In 2012, the adeno-associated virus (AAV)-mediated delivery of a functional gene to treat the rare disease lipoprotein lipase deficiency became the first gene therapy product based on viral gene-transfer technology to receive marketing approval in Europe.

In addition, RNA modification therapies, such as RNA interference (RNAi) and antisense oligonucleotides (ASOs), which silence the mRNA transcribed from disease genes, are moving forward in clinical trials[12,13]. An ASO-based product has received US Food and Drug Administration approval for use in patients with homozygous familial hypercholesterolaemia, and other RNA modification therapies are in advanced stages of development; for example, alicaforsen for pouchitis (see Ionis Pharmaceuticals' pipeline). RNAi is being evaluated in one phase III clinical trial targeting transthyretin-mediated amyloidosis, as well as in multiple phase I/II trials[10].

However, despite the remarkable progress and therapeutic promise of ASO and RNAi technologies, as well as AAV-facilitated gene transfer, these approaches have fundamental limitations. In principle, ASOs and RNAi can induce the knockdown of any target protein; however, delivery barriers have limited their clinical application to

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a subset of organs and tissue types[10,12]. Other challenges include incomplete suppression of disease proteins, off-target effects and safety concerns[12,13]. Stably expressing therapeutic genes in replicating cells via integration into the genome is associated with the risk of mutagenesis and oncogenesis[14]. AAVs facilitate gene transfer and episomal expression in non-dividing cells, but suffer from the limits associated with the size of encapsulated transgenes, pre-existing immunity against AAV vectors and CD8+ T cell-mediated adaptive response against the AAV capsid[11,15]. Moreover, although AAVs are stable in non-dividing cells, their expression levels may decrease over time owing to the turnover of the infected cells[16]. In addition, paediatric patients may experience loss of episomal genomes in growing tissues[17]. Importantly, it is difficult to use gene-transfer approaches for conditions requiring precise control of therapeutic genes or for gain-of-function genetic diseases[18].

A rapidly developing alternative technology to manipulate gene expression is genome editing, which, in contrast to gene-transfer approaches, uses programmable DNA nucleases[19]. Four genome-editing platforms currently predominate the field: meganucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the CRISPR–Cas system (reviewed in REFS 19–22). In principle, it is possible to correct many genetic diseases with genome-editing technologies. However, many hurdles need to be overcome to realize this tremendous therapeutic potential. In particular, the most substantial challenge to overcome is the safe and efficient delivery of genome-editing biomacromolecules.

This Review provides an overview of the different programmable nucleases and mechanisms of genome editing, focusing on the principles of biomacromolecule delivery, relevant delivery methods and associated delivery challenges. Preclinical studies and the current status of clinical trials involving genome-editing technologies are also discussed.

Genome-editing platforms

Genome-editing nucleases recognize and cut at specific sequences in the genome. Double-strand breaks (DSBs) can be repaired by one of two cellular endogenous DNA repair pathways: non-homologous end joining (NHEJ) or homology-directed repair (HDR) with template (donor) DNA[23] (FIG. 1). NHEJ is efficient but error-prone; thus, repeated repair of the same break site results in small insertions or deletions (indels) at the desired genomic locus[24]. These indels can disrupt target genes by shifting the reading frame, resulting in mRNA degradation or the production of nonfunctional proteins[25]. Programmable nuclease-mediated NHEJ can disrupt disease-causing genes permanently. Furthermore, it is also possible to use NHEJ to restore the reading frame of a dysfunctional gene to treat a disease: for example, the gene encoding dystrophin (*DMD*) to treat Duchenne muscular dystrophy[26–30]. By contrast, HDR requires exogenously delivered template DNA containing a sequence homologous to the DSBs. The different sequence in the template DNA can be incorporated into the endogenous locus, enabling precise modification of the genomic sequences[23]. Thus, HDR-based gene editing can be used to repair disease-causing mutations or to knock in sequences at specific loci to induce predictable expression patterns[31–33].

Four major platforms of targeted nucleases are currently used to introduce DSBs in the genome: meganucleases, ZFNs, TALENs and the CRISPR–Cas system[19–22] (FIG. 1). Meganucleases, ZFNs and TALENs generate sequence recognition specificity via protein–DNA interactions. Although meganucleases can target various sequences, the engineering required to target new sequences and unseparated DNA-binding and cleavage domains (that is, DNA recognition and cleavage sites that are linked closely in a single domain) restrict the broad application of these nucleases[20]. ZFN technology creates a chimeric protein via the fusion of a zinc-finger domain for DNA binding with the cleavage domain of the FokI nuclease[21]. To target new sequences, ZFNs require complicated protein engineering of the zinc-finger domain[34,35]. Recent studies have indicated that ZFNs recognize an average of one targeting site in ~4–30 bp in the genome[36,37].

TALENs are composed of a transcription activator-like effector (TALE) for DNA binding and a FokI nuclease[22]. Although molecular cloning is still required, TALENs can be rapidly designed and assembled with flexible targeting sequences with potentially high potency and specificity[22]. Distinctly different from these programmable nucleases, the CRISPR–Cas system uses an RNA molecule (guide RNA) to base pair with the target DNA as an RNA-guided nuclease[19]. By simply changing
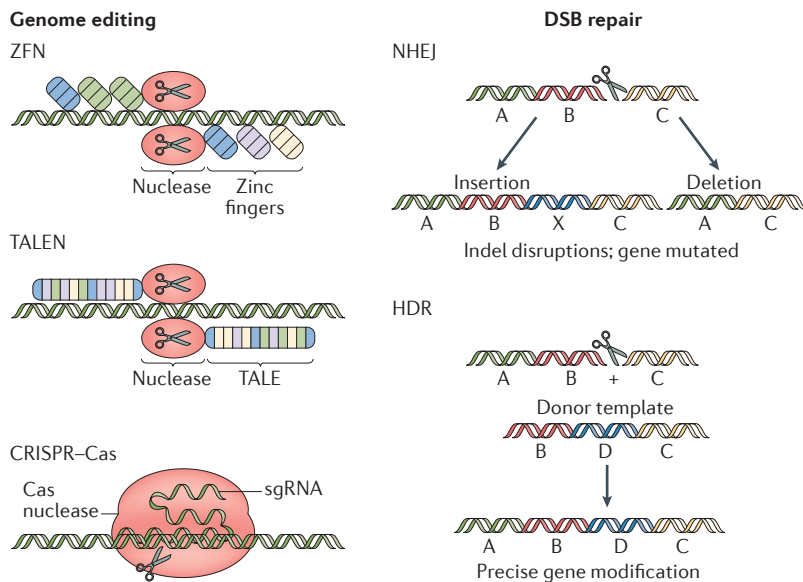

Figure 1 | **The mechanisms of genome editing and DSB repair.** Zinc-finger nuclease (ZFN), transcription activator-like effector (TALE) nuclease (TALEN) and CRISPR–Cas systems can induce double-strand breaks (DSBs) in DNA. One of two mechanisms repairs the DSB to achieve genome editing: non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ disrupts the target gene through insertions or deletions, whereas HDR inserts donor DNA template into the target genomic region to install insertions, deletions or alterations of genomic sequences. sgRNA, single guide RNA.

a portion of the guide RNA sequence, CRISPR–Cas can be engineered to target genomic sequences next to a protospacer adjacent motif sequence[19].

In general, meganucleases and ZFNs have reportedly relatively low success rates and efficiencies in inducing site-specific DNA breaks[38,20]. By contrast, TALENs and CRISPR have ~80–90% success rates in mammalian cells to induce >1% mutation frequencies[38–41], indicating that most guide RNAs of the CRISPR system or designed TALEN pairs are capable of inducing substantial DSBs in mammalian cells. Moreover, the average mutation rate of TALEN and CRISPR systems was reportedly substantially higher than that of meganucleases and ZFNs[38]. All four of these genome-editing platforms can cause off-target mutations. Multiple new methods to evaluate off-target effects[42–47] and to improve the specificity of the CRISPR system[48–55] have been developed. Although ZFNs and TALENs have been applied for multiplex gene editing, mismatched dimers can form when more than one pair are in cells, which introduces off-target effects[56]. By contrast, CRISPR can simultaneously target multiple loci in the genome with high efficiency and without substantially increasing the required dose[57,58].

All components in these genome-editing systems are biomacromolecules that must overcome several barriers to be delivered inside target cells to function (FIG. 2). The nucleases, delivered in the format of DNA, mRNA or protein, must access the genome of the target cells. For the CRISPR–Cas system, the guide RNA, in the format of DNA or RNA, must be delivered. For HDR-mediated gene editing, it is also necessary to provide a donor DNA template, usually a single- or double-stranded DNA. The relatively small size of ZFNs (two molecules ~1 kb each) and meganucleases (~1 kb) provide an advantage over TALENs (two molecules ~3 kb each) and CRISPR (~3.5–4.5 kb). The size of the biomacromolecule payload is particularly pertinent for viral-mediated delivery because viral vectors have a maximum limit to the size of genetic material that they can encapsulate[38]. Below, we discuss the various methods that are being developed for the efficient and safe delivery of genome-editing systems and consider the associated challenges and limitations.

Delivery of biomacromolecules

The intracellular delivery of biomacromolecules has been a focus of much research, and multiple methods have been developed to facilitate the delivery of a range of payloads, including DNA, RNA, ASOs and proteins[10,11]. These delivery methods can be generally categorized as viral (TABLE 1) or non-viral (BOX 1; FIGS 3,4). Viral delivery utilizes a viral vector (for example, AAVs[59], lentiviruses[60] and adenoviruses[61]) to encapsulate a gene, in RNA or DNA form, to facilitate efficient delivery. Non-viral delivery includes physical methods (for example, electroporation[62,63] and microfluidic-based technologies[64,65]), nanomaterial-based methods (for example, cationic lipids[66,67] and cell-penetrating peptides (CPPs)[68]) and self-assembled nanoparticles[69].
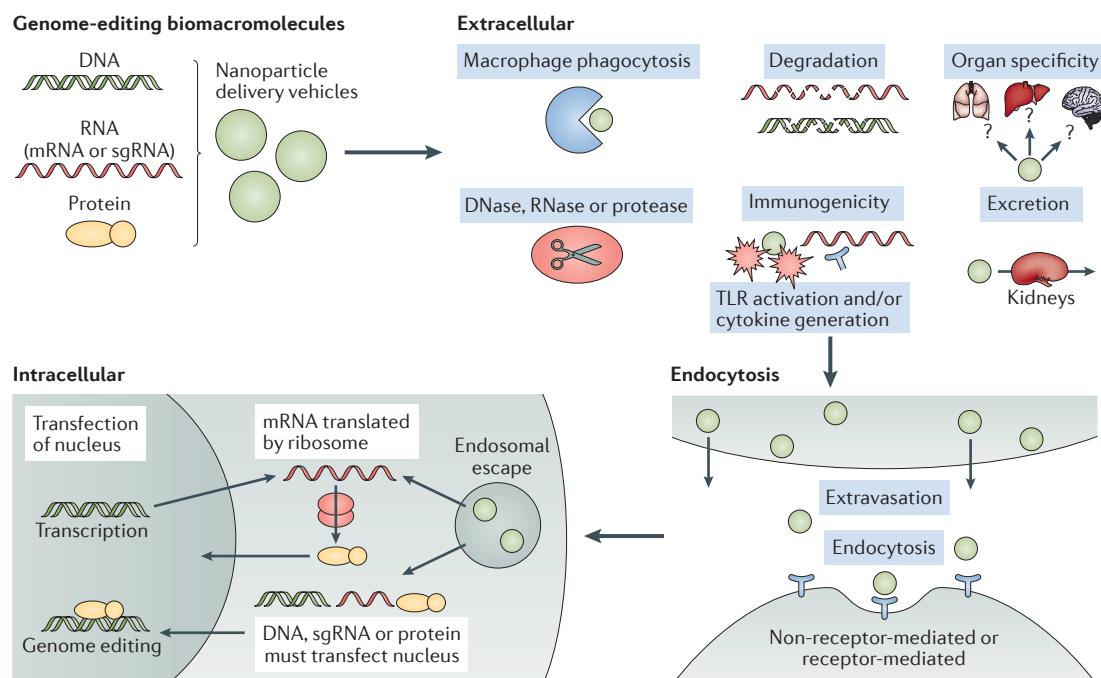

Figure 2 | **Barriers to delivery of genome-editing components.** Genome-editing biomacromolecules (DNA, mRNA, single guide RNA (sgRNA) and/or protein) or nanoparticles containing these biomacromolecules must first avoid extracellular barriers. These barriers include phagocytosis by macrophages or other phagocytes, degradation through enzymatic or hydrolytic means, and the potential for induction of an immune response and the generation of cytokines. If these biomacromolecules and nanoparticles can avoid excretion, localize to the proper organ and extravasate out of the bloodstream, they must then be endocytosed by the target cell. Once inside the cell, the biomacromolecules and nanoparticles must escape the endosome and localize to the cytoplasm (mRNA) or nucleus (DNA, sgRNA or nuclease protein) to successfully edit the target gene. TLR, Toll-like receptor.

Table 1 | **Characteristics of the three main types of viruses used for genome editing**

Virus	Genome	Packaging capacity	Capsid diameter	Duration of expression	Immunogenicity	Primary setting for genome editing	*In vivo* tropism	Refs
Integration-deficient lentiviruses	Single-stranded RNA (non-integrating)	~8 kb	~100 nm	Transient (depends on rate of cell division)	Low	*In vitro* and *ex vivo*	Not applicable	139,140
Adeno-associated viruses	Single-stranded DNA (non-integrating)	~5 kb	~25 nm	Long term (years) in quiescent cells*	Low	*In vitro*, *ex vivo* and *in vivo*	Broad, serotype-dependent‡	89, 139,141
Adeno-viruses	Double-stranded DNA (non-integrating)	~8 kb or ~36 kb (gutless)	~100 nm	Long term (weeks to months)	High	*In vitro*, *ex vivo* and *in vivo*	Primarily liver§	140,142

*Expression levels drop in proliferating cells. ‡Liver, brain, skeletal, kidney, retina, lung and vascular tissue. §Also demonstrated in muscle and the central nervous system.

For some tissues, genome-editing systems can be delivered *ex vivo* by extracting primary cells from patients, gene editing these cells and then transplanting the modified cells back into the patient. One potential target tissue is the haematopoietic system, including haematopoietic stem cells (HSCs) and primary lymphocytes[18,21,60,70]. *Ex vivo* transfection enables the use of physical methods that are not suitable for systemic application. For example, *ex vivo* electroporation of plasmid DNA or mRNA into HSCs can provide relatively high transfection efficiency[60,63]. However, cellular toxicity is associated with the electroporation of plasmid DNA into HSCs[60]. Retroviral and lentiviral vectors can also be used for *ex vivo* delivery into HSCs or primary lymphocytes to stably express therapeutic genes[11,60]. Details and more examples of successful *ex vivo* delivery are discussed below. Some of the methods commonly used for transfection of model cell lines (for example, HEK293T cells), including some nanomaterial systems and integrating lentiviral vectors, may not be suitable for delivery to primary blood cells for clinical use owing to low efficiency or safety concerns[71].

In vivo genome editing faces additional delivery hurdles relative to *ex vivo* therapies[10] (FIG. 2), and the challenges differ between viral and non-viral systems[10,11]. A range of viral systems have been investigated for their potential to deliver genetic payloads to transduce various types of tissues, including liver, eye, skeletal and cardiac muscle[11]. Indeed, this broad tropism makes viral vectors particularly attractive for the delivery of programmable nucleases *in vivo*. However, these viral systems can induce long-term transgene expression in humans with a single injection[72]; thus, the effects of long-term expression of transgenic nucleases on genome stability and the potential induction of antigenicity in corrected tissue will need further investigation.

Lipid-based and polymer-based nanoparticles and conjugate technologies have facilitated RNA *in vivo* delivery, and are in various stages of clinical development[10]. These non-viral platforms can also be used to deliver programmable nucleases to enable transient expression in desired tissues[73].

Given the different challenges faced by *in vivo* versus *ex vivo* delivery, the approaches and optimal procedures for these applications will differ. However, therapeutic genome editing of most tissues cannot be performed *ex vivo*, and effective *in vivo* delivery will be required to enable the broad application of genome-editing therapies (for examples, see REFS 33,74,75). In the following sections, viral and non-viral genome-editing systems will be discussed in greater detail, and specific examples from the recent literature of *in vitro*, *ex vivo* and *in vivo* delivery will be provided.

Viral delivery of genome-editing systems

Viral vectors are commonly used to transfer genes into cells and/or tissue. Refined vector systems, improved production methods, enhanced transduction efficiency and better safety profiles have been achieved during the past decade[11]. Retroviral vectors, adenoviral vectors and AAV vectors have been extensively studied in preclinical models, and they have been tested in several clinical trials[11]. Viral vector systems may provide useful tools to deliver genome-editing systems for both research and clinical applications *in vitro*, *ex vivo* and *in vivo*.

In vitro *and* ex vivo *approaches*

The key properties of the three most frequently used viral systems for *in vitro* and *ex vivo* genome-editing applications, integrase-defective lentiviral vectors (IDLVs; a type of retroviral vector), AAVs and adenoviruses, are presented in TABLE 1. Below, we discuss their application for generating gene-edited HSCs and T cells for genetic diseases, viral infections and cancer.

Integrase-defective lentiviral vectors. IDLVs have been used to deliver both ZFNs and donor templates *in vitro*[76]. The IDLV genome remains mostly episomal and gradually diminishes by dilution in dividing cells, leading to the short-lived expression of ZFNs[76]. IDLV-mediated delivery can produce significant gene editing in various types of cells, including HEK293T cells or Epstein–Barr virus-transformed B lymphocytes[76]. However, for CD34+ haematopoietic stem and progenitor cells (HSPCs), the ratio of gene addition at the C-C chemokine receptor 5 (CCR5) site was low: ~0.1% of total cells[76]. Subsequently, an average of 6% gene addition was achieved in HSPCs at either a mutational hotspot of interleukin-2 receptor subunit gamma (*IL2RG*) or the adeno-associated virus integration site 1 (AAVS1)[60]. This increase in gene

Tropism
The ability of a virus to specifically target particular cells or tissues.

Box 1 | Principles of gene delivery vectors

Plasmids encoding genome editing-associated proteins must successfully transfect the nucleus of the cell for their genes to be transcribed and eventually translated. The cellular, endosomal and nuclear membranes all present physical barriers to entry; large, hydrophilic molecules such as DNA, RNA and proteins cannot efficiently pass through these hydrophobic membranes. Furthermore, the *in vivo* environment presents further complications to nucleic acid delivery: bare nucleic acid is subject to degradation by endogenous nucleases in the blood, could potentially activate the immune system as a foreign nucleic acid, and should preferably only transfect the targeted cell type. For these reasons, delivery vectors are often used to protect the nucleic acid or protein cargo via encapsulation, target a specific cell and shuttle the payload across membrane barriers.

Viral vectors. Three main classes of viruses, which have been reviewed extensively elsewhere[11], have been engineered to deliver genetic material therapeutically: retroviruses, adenoviruses and adeno-associated viruses (AAVs). Retroviral vectors are characterized by replication via reverse transcription; for example, lentiviral vectors, a subclass of retroviral vectors, can integrate viral DNA into cells that do not undergo cell division, such as neurons or muscle cells. Adenoviral vectors merely deliver viral double-stranded DNA rather than incorporate it into the host cell genome, making them useful for applications requiring transient protein expression. AAV vectors are small, simple, episomal viruses capable of transducing both dividing and non-dividing cells with rare genomic integration[136]. In clinical trials, some viral vectors have demonstrated efficient gene delivery, but the history of viral gene therapy has also been plagued by serious safety concerns: in one study, 25% of patients treated with mouse Moloney retroviruses developed leukaemia[137,138], and in another study a patient treated with adenovirus died of a massive inflammatory response to the vector. AAVs, which are nonpathogenic, hold promise as safe gene delivery vectors but the durability of expression in humans and their long-term effects require further investigation.

Non-viral vectors. Delivery vectors based on non-viral materials have the potential to be less toxic and immunogenic than viral vectors, but present their own set of delivery challenges. Negatively charged DNA and other nucleic acids can be electrostatically complexed to cationic materials to form nanoparticles that can subsequently be endocytosed by cells through various mechanisms, including receptor-mediated endocytosis and phagocytosis. The most successful classes of cationic materials used so far for nucleic acid delivery are naturally occurring and synthetic polymers (for example, polyethylenimine, cyclodextrin and poly(β-amino esters)) and lipids (for example, lipofectamine, rationally designed lipids and lipid-like materials discovered through combinatorial libraries). Ideally, any non-viral delivery material for genome editing should be well tolerated (biocompatible, non-immunogenic) and capable of delivering payloads to the nucleus.

Non-viral physical delivery (delivery without a vector). In some instances, delivery vectors are not necessary for genome editing. In *ex vivo* therapies, mechanical deformation or electroporation can create transient holes in cell membranes, allowing nucleic acids and proteins to enter the cell. For *in vivo* therapies, a high-volume, hydrodynamic tail-vein injection of naked nucleic acid can efficiently transfect hepatocytes in mice, but safety concerns over lethal acute side effects have limited hydrodynamic injection in humans. Nucleic acid can also be injected directly into the embryo or zygote, but genome editing in human embryos remains highly controversial. In light of these limitations, clinically translatable *in vivo* therapies for genome editing are likely to require a delivery vector.

addition was achieved through the combination of IDLV infection to deliver donor template and electroporation of ZFN mRNA, as well as the optimization of the timing between the two deliveries and the culture conditions. Importantly, HSCs with corrected copies of *IL2RG* generated functional lymphoid cells with a growth advantage in an X-linked severe combined immunodeficiency (SCID-X1) mouse model, demonstrating therapeutic potential[60].

Adenoviral and AAV vectors. Adenoviral and AAV vectors have also been investigated for their potential use in genome editing *in vitro* and *ex vivo*. For example, various methods of delivering ZFNs in HSCs have been compared, with efficacy (defined as the percentage of cells expressing the transgene and the mean intensity of transgene expression in each cell) reported in the following order (from most to least efficacious): mRNA electroporation, plasmid electroporation, adenoviral vector and IDLV[60]. Recently, edited HSPCs were generated by combining electroporation of ZFN mRNA with AAV6-mediated delivery of the donor template[77,78]. Approximately 20–40% gene addition was achieved in

HSPCs using AAV6 as the donor vehicle, an efficiency that was higher than that observed in a previous study that used an IDLV[60,77,78]. Another study showed that an adenoviral vector more efficiently expressed ZFNs in primary T cells than an IDLV[79]. In parallel, megaTAL, which combines a TALE DNA-binding domain with a sequence-specific homing endonuclease, was delivered in an mRNA format. Pairing megaTAL with AAV-mediated delivery of a CCR5-targeting template achieved 8–60% rates of HDR in primary human T cells[80]. In addition to efficient delivery, the off-target effects of different viral vectors for delivering donor DNA will be a key consideration when choosing an appropriate vector for specific applications. Protein-capped adenoviral vectors seem to exhibit greater specificity and accuracy than free-ended IDLVs and plasmid DNA donors in human cell lines[81].

Applications of *ex vivo* gene editing. Because HSCs can generate all haematopoietic cell types, gene-edited HSCs could be used to treat a range of genetic blood disorders[82]. Moreover, modified primary T cells have become a promising tool for cancer immunotherapies[83]. T cells engineered to express synthetic chimeric antigen
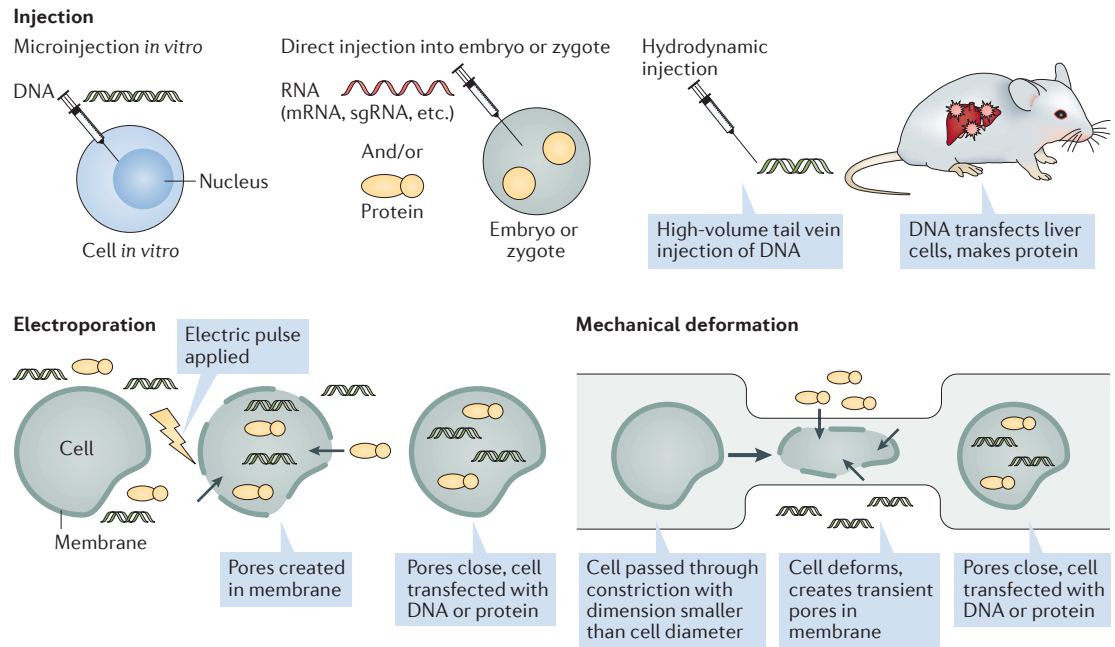
Figure 3 | Physical methods used for delivery of genome-editing biomacromolecules. Physical methods can be used for genome editing to deliver biomacromolecules intracellularly without the use of nanoparticles. Microinjections *in vitro*, direct injection into the embryo or zygote *ex vivo*, or hydrodynamic injection *in vivo* can be performed. Alternatively, electroporation or mechanical deformation can create transient pores in the cellular membrane to facilitate the entry of biomacromolecules for genome editing. sgRNA, single guide RNA.

receptors (CARs) recognize specific epitopes on tumour cells[83]. CAR T cell immunotherapy has achieved positive therapeutic outcomes in clinical trials for melanoma, leukaemia and lymphoma[83]. Despite these successes, CAR T cell therapy could be further improved by genome-editing tools. Currently, integrating viral vectors are applied to insert CARs into the genome to provide long-term effects. However, the semi-random pattern of vector integration has led to insertional mutagenesis events, raising safety concerns[71]. These concerns can be addressed by applying genome-editing tools to insert CARs specifically at a designated site, such as a safe harbour locus[84]. Moreover, genome-editing tools can increase the potency of tumour-specific T cells by knocking out the endogenous T cell receptor, which otherwise can dimerize with exogenous antitumour T cell receptors to decrease the specificity of recognition of the targeted epitope and thus decrease therapeutic potency[79,85]. The potency of CAR T cells can also be dampened by immune-checkpoint blockade by binding to specific surface receptors on those cells, such as the well-studied programmed cell death protein 1 (PD1) receptor[83]. Genome-editing tools have therefore been used to delete PD1 in T cells, which resulted in enhanced T cell effector function[86].

Currently, autologous T cells for immunotherapy are engineered *ex vivo* and then transferred back into patients[86]. However, such a personalized process is expensive, and it can be difficult to generate high numbers of healthy T cells from a number of patients with cancer[86]. Genome-editing tools may be applied to allogeneic T cells

to reduce the potential for immune rejection by knocking out genes involved in immune surveillance, such as the human leukocyte antigen (HLA)[87]. These universal T cells may enable increased consistency, availability, potency and safety[87,88]. Alternatively, electroporation of mRNA encoding genome-editing nucleases and AAVs as the donor template has been reported to induce effective levels of HDR in human primary T cells[89].
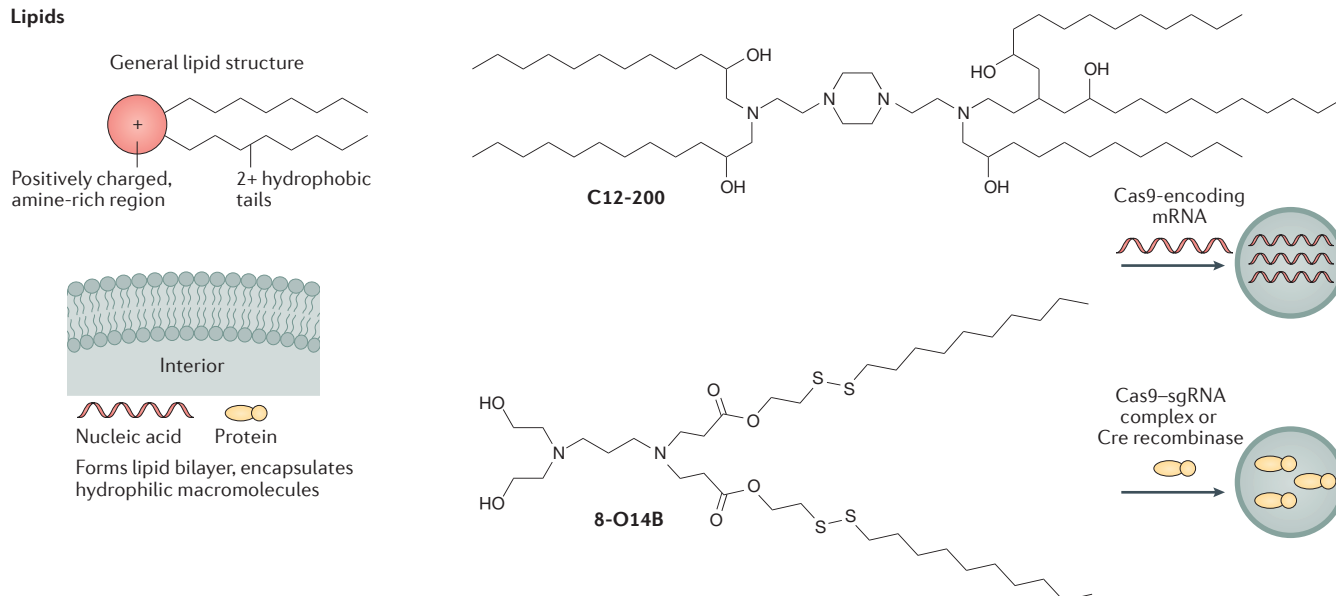
In vivo *approaches*

The potential of viral vectors to facilitate *in vivo* genome editing has been examined in several different model systems[26,29,32,61,75,90,91]. The nature and size of the nuclease is one determinant in selecting an appropriate viral delivery system (TABLE 1).

ZFN systems. In the case of ZFN systems, the small size of ZFNs facilitates their packing into AAVs. The AAV serotype 8 delivery of ZFNs and donor template successfully induced gene targeting in the liver to genetically correct mutated blood coagulation factor IX (FIX)[32]. Importantly, this system resulted in a significant, stable improvement of disease phenotype in a neonatal haemophilia B mouse model. In a follow up study, the AAV-mediated delivery of ZFNs and corrective donor template with homology arms flanking the targeted locus enabled the production of FIX in adult haemophilic mice[92]. Interestingly, the same study[92] also indicated that substantial levels of genome editing can be achieved without such homology arms in the donor carried by the AAV, indicative of NHEJ-mediated knock-in.

Allogeneic
From the same species but not genetically compatible; that is, will induce an immune response.

a Lipids

General lipid structure

Positively charged, amine-rich region

2+ hydrophobic tails

C12-200

8-O14B

Interior

Nucleic acid   Protein

Forms lipid bilayer, encapsulates hydrophilic macromolecules

Cas9-encoding mRNA

Cas9–sgRNA complex or Cre recombinase

b Polymers

Chitosan

PLGA

PLGA nanoparticle

Chitosan   ZFN-encoding mRNA

Linear PEI

Branched PEI

DNA nanoclew

DNA

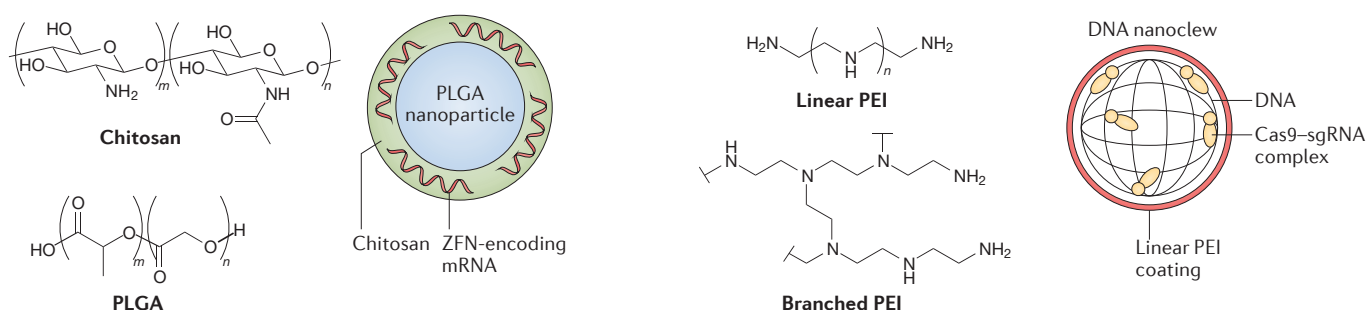Cas9–sgRNA complex

Linear PEI coating

Figure 4 | **Non-viral nanoparticles for delivery of genome-editing biomacromolecules.** Two main classes of non-viral nanoparticles are made from cationic materials: lipids (part **a**) and polymers (part **b**). Such nanoparticles enable the facile complexation with, and encapsulation of, negatively charged nucleic acids or ribonucleoprotein complexes. Ionizable lipids C12-200 (REF. 73) and 8-O14B[111] have been formulated into lipid nanoparticles encpasulating Cas9-encoding mRNA or ribonucleoprotein complexes. In addition, genome-editing biomacromolecules have been encapsulated into chitosan-coated PLGA nanoparticles[110] and PEI-coated self-assembled DNA nanoparticles called 'DNA nanoclews' (REF. 69) PEI, polyethylenimine; PLGA, poly (lactic-co-glycolic) acid; sgRNA, single guide RNA; ZFN, zinc-finger nuclease.

A general strategy for replacing liver-directed secreted proteins was demonstrated using AAV-mediated delivery of donor template and ZFNs targeting the albumin gene locus[93]. Albumin is highly expressed in hepatocytes; therefore, integration into this locus enables high transcriptional activity of therapeutic genes. Long-term expression of therapeutic levels of factors VIII and IX was achieved in mouse models of haemophilia A and B, respectively. This approach may also be useful for the treatment of lysosomal enzyme deficiencies[93].

CRISPR–Cas systems. Viral vectors have been investigated for their potential to facilitate the *in vivo* delivery of CRISPR–Cas genome-editing systems. With a minimal polyadenylation signal and a truncated version of the neuron-specific promoter, it has become possible to pack *Streptococcus pyogenes* Cas9 (SpCas9), the most commonly used Cas9, in an AAV vector[75]. Consequently, a dual-vector system was generated by packaging SpCas9 and guide RNA expression cassettes into two separate viral vectors. The stereotactic injection of this dual-vector system into the mouse brain enabled the depletion of targeted proteins in postmitotic neurons[75]. Although it is technically feasible to package SpCas9 into an AAV vector, this approach limits the use of additional control elements. A split Cas9 system was developed by separating SpCas9 into two fragments, followed by dimerization with a chemically inducible system[94,95], enabling editing with reduced efficacy relative to the single payload system. Cas9 from *Staphylococcus aureus* (SaCas9) is ~1 kb shorter than SpCas9 and can edit the mammalian genome[96]. Using a thyroxine-binding globulin promoter driving SaCas9 and its single guide RNA (sgRNA) expression cassette, these components can be packaged

into a single AAV vector. Injection of a hepatocyte-tropic AAV serotype 8 vector carrying an sgRNA targeting proprotein convertase subtilisin/kexin type 9 (PCSK9), the loss of which reduces low-density lipoprotein cholesterol levels, led to >40% indel formation at the *Pcsk9* locus in the liver tissue[96]. This indel formation was accompanied by a significant decrease in serum PCSK9 and total cholesterol[96].

An adenoviral system has also been used to deliver SpCas9 and an sgRNA targeting PCSK9 (REF. 91). The indel rates in the liver were >50%, resulting in decreased plasma PCSK9 and cholesterol levels. Subsequently, this group used the same approach to target the human *PCSK9* gene in the liver of humanized mice[90]. This study showed a similar rate of mutagenesis in human cells and reduced human PCSK9 protein levels, suggesting that humanized animals may provide a platform for the assessment of applications for somatic genome editing.

Duchenne muscular dystrophy, a severe muscle degenerative disease caused by defects in *DMD*, has been a major focus of genome-editing attempts. Because several regions of this protein are dispensable, exon-skipping strategies can benefit certain patients with this condition. Using adenovirus-mediated delivery[30] or dual AAV vectors to deliver two sgRNAs and SaCas9 (REFS 28,29) or SpCas9 (REF. 26), the mutated exon 23 of *DMD* was deleted, partially restoring *DMD* expression in skeletal muscle and enhancing skeletal muscle function. Intravenous infusion of two AAVs, one expressing SaCas9 and the other carrying a guide RNA and the DNA donor, into newborn mice reversed the mutations of the ornithine transcarbamylase gene in a substantial proportion of hepatocytes and increased survival of the animals[97].

Non-viral delivery of genome-editing systems

Non-viral delivery methods have the potential to transfer large genetic payloads[10]. Despite the rapid discovery and recent development of various synthetic vectors, only a small fraction of non-viral vectors and a few physical methods have been used in clinical stages of research[10]. Nevertheless, non-viral methods may provide unique advantages over viral vectors owing to their transient expression patterns and the potential for their repeated administration and improved efficacy.

In vitro *and* ex vivo *approaches*

Physical methods. One category of non-viral gene delivery systems for *in vitro* and *ex vivo* settings are those that use physical means to breach or deform the cellular membrane to facilitate biomacromolecule transfection. The most widely investigated physical transfection method is electroporation, in which an electrical pulse permits small molecules or macromolecules to enter cells. Current models of electroporation indicate that DNA associates with the destabilized membrane by controlled electric fields, passes through the membrane and then enters the nucleus[98] (FIG. 3).

Electroporation has also been investigated as a tool for *ex vivo* genome editing. For example, electroporation of Cas9 and two different sgRNA-encoding plasmids introduced significant gene deletion in HSPCs and in primary T cells[63]. Incubation of Cas9 protein with *in vitro* transcribed guide RNA forms a ribonucleoprotein (RNP) complex, and the electroporation of RNPs is reportedly superior to that of DNA. Compared to DNA, electroporation of RNPs resulted in higher efficiency genome editing, decreased off-target effects and less cellular toxicity in human cell lines, pluripotent stem cells and primary T cells[62,99]. Electroporation of Cas9 mRNA and sgRNA was reported to result in modest gene editing, possibly due to the instability of unmodified sgRNA[100]. By contrast, partially chemically modified sgRNAs significantly increased the editing efficiency via co-delivery with either Cas9 mRNA or protein[100]. Clinical-grade electroporators have been developed, making electroporation a suitable platform for genome-editing applications[101].

In addition to electric fields, mechanical deformation of the cell can transiently disrupt the cellular membrane to facilitate macromolecule entry into cells[65]. Microfluidic devices can be engineered to feature a dimension smaller in length than the diameter of specific cells. This system induces cells to mechanically deform (squeeze) through the constricting channels and thus allow target materials to diffuse into cells through transient pores created in the cellular membrane[65] (FIG. 3). Current data indicate that the engraftment efficiency of edited HSPCs through the 'squeeze' delivery of RNPs is significantly higher than electroporation and with less toxicity[102]. A separate study demonstrated mechanical deformation of cells for CRISPR-mediated efficient gene editing[64].

Direct intracellular protein delivery has been achieved using a combination of hypertonicity-induced micropinocytosis and a transduction compound named propanebetaine[103]. This method, termed iTOP, was used to induce the efficient transfection of proteins into a range of primary cells[103]. iTOP-mediated delivery of recombinant Cas9 protein and guide RNA resulted in high levels of indel formation in cells[103].

Nanoparticle-mediated delivery. In addition to physical delivery methods, non-viral synthetic lipid-based or polymer-based delivery vectors can be used to transfect cells *in vitro* and *ex vivo* (FIG. 4). Commercially available transfection reagents are routinely used for delivering DNA and RNA in cell culture[10]. Cationic liposomes that encapsulate nucleic acids have been used to deliver Cas9 and sgRNA-coding plasmids in mammalian cell lines[57,104], in adult intestinal stem cells[105] and in many other cell types (FIG. 4a). Cationic lipids in complex with RNPs or engineered negatively charged TALE proteins have been reported to provide higher gene-editing efficiency than with nucleic acid complexes[66,99]. Conjugating TALEN or Cas9 proteins and sgRNAs to CPPs may facilitate intracellular delivery, and reportedly results in gene deletion at micromolar concentrations[68,106]. The efficiency of these described cationic lipid or CPP-based methods is often dependent on the type and status of the cell. The delivery of ZFNs without any delivery vectors was reported to disrupt endogenous genes in several cell lines and in primary CD4+ T cells[107]. One hypothesis for this result is that some ZFNs have intrinsic

Ribonucleoprotein
(RNP). Any biomacromolecule consisting of an RNA in complex with a protein.

cell-penetrating capabilities, although their effective concentration is at micromolar levels, a higher than desired dose[66,107].

In vivo approaches

The delivery of genome-editing systems *in vivo* with a combination of viral, non-viral and physical methods has been reported.

Physical and combinational methods. The first demonstration of the use of CRISPR to facilitate the HDR repair of a disease gene *in vivo* and to rescue animals from a lethal phenotype was in hereditary tyrosinae-mia[33]. In this proof-of-concept study, CRISPR and repair templates were delivered via hydrodynamic injection. Hydrodynamic injection induces transient tissue damage and its use in clinical trials has currently been limited owing to its toxicity and low transduction efficiency[108,109]. Approximately 0.4% of hepatocytes were corrected, and this rate was sufficient to rescue hereditary tyrosinaemia[33] because the repaired hepatocytes have a growth advantage over diseased cells. The long-term expression of nuclease may increase the risk of off-target cleavage, genome instability and antigenicity to bacterial proteins. To address this challenge, and the potential toxicity associated with hydrodynamic injection, lipid nanoparticle-mediated delivery of Cas9 mRNA was combined with an AAV encoding an sgRNA and the donor template[73]. In that study, HDR-mediated correction occurred in >6% of liver cells, which is 15-fold higher than that achieved with hydrodynamic injection[73]. Importantly, off-target nuclease activity was below the limit of detection using an unbiased genome-wide analysis[73]. In addition to hydrodynamic injection, other physical methods have been used *in vivo* for genome editing. For example, electroporation-mediated transfection of Cas9 and guide RNA plasmid into skeletal muscles led to restored function of local tissue in a mouse model of Duchenne muscular dystrophy[30].

Nanoparticle-mediated delivery. Polymeric nanoparticles have also been used to deliver genome-editing systems *in vivo* (FIG. 4b). Delivery of ZFNs was able to prolong the survival time of animals in a transgenic mouse model of surfactant protein B (SPB) deficiency[110]. In that study, the authors intratracheally delivered chitosan-coated poly(lactic-co-glycolic) acid (PLGA) nanoparticles encapsulating ZFN mRNA and an AAV encoding a constitutive CAG promoter to target the site where the *SPB* cDNA was under the control of an inducible promoter[110]. Meanwhile, a polymer-based Cas9 protein delivery has been reported, in which the Cas9–sgRNA complex was loaded into DNA-based self-assembled nanoparticles called 'DNA nanoclews' coated with the cationic polymer polyethylenimine[69]. The potency of this polymeric DNA nanoclew nanoparticle-mediated delivery was reported in both model cell lines and in xenograft tumours[69].

Another nanoparticle-mediated delivery approach involves fusing therapeutic proteins to negatively charged proteins, which facilitates the electrostatic-driven

formation of complexes with cationic transfection reagents[66]. Applying this concept, proteins of Cre or TALE transcription activators tagged with negatively charged GFP were first delivered into cells *in vitro* at nanomolar concentrations using lipofectamine[66]. Subsequently, local injections of Cas9–sgRNA and lipofectamine complexes were shown to efficiently induce genetic modifications of inner ear cells *in vivo*[66]. Injection of the lipid–Cre protein nanocomplexes into mouse brain cells *in vivo* induced gene recombination, which suggests that a similar method could be used for the delivery of Cas9–sgRNA complexes[111].

Embryonic injection. Injection of genome-editing systems into animal embryos and zygotes has also been performed. One-step methods using CRISPR for the generation of experimental animals carrying multiple mutations have been developed[112] and are now adopted in many transgenic cores to generate animal models for biomedical research. Microinjection of Cas9 mRNA and sgRNAs into one-cell mouse embryos produce a high frequency of postnatal mice carrying biallelic mutations in one or multiple targeted genes[112]. Co-injection of DNA donors with Cas9 mRNA and sgRNAs, generate reporter and conditional mutant mice[58]. The same strategy could be used in pets or farm animals for veterinary medicine or to eliminate endogenous retroviruses in pig embryos to produce a suitable non-human organ donor[113]. CRISPR–Cas9-mediated gene editing in human embryos was performed and showed effective cleavage at the endogenous *HBB* locus (which encodes haemoglobin subunit-β) with detectable off-target cleavage[114]. Although such methods may have medical benefits under some circumstances, debate continues regarding the ethics of human embryonic gene editing[115].

Current status of genome-editing clinical trials

Several ongoing clinical trials are evaluating ZFNs to target monogenic and infectious diseases, such as the modification of CCR5 for the treatment of HIV (TABLE 2). Sangamo BioSciences is currently evaluating the safety and tolerability of ZFN-mediated *CCR5*-modified T cells in HIV-infected patients in a phase II trial[116]. In an earlier phase I trial, CD4+-enriched, CD3-specific and CD28-specific antibody activated T cells were transduced with a replication-deficient adenovirus encoding a ZFN targeting CCR5 (REFS 117,118). Within the limits of the phase I trial, the infusions of autologous CD4+ T cells with modified CCR5 were safe in patients, and partial acquired resistance to HIV infection was observed in a few patients[118]. In a separate clinical trial, ZFN mRNAs targeting CCR5 were delivered into T cells via electroporation[119]. This trial was initiated on the basis of preclinical studies showing higher efficiency and a better safety profile of ZFN mRNA delivery than IDLV and adenovirus[60]. Another phase I clinical programme is reported to be investigating this approach for CCR5 in HSPCs[120].

In addition to *CCR5*, clinical trials investigating the ZFN-mediated editing of other genes are underway (TABLE 2). For example, the AAV-mediated delivery of

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Hydrodynamic injection
A rapid, high-volume intravenous infusion.

Monogenic
Under the control of a single gene.

Table 2 | **Clinical trials of genome editing**

Drug	Delivery system	Gene target	Disease	Phase	Status	ClinicalTrials.gov identifier	Refs
SB-318	rAAV2/6 (*in vivo*)	*IDUA*	Mucopolysaccharidosis I	Phase I	Not yet open	NCT02702115	122
SB-913	rAAV2/6 (*in vivo*)	*IDS*	Mucopolysaccharidosis II	Phase I	Not yet open	NCT03041324	143
SB-FIX	rAAV2/6 (*in vivo*)	*F9*	Haemophilia B	Phase I	Not yet open	NCT02695160	121
SB-728-T	Adenoviral delivery of ZFNs to T cells (*ex vivo*)	*CCR5*	HIV	Phase I	Completed	NCT00842634	117,118
SB-728-T	Adenoviral delivery of ZFNs to T cells (*ex vivo*)	*CCR5*	HIV	Phase I/II	Completed	NCT01252641	144
SB-728mR-T	mRNA delivery of ZFNs to T cells (*ex vivo*)	*CCR5*	HIV	Phase I/II	Ongoing	NCT02225665	119
SB-728mR-HSPC	mRNA delivery of ZFNs to CD34+ HSPCs (*ex vivo*)	*CCR5*	HIV	Phase I	Recruiting	NCT02500849	120
SB-509	Plasmid	*VEGFA*	Diabetes (type 1 and, type 2), diabetic polyneuropathy	Phase II	Terminated	NCT01079325	145
SB-509	Plasmid	*VEGFA*	Amyotrophic lateral sclerosis	Phase II	Terminated	NCT00748501	146

All trials listed in this table are sponsored by Sangamo Biosciences. *CCR5*, C-C chemokine receptor type 5; *F9*, factor IX; HSPCs, haematopoietic stem and progenitor cells; *IDUA*, leukocyte and plasma iduronidase; *IDS*, iduronate 2-sulfatase; rAAV, recombinant adeno-associated virus; *VEGFA*, vascular endothelial growth factor A; ZFNs, zinc-finger nucleases.

a ZFN and the donor template to insert a correct copy of the factor IX gene into the albumin gene locus is currently being tested in a phase I trial in subjects with severe haemophilia B[121]. Using this same platform, Sangamo BioSciences aims to treat Hurler syndrome (also known as mucopolysaccharidosis I) by inducing the liver of the patient to produce a sufficient amount of corrective enzyme[122].

At the time of writing this Review, companies such as CRISPR Therapeutics, Intellia Therapeutics and Editas Medicine were working to translate CRISPR–Cas9 technologies to human therapeutics. Intellia Therapeutics is using existing delivery technology, such as lipid nanoparticles, for the *in vivo* treatment of liver diseases such as transthyretin amyloidosis, α1-antitrypsin deficiency, hepatitis B virus and inborn errors of metabolism (see the Intellia Therapeutics website). Intellia Therapeutics is also developing *ex vivo* HSCs and CAR T cell programmes using electroporation-mediated delivery. Meanwhile, Editas Medicine is actively pursuing gene editing in the eye through AAV-mediated local delivery and is also targeting CAR T cells and potentially other disease areas (see the Editas Medicine website). CRISPR Therapeutics is evaluating both *in vivo* and *ex vivo* treatments using the CRISPR–Cas9 system (see the CRISPR Therapeutics website), with potential targets for *ex vivo* treatment including haemoglobinopathies, immuno-deficiencies and immune therapies. According to the information provided on CRISPR Therapeutic's website, *in vivo* treatment may be explored for diseases in the liver, eye, lung and other organs.

Considerations for therapeutic genome editing

Genome editing has tremendous promise for both research and clinical applications. The ability to precisely modify the genome of living cells and tissues offers the potential to treat and perhaps even cure a range of important diseases via the deletion of disease-prone sequences, the correction of mutations or the site-specific insertion of therapeutic genes. Gene editing can also be applied as an antiviral strategy to either remove viral genomes or knock out key genes involved in viral infection[118]. However, there are multiple factors that may influence clinical outcomes of therapeutic genome editing that require consideration.

A key issue for therapeutic genome-editing technologies is the potential for off-target effects. As a permanent genetic modification, off-target cleavage could introduce unwanted mutations and potential toxicity. For example, mutation of oncogenes, tumour suppressors and DNA repair genes may lead to cancer, producing toxicity from a small number of mutated cells[123]. There have been several efforts to evaluate off-target effects[42–47] and increase the specificity[48–55] of genome-editing systems. It is worth noting that this specificity may also be affected by the dosage[47] and expression pattern[73] of genome-editing systems, as well as the number, type and stage of edited cells[73].

It is important to understand the desired efficiency of genomic modification required to achieve significant clinical benefit. The treatment of highly penetrant monogenic diseases will be more straightforward than complex, multigenic diseases, and each monogenic disease

may require various efficiencies of editing. For example, restoration of 3–7% functional factor IX has been reported to reverse disease symptoms of haemophilia B[32], whereas α1-antitrypsin deficiency may require >30% gene correction in the liver[124]. Currently, indel formation is more frequent than HDR repair, and obtaining high levels of HDR repair *in vivo* remains challenging[32,73,125].

The nature of the cellular target can also affect the durability of genome editing-based therapies. At least 5 years of clinical benefit was observed in an earlier CD34+ HSC gene therapy trial using a retroviral vector to treat Wiskott–Aldrich syndrome, an X-linked, primary immunodeficiency[71]. Although the use of retroviral vectors to transfer therapeutic genes is associated with a high risk of leukaemogenesis, the long-term effectiveness of HSC therapy was nonetheless demonstrated. It is anticipated that gene-edited HSCs would remain in the human body for a long period, thereby treating diseases such as SCID with a selective advantage relative to unedited cells. The expansion of gene-edited HSCs has been shown in rodent models, and therefore may provide a durable treatment for some diseases[60]. By contrast, editing mature airway epithelial cells may not provide a long-term clinical benefit for lung diseases such as cystic fibrosis or SPB deficiency due to the rapid turnover rate of these cells in disease stages[110].

Key to the broad implementation of genome-editing technologies is the need for safe and effective delivery methods. This delivery challenge differs from that facing classical gene replacement therapy, which requires long-term transgene expression, or RNAi therapy, which requires repeated dosing. In principle, the delivery of one or a limited number of doses of programmable nucleases, in a transient manner, could provide sufficient clinical benefits. However, multiple components must be delivered in the nucleus of one cell: for ZFNs and TALENs, it requires a pair of proteins; for CRISPR–Cas, it requires Cas protein and a guide RNA; and, for precise gene editing, it may also require an additional DNA donor template. Despite these challenges, there is growing evidence to indicate that functional levels of genome editing can be obtained in a range of tissues using different approaches[26,28–30,32,73,78,110,126].

IDLVs significantly reduce the risk of introducing insertional mutagenesis by abolishing its insertion mechanisms. IDLVs can deliver programmable nucleases to various human cell types[76]. However, the IDLV genome is free-ended, double-stranded and can result in insertion at off-target sites or spontaneous DSBs. Both AAVs and IDLVs have been used to deliver donor templates, inducing HDR in cells[127]. The clinical development of these systems will certainly include consideration of off-target genetic modifications and approaches to reduce its risk. The non-viral delivery of programmable nucleases in mRNA or protein form offers several potential advantages. First, non-viral delivery may enable more precise control of the duration of dosing than plasmid DNA or viral vectors because it avoids the transcription process. Second, concerns of long-term expression of programmable nuclease are diminished because proteins and mRNA are rapidly degraded and

are not likely to integrate into the genome[73]. The limited exposure to programmable nuclease would decrease the risk of off-target effects, thereby minimizing potential side effects such as tumorigenesis[73]. Third, the immunogenicity of adenoviruses and T cell responses to AAV capsids continue to increase risk and limit the opportunity for multiple doses per patient[11,61]. These concerns are reduced with the use of non-viral delivery systems. It may be more desirable to perform repeated injections of non-viral nanoparticles than with AAVs in patients because of a potentially lower adaptive immune response against synthetic materials[10]. Nucleotide chemical modifications, which have been used for decreasing the immune response of therapeutically delivered small interfering RNA, hold potential to decrease the immune response for non-viral delivery of nucleic acid-based genome-editing tools[10]. In addition, viral nanoparticles engineered based on native viral structures could result in a reduced immune response[128].

Many diseases requiring gene correction or addition may prove to be more difficult to treat than those benefiting from gene inactivation. In most cases, HDR is less frequent than NHEJ and is largely restricted to cells at S/G2 phases[129]. Thus, the nature of HDR limits the diseases requiring precise genome editing in postmitotic cells[129]. HDR-mediated small insertions or substitutions occur at a higher rate than large insertions[130]. Viral vectors with single-stranded DNA may generate higher HDR rates than double-stranded DNA or plasmid DNA[93,131].

Suppressing NHEJ moderately increases HDR in cells, although the safety of such strategies is currently unknown[132,133]. Optimization of the conditions of delivery (for example, the timing of providing the donor template[78], the format and dose of the donor template[93,131] and synchronization of the cell cycle[40,63]) may facilitate enhanced HDR. Further research into strategies to improve HDR efficiency will be important to address a broad range of diseases for *in vivo* applications.

Finally, a potentially important concern for clinical translation of genome-editing technologies is the immune response to the corrected genes. If a target gene has not been properly expressed in tissues due to gene deficiency, it is possible that the proteins produced by the correct copy could induce an adaptive immune response. For example, ~30% of patients with haemophilia A who received protein therapy developed inhibitory antibodies[134]. The broad application of gene correction technologies may also require further investigation into the development of targeted tolerance[135].

Conclusion

In summary, progress in the field of genome editing has been remarkable, and there is growing evidence to indicate that the delivery of genome-editing materials can be achieved *in vitro*, *ex vivo* and *in vivo* in a range of tissues. Advances in both viral and non-viral delivery of macromolecules, as well as progress in understanding the nature and function of programmable nucleases, continue, and we are optimistic that the near future will include therapies whereby precise genome editing provides new therapeutic approaches for currently untreatable diseases.

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