Clinical applications of CRISPR-based genome editing and diagnostics

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Clustered regularly interspaced short palindromic repeats (CRISPR)-driven genome editing has rapidly transformed preclinical biomedical research by eliminating the underlying genetic basis of many diseases in model systems and facilitating the study of disease etiology. Translation to the clinic is under way, with announced or impending clinical trials utilizing ex vivo strategies for anticancer immunotherapy or correction of hemoglobinopathies. These exciting applications represent just a fraction of what is theoretically possible for this emerging technology, but many technical hurdles must be overcome before CRISPR-based genome editing technology can reach its full potential. One exciting recent development is the use of CRISPR systems for diagnostic detection of genetic sequences associated with pathogens or cancer. We review the biologic origins and functional mechanism of CRISPR systems and highlight several current and future clinical applications of genome editing.

Genome editing, the process of deliberately changing the DNA sequence of a living cell or organism, is an immensely powerful research tool with remarkable promise as a future therapy for genetic diseases, cancer, and beyond. As early as 2012, it became apparent that the ability to program a clustered regularly interspaced short palindromic repeats (CRISPR) nuclease simply by changing its RNA guide would make it extraordinarily useful for genome editing.[1] The major genome editing tools used at the time, zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), recognize their genomic DNA target through protein-DNA interactions.[2] To edit a new DNA site of interest, researchers had to engineer a new protein. The CRISPR-Cas9 system, an RNA-guided genome editing tool, greatly facilitates genome editing because it can be reprogrammed quickly and inexpensively using readily available synthetic RNA molecules.

ABBREVIATIONS: CAR = chimeric antigen receptor; CRISPR = clustered regularly interspaced short palindromic repeats; crRNAs = CRISPR RNAs; gRNA = guide RNA; HDR = homology-directed repair; HSPCs = hematopoietic stem/progenitor cells; indel = insertion or deletion; OTEs = off-target effects; RNP = ribonucleoprotein; SCD = sickle cell disease; TALENs = transcription activator-like effector nucleases; ZFNs = zinc-finger nucleases.

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This work was supported by the Innovative Genomics Institute.

Received for publication September 12, 2018; revision received November 14, 2018, and accepted November 14, 2018.

doi:10.1111/trf.15126

TRANSFUSION 2018;00;1–11

In early 2013, scientists used a specific CRISPR-associated (Cas) protein, Cas9, to edit the genomes of human cells.[3–5] In the following months, labs used the CRISPR-Cas9 system to edit organisms like zebrafish, yeast, mice, nematodes, fruit flies, rice, *Arabidopsis,* wheat, and bacteria including *Escherichia coli.*[6–13] Since then, CRISPR technology has opened the door to brand new avenues of biologic research in diverse organisms like ferrets, coral, and cacao.[14–16] Scientists can manipulate the genomes of cultured cells to knock out genes, fluorescently tag endogenous proteins, make specific sets of mutations to model disease, and more.[17,18]

CRISPR has revolutionized biological research and the next frontier is medicine. Humans are afflicted with over 5000 different single-gene disorders.[19] For the vast majority, treatments are only palliative and there are no therapies that address the underlying genetic cause. Furthermore, many genetically complicated or infectious diseases (e.g., HIV infection) could be ameliorated, cured, or prevented with a genetic correction. Indeed, gene therapy has begun to fulfill its potential in recent years, emerging as a viable treatment option for some genetic conditions and cancers.

Researchers have used CRISPR-based editing to correct the genetic basis of many diseases in isolated cells or animal models.[20–29] The first wave of clinical trials using CRISPR enzymes to treat inherited disorders in humans involve removing a patient's cells, editing ex vivo, and reinfusing the corrected cells.[30] Such ex vivo genome editing is currently the most technically feasible approach, and has the potential to treat devastating blood disorders like sickle cell disease and β-thalassemia. The ex vivo strategy also underlies cancer immunotherapies. CRISPR-based cancer trials have already begun in China and may soon begin in the United States.[31] While awaiting the first news of how CRISPR genome editing performs in the clinic, researchers are working to surmount some of the technique's known limitations. Namely, there is a dearth of sufficient methods for delivering genome-editing molecules into disease-affected patient tissues; desired edits must be performed without causing any genetic collateral damage; and the possibility of preexisting immunity or a post-treatment immune reaction to bacterial CRISPR proteins must be assessed.[32–34] As some scientists refine the technology and gear up for clinical use, others continue to find novel medical uses for CRISPR enzymes. Notably, researchers have utilized two recently discovered Cas proteins for rapid, ultra-sensitive detection of RNA or DNA, providing the foundation for an emerging strategy for point-of-care diagnostics.[35,36]

Here, we review the prokaryotic origins of CRISPR-Cas systems, their application as genome editing tools, progress toward CRISPR-based therapies, long-term possibilities and challenges in the field, and recent work exploiting Cas enzymes for diagnostic nucleic acid detection.

PROKARYOTES EVOLVED CRISPR-CAS ADAPTIVE IMMUNE SYSTEMS TO DEFEND AGAINST BACTERIOPHAGE INFECTION

Like many biotechnologies, CRISPR-Cas systems originated in the microbial world. Bacteria and archaea evolved Cas proteins to fend off bacteriophages, the viruses that infect them.[37] Unlike most prokaryotic defenses, including well-known restriction-modification systems, CRISPR immunity is adaptive.[38] A variety of diverse CRISPR-Cas systems that share many commonalities exist in nature (a fact that has proven useful for creative biotechnology applications).[39,40] Cas proteins capture small pieces of invading nucleic acid and store them in the host genome as a molecular record of prior infections.[41] The storehouse of viral memories is called the CRISPR or repeat-spacer array. It is a collection of unique DNA segments (typically 29–43 nucleotides long) separated by a repeated sequence (typically 24–37 nucleotides long).[42]

The CRISPR array is transcribed into one long precursor CRISPR RNA. Cas proteins, and sometimes other proteins, process the precursor CRISPR RNA (crRNAs) into mature crRNAs, each containing one virus-targeting spacer flanked by bits of the repeat sequence.[43–45] Finally, the mature crRNA assembles with one or more Cas proteins to form a surveillance complex. This RNA-guided complex scans nucleic acids via transient binding until it finds a complementary sequence to destroy.[46] If the surveillance complex successfully cleaves phage DNA, the phage can no longer replicate and the prokaryotic cell survives.

REPURPOSING CRISPR PROTEINS FOR GENOME EDITING

Genome editing is accomplished by programming a nuclease to cut at a specific DNA sequence, which results in a change in the genetic code when the break is imperfectly repaired.[2] Over the past 6 years, CRISPR proteins have become the dominant tools for genome editing. Most researchers use Cas9 or Cas12a, single-protein nucleases that are easily reprogrammed to target new DNA sites, simply by changing part of their guide RNA (gRNA) (Fig. 1).[3–5,47] The Cas9 protein from *Streptococcus pyogenes* was the first used for genome editing and remains the most popular.

The first step in designing a genome-editing experiment is to choose a target site and design a complementary gRNA. There are a variety of online tools for designing effective and accurate gRNAs.[48–52] If a Cas9 gRNA is partially complementary to a sequence of DNA, it will sometimes bind and even cut at this so-called off-target site.[53] Computational resources help researchers choose gRNAs that are likely to have minimal off-target effects (OTEs), a critical consideration for therapeutic development, discussed in a later section.[32] Researchers will often design a panel of gRNAs to test, since their

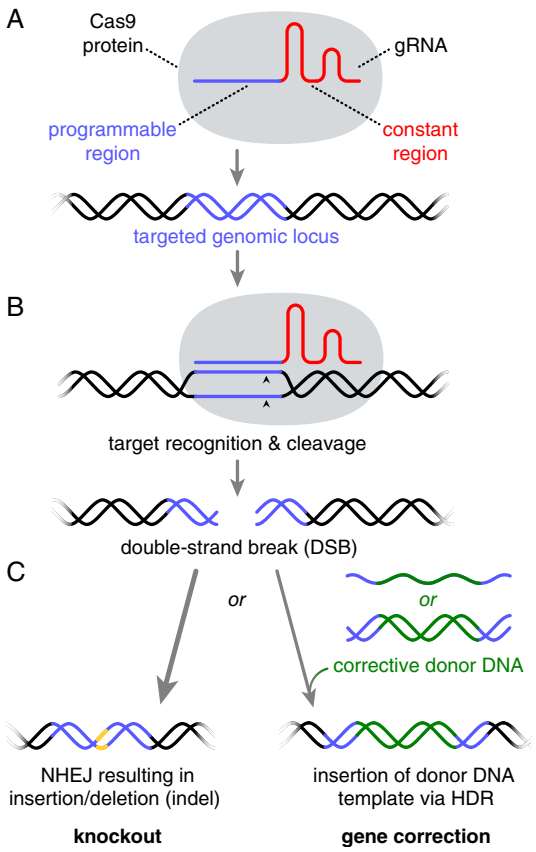

Fig. 1. The molecular mechanism of CRISPR enzymes driving genome editing. (A) Cas9, a prototypical CRISPR-Cas enzyme comprises a protein component (gray) that is responsible for cleaving DNA as well as a guide RNA component (red and blue) that mediates DNA targeting through a stretch of 20 bases (programmable region, blue). This region of gRNA is designed to match a genomic DNA sequence (also blue) to be targeted in an editing experiment. (B) If Cas9 finds a region of DNA with complementarity to the gRNA, the enzyme is activated for cleavage, creating a double-strand break in the genome. (C) Cellular repair pathways are activated to repair the break, which can have multiple outcomes. The most common result (left) is an error-prone repair pathway that typically results in disruption (yellow) of the targeted gene, knocking it out. It is also possible to provide a single- or double-stranded corrective DNA template (green) that can be incorporated into the break site at low frequencies (right) based on regions of homology (blue) between the donor and the cut site, allowing insertion of restorative or novel DNA.

individual effectiveness can vary by target site, cell type, experimental setup, and so on.

After designing a gRNA, the experimenter must prepare and deliver the genome-editing reagents. Cas9, gRNA, and any other components can be delivered in a variety of ways, with therapeutic strategies discussed in the next section.[33] For laboratory manipulation of cells, DNA instructions for Cas9 components can be introduced into the cells by chemical transfection or electroporation. Alternatively, the Cas9 enzyme can be preassembled from its protein and RNA components, and this tight complex can be introduced to the cell via electroporation. Inside the cell, the enzyme will diffuse throughout the cell's nucleus, scanning the genome in the hunt for a match: a region of DNA capable of base-pairing with the Cas9 gRNA. Once a sufficiently complementary genomic target is found, Cas9 will cut each strand of the DNA, producing a double-strand break (Fig. 1).

Following a double-strand break, the cell must repair the chromosomal disruption to survive. DNA repair is the process that actually produces genomic "edits," but it is largely outside of the experimenter's control. The precise mechanisms responsible for repair of Cas9-induced DNA breaks are not fully characterized, but eukaryotic cells typically follow one of two general pathways.[54–56] The predominant route is nonhomologous end joining (NHEJ), which eventually leads to formation of an insertion or deletion (indel) (Fig. 1). Indels are typically small, no more than 1–15 nucleotides long.[57,58] These apparently minor genetic changes are usually adequate to induce a crippling frameshift (indels of 1–2 nucleotides are most common) or premature stop codon, effectively knocking out gene function. Experimenters test the levels of editing at the target site by isolating the DNA from the cells or tissue and performing sequence analysis of amplicons through methods such as targeted next-generation sequencing, tracking of indels by decomposition,[59] or less reliable heteroduplex assays such as T7E1.[60] Within each experiment, the levels of editing at the target site are compared to the levels of editing observed at the computationally predicted off-target sites for that gRNA. Genome-wide sequence analysis permits monitoring of broader OTEs via methods such as Digenome-seq,[61] GUIDE-Seq,[62] CIRCLE-Seq,[63] and AMP-Seq.[64] The therapeutically tolerable frequency of OTEs associated with a candidate therapy is a topic of open debate. Because OTE frequencies resulting from a given genome-editing approach will often be comparable to—or below—the natural mutation rate of a targeted cell type, it is not mandatory that an approach be free of OTEs. Low levels of off-target editing are therefore considered acceptable for preclinical research. That said, the genetic position and editing outcome at each site will contribute to the biologic impact of a given OTE, so each gRNA and experimental design requires extensive analysis.

If a genome editor wishes to replace a faulty genetic sequence with a corrected version, they must provide a segment of "donor" DNA, which is used as a template for repair, allowing insertion of novel genetic information. The cellular repair machinery may insert experimenter-provided DNA at the site of the break through a pathway called homology-directed repair (HDR), but this process is rare compared to NHEJ (Fig. 1).[54] The donor is typically a single- or double-stranded DNA bearing "homology arms" that

match either side of the Cas9 cut site.[65] The likelihood of successful insertion can be improved using a few different strategies, but the general inefficiency of this gene replacement approach represents a substantial challenge in the field.[65–72] As our ability to carefully manipulate DNA grows, so will the clinical potential of CRISPR technologies.[17]

CURRENT AND NEAR-TERM THERAPEUTIC APPLICATIONS

Genetic therapies offer hope for treating and effectively curing monogenic diseases by reversing the underlying genetic cause. Furthermore, genome manipulation is a powerful tool to treat or cure cancer as well as degenerative diseases or HIV infection. Technologies previously developed for genetic therapy may soon be eclipsed by the promise of CRISPR-Cas systems for precise genome editing of human cells. Cas enzymes have the particularly valuable ability to target specific sites in the genome for editing, which is an improvement on the viral technology used to incorporate helpful genes in traditional gene therapy. Furthermore, CRISPR-Cas systems are more flexible and convenient than site-specific endonucleases such as ZFNs and TALENs.[2] There has been exciting progress applying CRISPR-Cas systems in preclinical models of disease such as cultured human cells and animal models, justifying the transition to therapeutic use.

Although CRISPR genome editing offers great promise for therapeutic applications, several challenges must be addressed before widespread clinical application can be considered. One of the largest barriers facing the translation of CRISPR-Cas genome-editing technologies into the clinic is effective delivery of the genome-editing enzymes to the cells in need of correction. In the case of template-directed HDR, the donor DNA template must be codelivered, further complicating the task. The major strategies for delivery of CRISPR-Cas systems into cells and tissues include viral vectors that deliver DNA encoding the enzyme and the guide RNA; lipid nanoparticles that deliver the mRNA encoding Cas9 and the gRNA; and the Cas9 enzyme with its gRNA as a preformed ribonucleoprotein (RNP) complex (Fig. 2). Viral approaches are the closest to clinical application, as these have been in clinical development for decades for use in classical gene therapy paradigms. All three of these approaches present specific advantages and disadvantages, and the most appropriate approach will depend on the tissue in need of correction, the route of administration, and the type of editing required.[33]

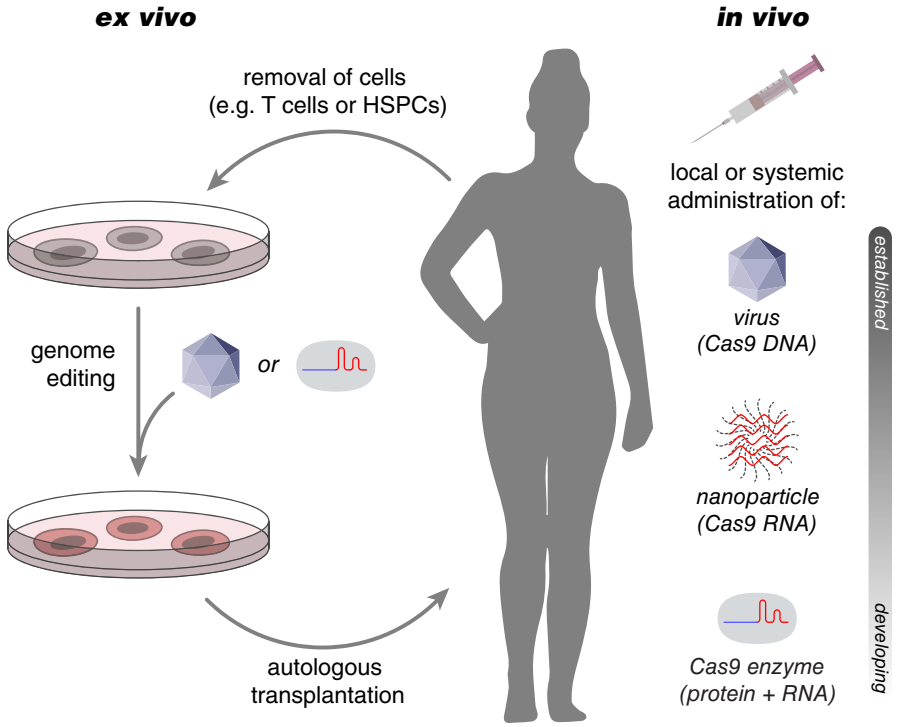

Fig. 2. Strategies for therapeutic genome editing. For ex vivo therapies (left), a patient's cells are removed for genetic correction using a CRISPR-Cas enzyme delivered either by virus encoding the enzyme or as a preformed RNA-protein (RNP) complex. Once the cells have been edited, they are returned to the patient. In vivo clinical use (right) relies on administration of the genome-editing therapeutic either locally or systemically. Viral delivery of DNA encoding the enzyme components is the most well-developed delivery technology. Other delivery platforms are under development: nanoparticles carrying the RNA components or delivery of the CRISPR-Cas RNP.

The field of CRISPR-based therapeutics is off to a quick start, thanks to decades of gene therapy research with other technologies, especially lentiviral vectors, ZFNs, and TALENs. More than 10 clinical trials are ongoing worldwide involving CRISPR-Cas9 genome editing, almost all of which involve ex vivo genome editing of human cells, as explained in the subsequent section. The first US and European clinical trials are both due to begin enrolling patients in 2018. The biotechnology sector's multibillion-dollar investment in developing CRISPR-based therapeutics strongly suggests that CRISPR will find its way to the clinic, and hopes are high that these initial clinical studies will pave the way for an expanding range of CRISPR-based treatments for genetic diseases.

Ex vivo approaches

Ex vivo therapeutic applications of CRISPR systems are closest to the clinic because they rely on the genetic manipulation performed in a laboratory setting. Ex vivo therapies involve removing cells from the body, performing CRISPR-based genome editing, and putting the edited cells back into the patient via autologous transplantation.[73] For blood- and immune-based diseases, this requires removing blood and/or bone marrow from the patient to edit either immune cells or hematopoietic stem/progenitor cells (HSPCs). HSPCs are found in the bone marrow and give rise to all blood and immune cells in circulation. Radiation or chemotherapy is typically used to ablate the endogenous population of HSPCs (a process known as conditioning) to promote subsequent engraftment of the corrected cells.

The precise editing to be performed depends on the disease target. For example, monogenic inflammatory disease has been a target for traditional gene therapy via autologous transplantation of HSPCs, as exemplified by Wiskott-Aldrich syndrome,[74] and progress will likely be accelerated by current CRISPR-based genome-editing tools. In the case of the autoimmune disease rheumatoid arthritis, it may be preferable to edit regulatory T cells (instead of their progenitors) to inhibit the release of proinflammatory cytokines.[75]

Fortunately, T cells are readily edited ex vivo, and can be returned to the patient's bloodstream without the need for conditioning. Autologous transplantation of T cells is an established therapeutic avenue to combat HIV infection, as demonstrated by a clinical trial (NCT00842634) that successfully performed ZFN-mediated knockout of HIV host factor CCR5.[76] Similar future efforts will likely rely on CRISPR-based editors, and there is an ongoing push to excise the HIV provirus from infected cells using Cas9.[77] Initial applications of ex vivo therapeutics rely on autologous transplantation, but engineered "universal donor" stem or T cells from healthy donors are being developed using genome editing to enable allogeneic transplantation.[78,79] This approach is appealing because it would eliminate the need for the substantial infrastructure associated with autologous transplantation.

Immunotherapy

Adoptive cell transfer, a type of immunotherapy, represents fertile territory for utilization of genome-editing technology. A CRISPR-driven clinical trial relying on T cells bearing a knockout of programmed cell death protein 1, PD-1, has been completed in China (NCT02793856),[80] and a chimeric antigen receptor (CAR) T-cell therapy is the first US Food and Drug Administration–approved commercial gene transfer therapy.[81] In CAR-T therapy, T cells are removed from a patient and genetically engineered to express a CAR, which consists of an extracellular cell-targeting component and an intracellular signaling moiety. In this approach, the modified T cells are programmed to recognize cancerous cells through an engineered extracellular antigen-binding domain, which typically incorporates the variable region of an antibody with cancer specificity. In the current generation of CARs, the cytoplasmic portion includes a CD3ζ chain with two costimulatory domains, which activates the T cell for killing of a bound cancer cell.[81] There are more than 250 current or completed clinical trials for CAR–T-cell therapies, wherein each CAR directs detection of a specific type of cancer.[81] The first North American clinical trial for CRISPR-based CAR–T cell therapy began in 2018 for patients with multiple myeloma, melanoma, and sarcoma (NCT03399448). Several similar trials are also under way in China.[30]

CRISPR should make CAR–T-cell therapy a more viable therapeutic approach due to its precision as compared to viral approaches. Recent preclinical studies demonstrate enhanced efficacy when CRISPR-mediated HDR facilitates insertion of the CAR sequence specifically into the T-cell receptor alpha constant genetic locus, allowing expression under the control of the endogenous TCR promoter;[82] this is an improvement over viral strategies wherein the CAR is expressed in an unnatural genetic context. Viral vectors present other specific issues for clinical application, especially concerning the substantial manufacturing costs and risks of spurious genomic integration.[83,84] Exciting preclinical advancements in nonviral delivery of Cas9 into immune cells have been reported recently, where electroporation of the Cas9 RNP and donor DNA template allowed effective editing of patient T cells by Cas9-mediated HDR. Patients' T cells were redirected to bind the NY-ESO-1 tumor antigen, again by inserting the CAR into the T-cell receptor alpha constant locus.[85] These engineered cells were then administered to mice with a melanoma tumor xenograft, effectively reducing tumor size and demonstrating the utility of nonviral T cell editing.

Sickle cell disease and β-thalassemia

Sickle cell disease (SCD) and β-thalassemia are hemoglobinopathies caused by well-understood genetic aberrations. Despite this knowledge, treatments are woefully inadequate, resulting in severe symptoms and reduced life expectancy

for those afflicted. These devastating diseases afflict millions of people worldwide, and the only lasting curative treatment is a bone marrow transplant from a healthy, matched human leukocyte antigen donor. In a practical sense, this solution is untenable because greater than 80% of patients do not have access to a matched donor, and the transplant includes massive risks for the potentially fatal graft-versus-host disease.[86,87]

Both of these diseases are tempting targets for correction by genome editing because their genetic etiology is so well understood.[88] Template-directed gene replacement via CRISPR-Cas9 can reverse the single point mutation causing SCD in ex vivo HSPCs at levels that are therapeutically viable.[20,21] Another strategy relies on the "knockout" editing that Cas9 performs with high efficiency, but instead targets the transcription factor BCL11a, which is responsible for silencing expression of fetal hemoglobin.[89] Fetal hemoglobin can serve as a "backup" copy of hemoglobin, but it primarily lies dormant in adults. With a straightforward genetic edit at the BCL11a locus, fetal hemoglobin can be reactivated and can alleviate the symptoms of SCD.[90] Importantly, the editing to restore the corrected hemoglobin or upregulate fetal hemoglobin need not be extremely efficient; even moderate levels of editing and successful engraftment (2%–20%) will likely provide sufficient benefit to patients.[21,91] Targeting BCL11a currently appears more therapeutically viable than CRISPR-based gene correction, thus both Intellia/Novartis and CRISPR Therapeutics/Vertex (NCT03655678) are pursuing this strategy for treating β-thalassemia, with SCD trials expected to follow thereafter. Thus, it is likely that β-hemoglobinopathies will be the first monogenic diseases to be curable using CRISPR-Cas therapeutics.

FUTURE THERAPEUTIC APPLICATIONS

Genome editing is poised to solve critical issues in transfusion medicine, specifically in generating cell reagents in vitro to improve upon what is currently available from donors.[92] First, the ability to generate erythroid progenitor cells that are compatible for transfusion to a wide variety of donors would alleviate the impacts of alloimmunization when blood antigens are not matched between donor and patient (including minor antigens that are not included in routine screens). This is particularly critical for patients with blood disorders such as β-hemoglobinopathies, as the frequently required blood transfusions lead to increased side effects. CRISPR-based genome editing of an immortalized human erythroblast cell line (with subsequent erythroid differentiation) has allowed removal of five distinct antigens that would alleviate alloimmunization side effects in a subset of patients with rare RBC antigen profiles.[93] Similarly, customized platelets with rare alloantigenic epitopes (HPA-1b) have been generated by editing induced pluripotent stem cells and megakaryocyte-like cells, offering future diagnostic and therapeutic applications for thrombocytopenia.[94] Further, studies suggest that engineering induced pluripotent stem cells into reagent red cells with rare antigen phenotypes show promise for alleviating a great shortage of diagnostic cells for patients with complex alloantibody profiles.[95] The field is rapidly addressing challenges associated with differentiation, enucleation, and scalability to make genome-edited, customized blood cells a reality in transfusion medicine.[96]

The progress made in ex vivo CRISPR therapeutics will hopefully translate to in vivo–compatible administration of genome-editing platforms for a wide variety of diseases. There is an upcoming clinical trial for in vivo–administered Cas9-encoding plasmid directly to the cervix for treating human papillomavirus–associated malignancies through targeting parts of the viral genome associated with disease (NCT03057912). Extensive work in mice and human cell lines has demonstrated that CRISPR can be used to correct mutations causing Duchenne muscular dystrophy, which recently advanced to restoring dystrophin protein expression in a canine model of the disease through systemic and intramuscular delivery of adeno-associated virus–encoded Cas9.[22] CRISPR-based treatments are also being developed for several brain diseases,[26,97–99] eye diseases such as congenital blindness, and diseases of the liver.[100]

The ideal genome-editing therapeutic would allow a single dose of a transiently active nuclease that can be administered systemically but with tissue-selective uptake, editing only the specific cell type in need of correction. Editing only cells in need of correction is critical, both to limit the total dose and to avoid germline editing. Different delivery strategies display different propensities for tissue-specific delivery. For instance, nanoparticles display liver-specific uptake,[100] and a range of viral vectors display tropism for specific tissues.[101]

As the CRISPR-Cas systems are of prokaryotic origin, it remains to be seen whether innate or adaptive immune responses will hinder their clinical application. Both *Streptococcus pyogenes* and *Staphylococcus aureus* are human pathogens; preexisting antibodies against the Cas9 proteins from these bacterial species have been identified in humans.[34,102] The presence of humoral and cell-mediated immunity to Cas proteins is a challenge but could be mitigated by immunosuppression, limiting exposure to a single dose, and/or using Cas proteins from microbes that humans have not encountered and acquired immunity against.[103] Furthermore, targeted mutation of Cas9 has allowed removal of specific T-cell epitopes, thereby engineering an "immunosilenced" version of Cas9 while preserving function.[104] Editing organs that are relatively immune privileged, such as the brain or eye, may present fewer complications therapeutically.[105]

The gRNA is responsible for programming specificity based on complementarity with the DNA target sequence, but sometimes the Cas enzyme cuts nontargeted DNA sequences that pair with the gRNA in nearly all sequence

positions. Such unintended cutting results in OTEs, which are a critical issue, especially when editing stem cell populations that will repopulate the entirety of the cell type. This risk is mitigated in the CRISPR clinical trials that are under way, as a subset of the cells edited ex vivo will be analyzed for genome-wide OTEs before autologous transplantation of the edited cells into the patient. Furthermore, variants of Cas enzymes are being engineered with higher fidelity, which could limit OTEs to levels that are not of clinical concern.[106,107] The recent observation that Cas9 is capable of infrequently inducing larger genomic rearrangements stimulated significant apprehension.[108] Such events may be cell type-specific and could be preventable by using genome-editing approaches that do not induce double-strand breaks, such as nickases[109,110] or base editors.[111]

To minimize the incidence of OTEs and immune responses, it is critical to minimize the dose and lifetime of CRISPR editing reagents. Lentiviral administration involves gene integration, which promotes persisting expression of the Cas9 enzyme, although this can be minimized using integration-deficient lentivirus.[112] Adeno-associated virus vectors can also cause integration events, leading to genotoxicity.[83] Other delivery technologies, such as nanoparticle-based delivery of Cas9 mRNA/gRNA and delivery of the intact Cas9 RNP complex (Fig. 2), result in intracellular Cas9 that is only transiently present and can still perform efficient editing. It has been demonstrated that transient delivery of Cas9 as an RNP complex causes far fewer OTEs than does viral delivery of Cas9.[113] Both RNP and nanoparticle delivery technologies are in rapid preclinical development, especially for treating diseases of the brain and the liver, and thus there is great excitement for their transition into clinical studies.

APPLYING CRISPR ENZYMES FOR SENSITIVE NUCLEIC ACID DETECTION AND DIAGNOSTICS

There is a critical need for point-of-care diagnostics for detection of minute amounts of specific nucleic acid sequences.[114] Nucleic acids can serve as biomarkers of disease (e.g., an oncogenic mutation) or infection (e.g., viral or bacterial sequences). The ideal diagnostic assay is capable of highly sensitive, direct detection of sequences from a range of bodily fluids with a visual readout and minimal reliance on instrumentation. Scientists have leveraged recently identified CRISPR-Cas enzymes to create just such a technology.

Cas12a and Cas13 were discovered in 2015, and originally named Cpf1 and C2c2, respectively.[47,115] The next year, researchers found that Cas13 uses a crRNA guide to identify and cleave single-stranded RNA substrates.[116] Upon recognition of its target RNA, the enzyme indiscriminately cleaves any nearby single-stranded RNAs, regardless of sequence.[117] Just this year, scientists realized that Cas12a works in a similar manner, collaterally cleaving nearby single-stranded DNA after binding to complementary double-stranded DNA.[36]

This activity can be leveraged for in vitro detection of specific DNA or RNA using purified Cas12a or Cas13 enzymes (Fig. 3).[117,118] The experimenter designs a crRNA guide that is complementary to the nucleic acid of interest,

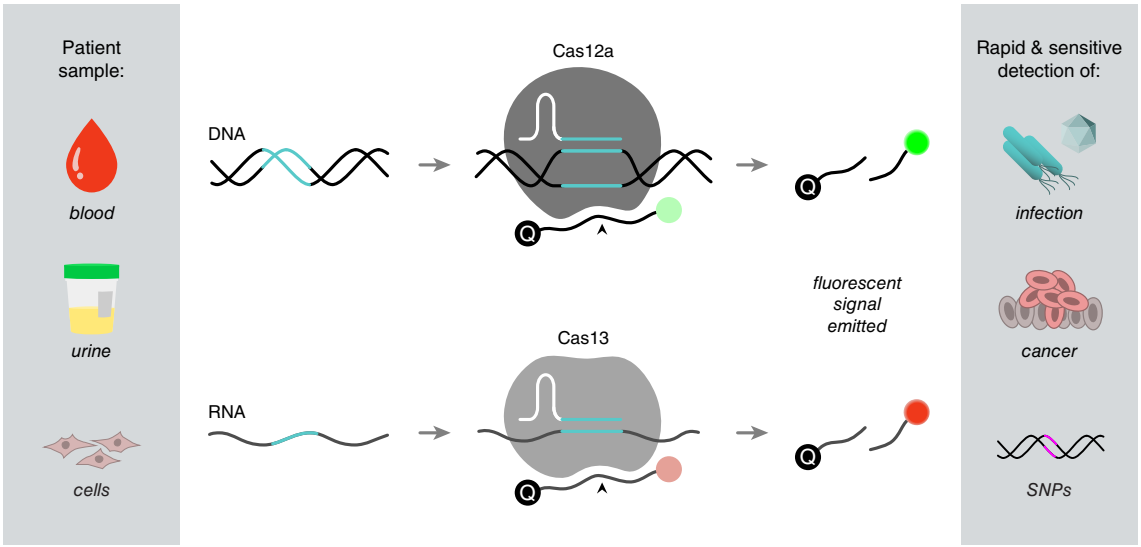

Fig. 3. Diagnostic applications of CRISPR-Cas enzymes. The ability to recognize precise genetic sequences allows use of CRISPR-Cas enzymes for biomedical testing. Cas12a (top) and Cas13 (bottom) can respectively be programmed to recognize DNA or RNA, activating the enzymes to cleave nearby nucleic acid molecules. Single-stranded DNA or RNA molecules bearing a fluorophore and a quencher (Q) are provided as part of the detection system, and these will result in a fluorescent signal following cleavage by Cas12a or Cas13, respectively. Because this signal will only result if the CRISPR-Cas enzyme finds a sequence matching its targeting RNA (cyan), this system can perform exquisitely sensitive detection of genetic sequences associated with pathogens or disease.

assembles it with a Cas enzyme, and supplies a reporter DNA or RNA. The reporter is typically a modified nucleic acid with a fluorophore at one end and a chemical "quencher" at the other. These components are combined with nucleic acids extracted from a biologic fluid sample. Upon detection of the target nucleic acid, the Cas enzyme will cleave the reporter, releasing the fluorophore from the quencher and allowing it to fluoresce. Researchers can quantify the resulting signal using equipment common to molecular biology laboratories, and simply switching the reporter to generate an antibody-detectable signal enables visual readout on commercial lateral flow test strips.[35,119]

Scientists have built upon this basic strategy, first by adding isothermal amplification of the target nucleic acids to enhance sensitivity. One such technology, dubbed SHERLOCK (specific high-sensitivity enzymatic reporter unlocking), initially employed Cas13, enabling detection of attomolar levels of Zika and dengue virus RNA. The other approach, DETECTR (DNA endonuclease-targeted CRISPR *trans* reporter), uses Cas12a and shows attomolar sensitivity for human papillomavirus.[119] A new application of SHERLOCK allows multiplexing using orthogonal CRISPR enzymes to report the presence of different targets in the same sample.[119] CRISPR-based systems have also been used to detect disease-causing mutations in patient biopsy samples.[35,119] Though still in their infancy, CRISPR-based diagnostic platforms have great potential for broad clinical applicability, and several groups are working to further develop the systems for commercial deployment.[120]

CONCLUSIONS AND OUTLOOK

In just 6 years, CRISPR-Cas systems have revolutionized how biomedical research is performed on living systems. At this stage, genetic knockouts are facile, but replacing sequences by HDR remains inefficient and technically challenging, although great strides are being made to improve the technique.[65–72] CRISPR systems have generated an extraordinary level of excitement for the clinical potential for curing human disease. In this nascent stage of CRISPR in the clinic, initial applications are treating diseases with the most accessible cells (immune cells and HSPCs edited ex vivo), which avoids the ongoing challenge of tissue-specific delivery in vivo. Ex vivo treatment carries the lowest risk for the first application of CRISPR in humans, since it largely mitigates the impacts of the technology's two largest hurdles: the host immune response and unintended editing events.

The initial clinical trials involving ex vivo CRISPR-based immunotherapies are being watched with much anticipation. Given that the research community is still optimizing the technology for editing human cells, some believe it is too early to enter clinical trials.[121] While every effort is being taken to ensure effectiveness and safety, much can only be determined by performing human trials. In addition to unpredictable outcomes, another major hurdle is in the cost of manufacturing CRISPR therapeutics at a scale that is economically feasible. This becomes particularly challenging at the scale of in vivo therapeutics, as the required doses are much higher than what is required to edit a subset of cells ex vivo. On the other hand, the substantial infrastructure required to administer ex vivo therapeutics makes their application infeasible in developing countries. CRISPR-based editing has not yet been tested for therapeutic use in vivo, but with the current enthusiastic global effort, it is likely that CRISPR will eventually realize its potential to cure a wide range of human genetic diseases.

CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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