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REVIEW

Applications of CRISPR technologies in research and beyond

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Programmable DNA cleavage using CRISPR–Cas9 enables efficient, site-specific genome engineering in single cells and whole organisms. In the research arena, versatile CRISPR-enabled genome editing has been used in various ways, such as controlling transcription, modifying epigenomes, conducting genome-wide screens and imaging chromosomes. CRISPR systems are already being used to alleviate genetic disorders in animals and are likely to be employed soon in the clinic to treat human diseases of the eye and blood. Two clinical trials using CRISPR-Cas9 for targeted cancer therapies have been approved in China and the United States. Beyond biomedical applications, these tools are now being used to expedite crop and livestock breeding, engineer new antimicrobials and control disease-carrying insects with gene drives.

CRISPR–Cas9 technology provides a precise and facile molecular mechanism for editing cells, tissues and whole organisms, with widespread uses in experimental and applied systems[1,2]. Derived from a prokaryotic adaptive immune system that provides DNA-encoded, RNA-mediated and sequence-specific protection against viruses (**Box 1**), CRISPR–Cas9 has been exploited to develop potent tools for genome manipulation in animals, plants and microorganisms. The RNA-guided Cas9 endonuclease first recognizes a 2- to 4-base-pair conserved sequence named the protospacer-adjacent motif (PAM) (R.B. and colleagues)[3,4], which flanks a target DNA site[3–6]. Upon binding to the PAM, Cas9 interrogates the flanking DNA sequences for base-pairing complementarity to a guide RNA (J.A.D. and colleagues)[7]. If there is complementarity between the first 12 base pairs (the 'seed' sequence)[8] of the guide RNA and the target DNA strand, RNA strand invasion accompanies local DNA unwinding to form an R-loop[9]. Precise cleavage of each DNA strand by the RuvC and HNH domains of Cas9 generates a blunt double-strand DNA (dsDNA) break (DSB) at a position three base pairs upstream of the 3′ edge of the protospacer sequence, measuring from the PAM (R.B. and colleagues[10]; see **Box 1** and **Fig. 1**). In addition to PAM-based DNA recognition and guide RNA–target DNA complementarity, Cas9 specificity is also affected by conformational control of DNA cleavage. Nuclear DNA is scanned by three-dimensional diffusion of Cas9, followed by differential binding residence time between off-target sequences (to which Cas9 binding is short-lived) and target sequences (to which Cas9 binding time is extended (J.A.D. and colleagues))[11]. All three mechanisms contribute to the specificity of target site cleavage (J.A.D. and colleagues)[12].

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Alternative technologies for genome engineering include those based on zinc-finger nucleases (ZFNs) and transcription activator-like effector nuclease (TALEN) proteins, programmable enzymes that use protein–DNA binding, rather than RNA–DNA hybridization, for site-specific genome editing[13]. Although still employed in some experiments, these earlier technologies require new protein design and validation for each experiment, restricting their wide adoption. The potential for RNA-guided genome alterations emerged in applications of group II self-splicing introns to integrate DNA sequences at specific genomic loci[14]. The CRISPR–Cas9 system, which is built upon the promise of these earlier findings, offers simplicity and efficacy in virtually all cell types (see **Fig. 2** for an example in butterflies), encompassing cells and animals of medical interest, plants and livestock species relevant for food and agriculture, and model organisms widely used by the scientific community[2,15]. Combined with lessons learned from other genome-editing systems, the properties of CRISPR–Cas9 ensured rapid adoption for genome engineering applications across biology (**Fig. 3**) (R.B.)[14,16]. Here, we review current and future applications of CRISPR–Cas9 technologies both inside and outside the laboratory, including therapeutics, xenotransplantation, and uses in livestock, crops, food organisms and industrial microbes. We also discuss the scientific, commercial, regulatory and ethical implications of this transformative set of technologies.

Engineering CRISPR systems

The first CRISPR–Cas9 genome-editing experiments exploited host cell machinery to repair the genome precisely at the site of the Cas9-generated DSB. Mutations can arise either by non-homologous end-joining (NHEJ) or homology-directed repair (HDR) of DSBs[13]. NHEJ produces small insertions or deletions (indels) at the cleavage site, whereas HDR uses a native (or engineered) DNA template to replace the targeted allele with an alternative sequence by recombination. Additional DNA repair pathways such as single-strand annealing, alternative end joining, microhomology-mediated joining, mismatch and base- and nucleotide-excision repair can also produce genome edits[13,17–20]. Inspired by genome-editing technologies that use ZFNs, meganucleases or TALENs, researchers have developed Cas9 variants

Box 1 CRISPR primer

Clustered regularly interspaced short palindromic repeats (CRISPRs), together with CRISPR-associated (Cas) proteins, provide adaptive immunity against viruses and plasmids in many bacterial species and most archaea[186,187] (R.B. *et al.*)[188,189]. CRISPR–Cas systems use DNA-encoded[190], RNA-mediated[191] sequence-specific cleavage[10] of foreign nucleic acids[192,193] (**Fig. 1**). Integration of short DNA sequences into CRISPR arrays (R.B. *et al.*)[190] provides organisms with a long-term genomic record of infections. Transcripts derived from these arrays can base-pair with complementary sequences, thereby recruiting Cas enzymes to bind and cleave incoming pathogenic nucleic acids (R.B. and colleagues)[194] (**Fig. 1**).

Mechanistic dissection of the Cas9 enzyme led to a system that could be employed for facile genome engineering (J.A.D., R.B. and colleagues)[195]. Using purified protein, RNA and DNA components, Cas9 was shown to function as an enzyme that uses RNA molecules to specify double-stranded DNA sequences for site-specific cleavage[196] (J.A.D., R.B. *et al.*)[10,194,195]. This finding led to the creation of a streamlined two-component system comprising a chimeric sgRNA (which includes the 20-nt target binding sequence and hairpin RNA structures that are required for DNA recognition and Cas9 protein assembly) and a Cas9 protein (J.A.D. and colleagues)[195].

Upon binding to a DNA sequence specified by the 20-nt target recognition segment in the guide RNA, Cas9 uses its HNH and RuvC catalytic domains to create a precise DSB (J.A.D., R.B. and colleagues)[194,195]. This mechanistic understanding led to the proposal that RNA-programmed Cas9 could be employed for genome engineering by triggering repair of double-stranded DNA breaks at desired sites (J.A.D., R.B. *et al.*)[195,197]. Using standard methods of protein and RNA expression and subcellular localization, RNA-programmed genome engineering technology was established in short order by multiple laboratories[198–202] (J.A.D. and colleagues)[203] and implemented thereafter in a wide range of cells and organisms[15,21,22] (S.H. Sternberg & J.A.D.)[204].

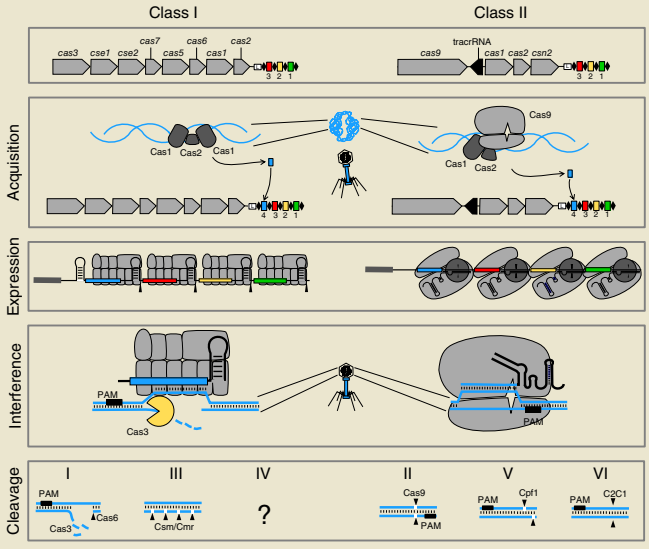


Figure 1 Mechanism of action of CRISPR–Cas immune systems. Left, class I CRISPR–Cas systems (using type I as a canonical example), which consist of repeat (black diamonds) and spacer (colored blocks) arrays, flanked by *cas* genes that encode the Cascade machinery. Right, class II CRISPR–Cas immune systems (using type II as a canonical example), which consist of repeat and spacer arrays flanked by *cas* genes that encode sequence-specific nucleases (e.g., Cas9) and ancillary RNAs (e.g., the tracrRNA). During CRISPR-mediated vaccination (top), the acquisition machinery (Cas1 and Cas2) copy and paste invader DNA sequences as novel spacers at the leader end of the CRISPR array. During the expression stage (center), the Cas machinery transcribes CRISPR arrays and generates mature small interfering crRNAs. During the interference stage (bottom), guide RNAs direct the Cas machinery toward complementary DNA flanked by PAM sequences and drive sequence-specific cleavage of target DNA.

derived from the *Streptococcus pyogenes* Cas9 (SpyCas9) for use as nickases, dual nickases or FokI fusion variants[15,21–28]. More recently, Cas9 orthologs (**Fig. 4**), and other nucleases derived from class II CRISPR–Cas systems including Cpf1 (ref. 29) and C2c1 (ref. 30), have been added to the CRISPR toolbox. These ongoing efforts to mine the abundant bacterial and archaeal CRISPR–Cas systems should increase the range of molecular tools available to researchers.

Beyond genome editing, the impact of CRISPR technologies extends to site-specific genomic control, including both transcriptional and epigenetic regulation (**Fig. 5**). Specifically, deactivated variants of Cas9 (dCas9) lacking DNA cleavage function convert the single guide RNA (sgRNA):Cas9 technology into a sequence-specific transcriptional regulator[31–34]. When fused to a transcriptional repressor (e.g., KRAB) or activator domain (e.g., VP64), dCas9 chimeras can reduce or increase gene expression, respectively[31,32,34]. Likewise, by fusion to fluorophores, dCas9 enables sequence-specific visualization of DNA and dynamic imaging of chromatin[35]. For example, it is now possible to simultaneously visualize up to six loci using CRISPRainbow[36]. Recent success using sgRNA:Cas9-based acetyltransferases[37] and demethylases[38] further extends this technology to engineering epigenetic changes in the genome[39].

The development of orthogonal (R.B. and colleagues)[40] sgRNA:Cas9 systems[40–43] will allow users to exploit different CRISPR tools for distinct applications in the same cell, including manipulating several pathways at once. Further improvements could include the use of chemically modified sgRNAs to enhance the stability and functionality of the sgRNA:Cas9 complex for improved genome editing[44]. In particular, rational chemical modifications of sgRNAs by incorporation of modified nucleotides such as 2′-*O*-methyl (2′-O-Me), 2′-fluoro (2′-F) and *S*-constrained ethyl (2′-cEt) can improve stability and decrease the RNAse susceptibility of CRISPR guides[45].

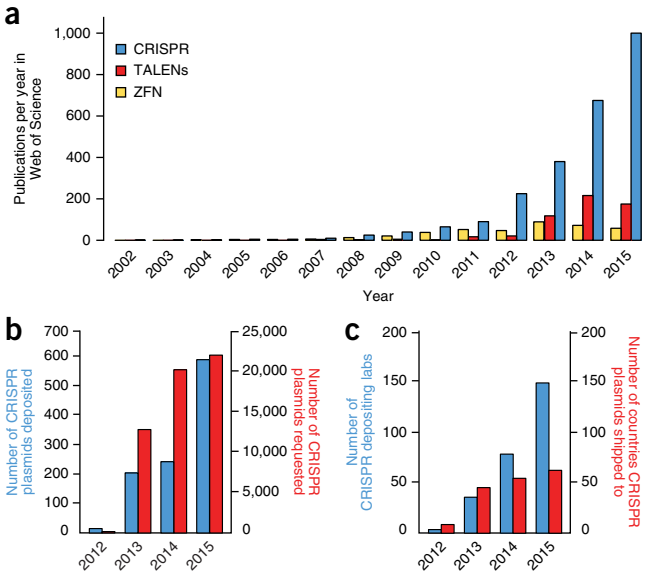
In common with any nascent technology, CRISPR-derived tools can be enhanced for particular applications. For example, the large size of Cas9 and related proteins impacts the efficiency of viral vector packaging, and PAM-constrained targeting and off-target activity can affect editing precision. To solve these problems, researchers have carried out bioinformatic analyses of native CRISPR–Cas systems that have identified smaller Cas9 homologs (**Fig. 4**). Some of these variants have different PAMs relative to SpyCas9, thereby offering alternative DNA targeting capabilities[46].

Structural data and biochemical studies have also been used to engineer Cas9 variants with altered PAM-targeting or modified DNA

Figure 2 Butterfly wing patterns altered using CRISPR–Cas9-mediated genome editing. This picture illustrates the power with which CRISPR has been used for altering animal traits. CRISPR works well in several species of butterflies[179] (*Papilla xuthus*, *Papilla machaon*); in this example, the wild type (bottom) is darker than the G_0 bi-allelic *yellow* gene knockout (top), and small patches of darker wild-type tissue are observed in the *yellow* knockout animal, while the wing pattern is maintained. (Photo courtesy of Michael Perry and Claude Desplan, New York University.)

affinity. Distinct segments of the sgRNA drive assembly and function of Cas9 proteins (R.B. and colleagues)[40], which contain two structural lobes that drive target recognition and endonucleolytic activity[6,47–49] (J.A.D. and colleagues)[50]. Crystallographic and electron-microscopy-based structures of Cas9 have revealed the nature of its interactions with guide RNA and target DNA, which can be exploited for the engineering of Cas9 variants with enhanced affinities and activities[6,9,47,51] (J.A.D. and colleagues)[50,52,53]. Similar efforts are under development using other CRISPR systems, such as efforts based on the Cpf1 structure[54,55]. The CRISPR–Cas9 toolbox is expanding to extend the PAM-dependent targeting space, to reduce non-specific interactions with target DNA, reduce off-target binding and increase specificity[56,57]. As additional natural Cas9 variants are analyzed, rationally engineering PAM recognition domains will likely become easier and may yield variants that can target any DNA sequence, regardless of G+C content and genomic context. Recent advances in understanding PAM composition and diversity (R.B. and colleagues)[58] will also enable improved predictions of on- versus off-target binding, and allow improved design and selection of CRISPR guides.

For regulatory control, split-Cas9 variants have been designed to enable controllable assembly in the presence of sgRNAs or other effector molecules (J.A.D. and colleagues[59])[60]. Such variants can also be used to enhance HDR ratios[61]. Another option is to tether programmable DNA-binding domains to Cas9 to increase precision by adding another binding requirement, in addition to the PAM and target DNA complementarity[62]. Likewise, transcriptional and functional control of Cas9-mediated genome editing can also be managed using optogenetics[63,64] and chemically inducible (doxycycline-regulated) approaches[65] to ensure transient Cas9-mediated DSB genesis and subsequent editing. These approaches open new avenues for skewing DNA-repair pathways by limiting efficient but imprecise mutagenesis by NHEJ and enhancing less frequent but predictable and user-defined designer edits by HDR. This can be achieved by targeting molecules driving NHEJ, such as KU70, KU80 and DNA ligase IV to increase HDR by many fold[66–68]. Alternatively, small molecules, such as azidothymidine and trifluridine, can be used in concert with CRISPR enzymes to selectively favor a repair pathway[69]. It is likely that combinatorial approaches will produce optimized Cas9 variants with enhanced specificity and activity, ensuring that CRISPR technologies advance rapidly toward utility in the clinic and beyond.



Genome-wide screens

Noting the success of RNA interference (RNAi)-based screens (R.B. *et al.*)[70], researchers have been using scaled-up sgRNA:Cas9 technology for genome-wide screening with large-scale pooled-guide libraries[31,68,71–78]. Recent studies have shown that loss-of-function screens exploiting libraries comprising tens-of-thousands of sgRNAs can be used to identify genes involved in tumor growth and metastasis[71]. Furthermore, screens based on transcriptional interference or repression (CRISPRi) and activation (CRISPRa) have harnessed Cas9-based technologies for use in genome-wide studies[31,68,71,72]. In addition, recent improvements in lentiviral library generation and propagation, as well as large-scale DNA and RNA synthesis, have allowed implementation of the sgRNA:Cas9 technology across multiple model platforms[68,71,72,74,79].

Figure 3 Genome-editing in the literature. (**a**) The number of manuscripts published per year since 2002 using CRISPR, ZFN and TALEN as keywords, according to Thomson Reuters' Web of Science. (**b**) The number of CRISPR constructs deposited and requested at Addgene. (**c**) The number of depositing laboratories and recipient countries.

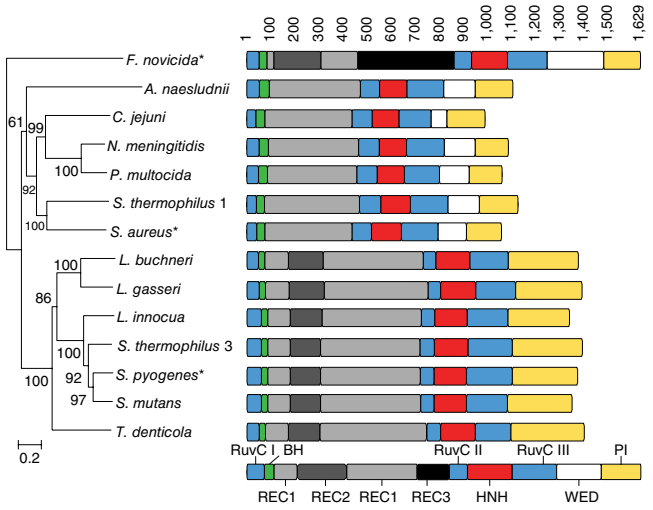
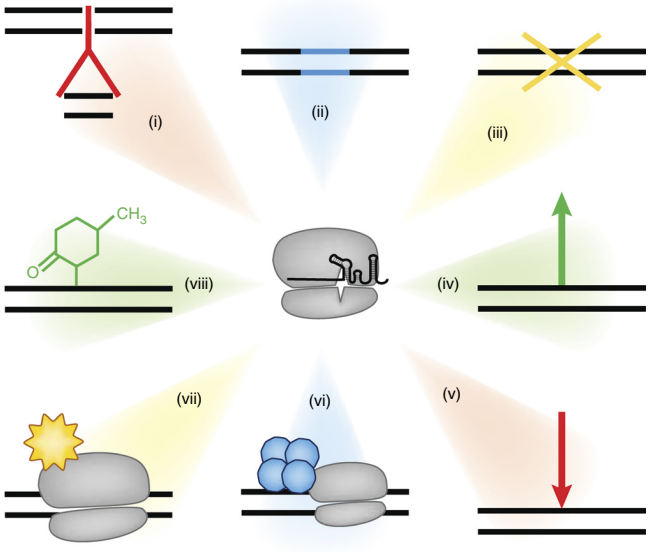


Figure 4 Cas9 diversity. A wide range of Cas9 proteins have been investigated and characterized to date, spanning multiple families that include the canonical SpyCas9 cluster (related to the Sth3 model system), and a set of smaller Cas9 proteins (related to the Sth1 model system, and the recently established Nme and Sau[180,181] orthologs). Diversity encompasses size and sequence variation across structural and functional domains labeled using a color scheme for which the key is labeled at the bottom, notably the REC lobe sections, the RuvC and HNH nickase domains, and the PAM-interacting (PI) segment. *Proteins for which crystal structures have been determined.

Previous genome-engineering technologies based on ZFNs and TALENs required constructing and testing newly designed proteins for each DNA target sequence to be modified. Therefore, the main advantage of CRISPR technologies is the ease with which ~80-nt CRISPR sgRNAs can be synthesized to direct Cas9 to different target sequences, which in turn means throughput can be increased. CRISPR-based screens have enabled identification of essential genes[80,81] and drug targets[82,83]. Using a CRISPR lentiviral library targeting protein-coding genes in five distinct human cell lines, a recent study identified almost 2,000 genes, including ~1,500 genes conserved across cell lines, as core fitness genes[84]. Screens like this could be used for comprehensive analysis of cancer mutation profiles and may one day highlight mutated proteins that could be targeted for therapeutic intervention; for instance, CRISPR–Cas9-mediated repair of a nonsense point mutation in the tumor suppressor ASXL1 in a mouse model of myeloid leukemia has been shown to extend lifespan[85]. Likewise, a study in mice using multiplexed mutagenesis in hepatocellular carcinoma and intrahepatic cholangiocarcinoma illustrates the potential of high-throughput CRISPR-enabled analysis of cancer cells for the genesis of future therapies[86].

Beyond functional and essential genes, CRISPR-based screening can be used to study non-coding sequences and characterize enhancer elements and regulatory sequences[87,88]. This type of analysis will be crucial to elucidate the roles of the so-called non-coding genome.

Going forward, however, there is a need to develop robust CRISPR-based assay technologies that generate consistent results across cells lines and types, regardless of reagents and manufacturers, and for more extensive validation of results across experimenters and platforms[81]. Another practical consideration is how to make screening technologies affordable and accessible; engineering short(er) sgRNAs could reduce reagent manufacturing costs and thereby further democratize the use of CRISPR-based technologies.



From small- to large-animal models

Genome editing will continue to be used to generate disease models and tissue donors, and to bridge the gap between therapeutic proof-of-concept validation in rodents and human clinical trials. The availability of somatic cell nuclear transfer will enable the study of cardiovascular, immune and metabolic systems in animals, such as pigs and primates, that mimic human physiology[89].

One exciting potential application of CRISPR technologies is to engineer large animals to study mechanisms of immune rejection and disease transmission across species barriers. The demonstration last year that endogenous retroviruses could be eliminated from porcine cells by CRISPR targeting indicates whole animals could be engineered with a reduced risk of transmitting disease, bringing xenotransplantation applications one step closer[90]. For xenotransplantation to become reality, not only would researchers working with engineered organs need to address zoonosis concerns, they would also need to demonstrate effective repression of hyperacute rejection, acute vascular rejection and chronic cellular xenograft rejection following transplantation across species. It is conceivable that genome editing could be used to both reduce immune barriers in the host and improve donor organ function.

CRISPR-mediated genome editing could also expedite the development of large animal models of human diseases, including in primates, and thereby accelerate the identification of suitable therapies[91], although mosaicism issues have to be addressed to ensure the genotype

Figure 5 Genome editing redefined. This figure illustrates the range of applications based on CRISPR–Cas9 technologies. (i) Deletions (using HDR with a template in which a deletion is engineered). (ii) Insertions (by providing a HDR template carrying a designer sequence). (iii) Knockouts (using NHEJ-mediated DSB repair). (iv) Transcriptional activation (CRISPRa, using dCas9 tethered to a transcriptional activator, such as VP64). (v) Transcriptional repression (CRISPRi, using dCas9, potentially fused to a transcriptional repressor such as KRAB). (vi) Fusion protein delivery (by direct or indirect recruiting of an effector molecule of interest, through fusion, tethering, or by the use of guides carrying protein-binding DNA sequences of interest). (vii) Imaging (using fluorophores). (viii) Epigenetic state alteration (using either epigenetic repressors such as the LSD1 histone demethylase for interaction with distal enhancers, or epigenetic activation using the p300 histone acetyltransferase).

of interest is consistently generated across tissues and cell types. CRISPR-mediated genome editing is being used to establish robust *in vitro* and *in vivo* models of human disease (R.B. & A.P. May)[92].

Cell therapy applications

Therapeutic uses of ZFNs and TALENs in human cells have paved the way for clinical applications of CRISPR-based technologies. The first proof-of-concept experiments that CRISPR-based genome editing can correct defective genotypes *in vitro* have recently been reported[93–99].

For example, editing of the cystic fibrosis transmembrane regulator sequence in patient-derived induced pluripotent stem cells (iPSCs) produced corrected cells that differentiated into mature airway epithelial cells *in vitro*[93]. Other studies focused on crystalline gamma c (*Crygc*)-associated cataract[94], dystrophin-related Duchenne muscular dystrophy[95] and Fanconi anemia[96], providing further evidence that CRISPR–Cas9-based approaches can correct disease alleles in cells. In addition, efforts to use CRISPR–Cas9-mediated genome editing to correct muscular dystrophy are setting the pace for clinical implementation[97–99]. These studies show that disease genotypes (exon 23 frame shifts) can be corrected to restore the dystrophin gene in *mdx* mouse models, thereby improving muscle function in myofibers, cardiocytes, muscle stem cells and live animals.

The next step will be to establish the efficacy of CRISPR-based therapies *in vivo* in model organisms. Several studies have shown that CRISPR therapies can be implemented *in vivo* (e.g., correction in adult mouse liver of fumarylacetoacetate hydrolase (*Fah*)-related tyrosinemia[100], and alteration of cholesterol metabolism by proprotein convertase subtilisin/kexin type 9 (PCSK9) editing in mouse hepatocytes[101]). More recently, a rhodopsin mutation involved in autosomal dominant retinitis pigmentosa was corrected in rats by means of subretinal injection of plasmids encoding CRISPR–Cas9 and suitable sgRNAs[102].

Ex vivo editing of iPSCs derived from human fibroblasts provide flexible alternatives to introduction of editing molecules to tissues *in vivo*, allowing the analysis and characterization of edited cells before delivery into patients. One noteworthy development is the production of organoids from engineered cells to model and recapitulate disease phenotypes in three-dimensional tissues[103]. This strategy provides a framework for both disease modeling and regenerative medicine based on the synthetic reconstitution of tissues with physiologically relevant structural and functional features that could be transplanted into patients. Work is underway on autism-spectrum disorders using iPSCs[104] (investigating the role of autism-related genes, such as *CHD8*), with long-term potential to address neurodegenerative diseases[105,106].

These *ex vivo* applications as well as hematopoietic-stem-cell-based approaches and T-cell-based immunotherapies are most likely to move genome engineering into the clinic. For example, complex diseases, such as liver cancer can already be readily recapitulated *in vivo* in wild-type mice, by targeting tumor suppressor genes, such as *PTEN* and *TP53* (refs. 107–109), accelerating the pace at which genetic contributors to cancer can be dissected, both individually and in combination.

Before CRISPR-based gene therapies can be tested in human clinical trials, several practical issues and technical challenges need to be overcome, including the following: first, setting and reaching targets of accuracy and efficiency of both cleavage and repair at the cell population level; second, achieving efficient delivery to particular cell types, tissues or organs; third, understanding how to control various repair pathways; and fourth, predictably defining the mutational outcome of the DNA repair after DSB genesis. Indeed, repairing faulty alleles with corrected genotypes will most often require surgical replacement through recombination rather than simple deletions.

Delivery of Cas9 into cells or tissues for therapeutic benefit is an ongoing challenge. Although initial experiments relied on delivering plasmids or viral vectors encoding Cas9 and sgRNAs[110], advances in non-DNA-dependent options such as pre-assembled protein–RNA complexes are opening new delivery avenues. Strategic delivery of Cas9 and sgRNAs in a time-controlled manner (J.A.D. and colleagues)[111] could be instrumental in co-opting specific repair pathways. Direct delivery of ribonucleoprotein complexes can provide high genome editing rates in T cells (J.A.D. and colleagues)[112]. Cas9 ribonucleoprotein delivery using lipid transfection in human cells and in mice[113] shows promise, and has been combined with single-stranded oligodeoxynucleotides to introduce mutations into human iPSCs[114]. Both ribonucleoproteins and mRNA-based Cas9 delivery are advantageous because they avoid DNA-based toxicity, and provide only transient functionality[112,115–117]. DNA 'nanoclews', advanced variants of nanocages and nanoparticles, have also been used to deliver CRISPR payloads[118]. Nevertheless, the initial focus for clinical applications will likely be on targeting cells and tissues for which delivery methods are readily available, such as blood, liver, eye and muscle.

Antimicrobial and antiviral applications

Eukaryotes maintain homeostasis in cells and tissues through multiple robust DNA repair pathways. By contrast, bacteria readily acquire genetic material, and keep mutation rates high enough to ensure evolutionary flexibility, because they have few and primitive DNA repair processes. This feature has paved the way for of the development of CRISPR–Cas systems that can program bacterial death (R.B. *et al.*)[119,120].

Engineering of self-targeting CRISPR–Cas systems has shown promise in anti-microbial applications[119–122]. Either native or engineered CRISPR–Cas systems—including type I systems (**Box 1**) that are associated with nucleases that process cleaved DNA following the generation of a DSB (R.B. and colleagues)[123,124]—can be programmed to target any bacterial species. The sequence-specific antibiotics thus generated can selectively modulate bacterial populations and eliminate pathogens[120].

One key advantage of sequence-specific antimicrobials is that they can be designed to precisely target clinical genotypes or epidemiological isolates. A second advantage is that sgRNAs can be engineered to enable a wide range of organisms (e.g., a whole genus) to be targeted at the same time. Precise eradication of pathogens would enable survival of beneficial commensal bacteria, thereby providing an advantage over broad-spectrum antibiotics. It is conceivable that selective antimicrobials might also work for the removal of eukaryotic parasites. One challenge that needs to be overcome before antimicrobials based on CRISPRs can enter clinical trials is to develop robust delivery options.

In the area of antivirals, work is underway to expand on their role as natural systems to block phage infection in bacteria. CRISPR-based therapies are being developed that target human viruses, including HIV-1 (refs. 125–131), herpes[132], papillomavirus[133–135] and hepatitis B virus[136–143]. Options for HIV therapy include either editing the host[128] or targeting the virus[125]. For example, co-disruption of genes encoding the HIV cell-surface receptor proteins chemokine (C-X-C) motif receptor 4 (CXCR4) and chemokine (C-C motif) receptor 5 (CCR5) shows promise in preventing HIV entry into $CD4^+$ T cells, and for antiviral therapy[126,127,131]. As noted above, CRISPR–Cas9 has

also been used to remove porcine endogenous retroviruses (PERVs) from the entire pig genome, thereby preventing the risk of PERV viral spread during tissue transplantation[90].

Agricultural applications

Trait improvement through classic breeding in livestock, such as cows, chickens and pigs, will be accelerated by CRISPR-based genome engineering. Animal breeders have already identified trait-associated chromosomal markers known as quantitative trait loci and use marker-assisted breeding to selectively advance valuable traits. This process will be accelerated using genome editing technologies, as recently shown in pigs[144] and dairy cattle[145], to protect against viruses and remove horns, respectively. Another application in animals is to engineer production of either medical products or tissues. For example, knock-in of human albumin cDNA into the pig *Alb* locus using CRISPR–Cas9 could enable the production of albumin using transgenic pigs[146].

CRISPR-enabled engineering is being used in commercial and model crops to increase yield, improve drought tolerance and increase growth in limited-nutrient conditions, and to breed crops with improved nutritional properties[147,148]. The use of CRISPR technology in corn[149] and soybean[150] illustrates the speed of adoption of CRISPR technology outside the laboratory. CRISPR-based gene targeting can also be harnessed to combat plant pathogens, as has been shown for the tomato yellow leaf curl virus in *Nicotiana benthamiana*[151].

CRISPR-based technologies have been implemented in diverse foods, and have received much attention given their permissive regulatory status. It was recently reported that CRISPR-edited mushrooms might not be construed as regulated products by regulatory agencies[152]. Furthermore, recent feedback from regulatory agencies regarding waxy corn has laid the groundwork for future uses and commercialization of CRISPR-based technologies in food and feed crops.

Applications in food and industrial biotechnology

To date, applications of CRISPR systems in bacteria include genotyping, vaccinating industrial cultures against viruses, controlling uptake and dissemination of antibiotic resistance genes by bacteria, and engineering probiotic cultures (K. Selle & R.B.)[153,154]. The commercial success of native CRISPR–Cas immune systems for the vaccination of *Streptococcus thermophilus* starter cultures used in dairy fermentations (yogurt and cheese) has paved the way for CRISPRs in food (R.B. *et al.*)[155,156]. Food-grade applications could be enabled by screening for natural CRISPR-based vaccination events, which generate otherwise isogenic and iso-functional starter cultures[155]. Recent work has also shown proof of the concept that beneficial bacteria may be generated that are immunized against the uptake and dissemination of genes that encode antibiotic resistance[10].

CRISPR technologies will have a broad impact on all industries related to bacteria, fungi and yeast, as we are on the cusp of the widespread use of genome editing in these organisms[157,158] (R.B. & J.P. Pijkeren)[159]. CRISPR–Cas9 is likely to be used to engineer industrial bacteria, yeast and fungi to manufacture green chemicals, including biofuels[160] and biomaterials. Synthetic biology approaches that incorporate CRISPR technologies could produce mosaic genomes that have been streamlined for minimal content by strategic deletions (R.B. *et al.*)[154,161]. A recent report also showed that CRISPR-mediated vaccination processes can be exploited as molecular recording events, with the ability to capture synthetic DNA sequences, useful for data storage, into bacterial, and possibly other, genomes[162].

Biological control applications

Cas9 has been used to create gene drives[163–165], in which acquisition of a trait and the Cas9 machinery are coupled to ensure rapid trait propagation through a population. Specifically, gene drives can be used in *Anopheles gambiae,* the mosquito vector for malaria, to drive a recessive female sterility genotype with transmission to progeny rates exceeding 90%. Such an approach has the potential to suppress the spread of malaria in humans[166]. Likewise, anti-*Plasmodium falciparum* CRISPR systems have been implemented in the Asian malaria vector *Anopheles stephensi*[164]. Notwithstanding the potential of CRISPR-based gene drives for controlling the spread of disease agents, as with any nascent technology, successful implementation on a broad scale will require both scientific advancement (notably regarding biological containment and drive efficiency), as well as regulatory approval and public acceptance.

Conclusions

The repurposing of bacterial CRISPR–Cas immune systems as genome engineering tools has heralded an era in which RNA-programmed genome editing is a democratized and broadly accessible technology. In the clinic, therapeutic success is likely to be attained in localized tissues (liver, blood, eye), with longer-term goals of targeting systemic diseases dependent on future delivery options. Screen-based drug discovery approaches, together with the ability to use RNA-programmed genome editing technology to produce disease-recapitulating cell line models and animals, will continue to identify potential therapeutic targets. The application of genome-wide, Cas9-based screens to complex diseases, such as leukemia, provides intriguing opportunities for the selection of therapeutic targets and the design of anti-cancer drugs[167].

Over the long term, the potential for CRISPR-enabled production of synthetic tissues or animals and immune-compatible donor organisms for xenotransplantation[168,169] is vast. In the short term, with the proof of concept already provided for the correction of genetic diseases, such as Duchenne muscular dystrophy[95,97,98,170,171] and beta-thalassemia[172–175], there is potential for gene and antiviral CRISPR-based therapies. Investigations of toxicity and safety will need to accompany advances in our understanding of CRISPR-system efficacy to ensure an appropriate risk-benefit profile for therapeutic interventions.

Notwithstanding the promise of RNA-programmed genome editing in somatic cell therapy, a key outstanding issue is whether applications in zygotes and human germline cells should be considered in the light of the associated ethical issues[176,177]. The pace of the science is faster than our grasp of the regulatory ramifications, an issue that is being addressed by the scientific community, together with key stakeholders[178–181]. This is timely, given a recent report of the use of CRISPR–Cas9 for human germline editing[182]. A consensus statement released after the International Summit on Gene Editing in Washington, DC (http://www.nationalacademies.org/gene-editing/Gene-Edit-Summit/index.htm) proposed that pre-implantation genetic diagnosis obviates the need for germline genetic modification in most cases. Exceptions might include couples in which both partners bear homozygous dominant mutations in a disease-causing locus, or recessive rare Mendelian disease. For multigenic diseases, the addition of naturally occurring protective variants could also be a justification for germline editing. As of writing, four countries (Sweden, the UK, Japan and China) have approved research applications of CRISPR-based genome editing in human embryos, with other jurisdictions considering such approval. In the meantime, ongoing clinical trials testing hematopoietic stem cell and iPSC-based therapies[183,184] set the stage for next-generation genome editing therapeutics, and shift the paradigm toward genome-editing-derived alternatives.

Beyond clinical applications (J.A.D.)[185], this disruptive technology is on the brink of transforming agriculture, livestock breeding, food manufacturing and the biotech industries, with uses in plants, animals, bacteria, yeast and fungi for trait enhancement, breeding and fermentation improvements. We note that the diversity of naturally occurring CRISPR–Cas systems in archaea and bacteria, including the widespread type I systems[178], portends an increase in the use of native CRISPR–Cas systems.

The development of CRISPR–Cas9 technologies underscores the contributions of foundational research to biotech. As RNA-programmed genome editing technology matures, it will not only serve as a fundamental component of the biologist's toolkit but could also affect almost every aspect of life, and provide inspiration for future technological breakthroughs.

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The authors declare competing financial interests: details are available in the online version of the paper.

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