

CRISPR-Cas Systems: Prospects for Use in Medicine

Marina V. Zaychikova, Valery N. Danilenko and Dmitry A. Maslov *

Laboratory of Bacterial Genetics, Vavilov Institute of General Genetics, Russian Academy of Sciences, 119333 Moscow, Russia; marinaz15@yandex.ru (M.V.Z.); valerid@vigg.ru (V.N.D.)

* Correspondence: Maslov_da@vigg.ru; Tel.: +7-(499)-135-1239

Received: 27 November 2020; Accepted: 15 December 2020; Published: 16 December 2020

Abstract: CRISPR-Cas systems, widespread in bacteria and archaea, are mainly responsible for adaptive cellular immunity against exogenous DNA (plasmid and phage). However, the latest research shows their involvement in other functions, such as gene expression regulation, DNA repair and virulence. In recent years, they have undergone intensive research as convenient tools for genomic editing, with Cas9 being the most commonly used nuclease. Gene editing may be of interest in biotechnology, medicine (treatment of inherited disorders, cancer, etc.), and in the development of model systems for various genetic diseases. The dCas9 system, based on a modified Cas9 devoid of nuclease activity, called CRISPRi, is widely used to control gene expression in bacteria for new drug biotargets validation and is also promising for therapy of genetic diseases. In addition to direct use for genomic editing in medicine, CRISPR-Cas can also be used in diagnostics, for microorganisms' genotyping, controlling the spread of drug resistance, or even directly as "smart" antibiotics. This review focuses on the main applications of CRISPR-Cas in medicine, and challenges and perspectives of these approaches.

Keywords: CRISPR-Cas; genome editing; gene therapy; drug resistance

1. Introduction

Many human diseases are genetically determined: as of today, over 6000 hereditary diseases are known to be caused by gene and chromosomal DNA mutations, both nuclear and mitochondrial [1]. The treatment of hereditary diseases has been mostly symptomatic, until recently gene therapy has emerged as a fundamentally new approach, aimed at eliminating directly the cause of the disease by correcting mutations (genome editing). CRISPR-Cas systems are the newest and most promising genome editing tools, with the 2020 Nobel Prize in Chemistry awarded for their application in genome editing.

Clustered regularly interspaced short palindromic repeats (CRISPR) are genetic elements that are widely spread among microorganisms, found in 40% bacterial and 90% archaeal genomes [2]. Together with CRISPR associated proteins (Cas), they form CRISPR-Cas systems, being a kind of adaptive prokaryotic immune system, storing information about contacts with foreign DNA (plasmid or viral). The immunity provided by the CRISPR-Cas systems has some features of the higher animals' adaptive immunity, such as specificity, versatility (the ability to provide protection against various foreign elements), and long-term memory [3]. However, unlike animal immunity, bacterial immunity can be inherited.

Short direct repeats (DRs), interspaced with unique regions (spacers), were first described back in 1987 in the *Escherichia coli* genome [4]. They were originally referred to as "short regularly spaced repeats" (SRSRs), but this designation was changed to "clustered regularly interspaced short palindromic repeats" afterwards. Though nothing was known about the function of these elements in the genome at that time, they have become widely used as convenient markers for bacterial

genotyping, since the DR set usually differed within one species. It was only in the 2000s when the role of CRISPR-Cas systems in the bacterial immunity was described [5,6].

These systems were well studied subsequently, with other functions, such as gene expression regulation, DNA repair, and even indirect participation in the process of host infection, described for them aside from providing adaptive immune response [7–12]. CRISPR-Cas systems evolve rather slowly, especially if compared to constantly changing viruses and mobile genetic elements, therefore, having only one function could have been shown to be evolutionarily disadvantageous [13]. Thus, in *Pseudomonas aeruginosa*, these systems have been shown to participate in biofilm formation and virulence [14]. In *Myxococcus xanthus*, Cas proteins are involved in the regulation of the fruiting body formation [15]. For *E. coli*, the involvement of CRISPR-Cas systems in the processes of DNA repair through the interaction of the Cas1 (YgbT) protein with the *recB*, *recC*, and *ruvB* genes, which are key components of the repair systems, has been demonstrated [8]. These systems can also be involved in virulence. In 2013, the involvement of CRISPR-Cas in post-transcriptional control in *Francisella novicida* was established, in which the gene encoding a virulence factor (lipoprotein that induces an inflammatory response in the host) is regulated by the Cas protein (namely Cas9) and CRISPR-associated small RNAs [16]. It is assumed that in this case Cas9 targets endogenous mRNA, rather than DNA. Currently, the relationship between CRISPR-Cas systems and the ability of bacterial strains to exhibit increased virulence or even drug resistance has been shown in a number of studies [17].

Although genome editing (including the medical field) is the most important application of CRISPR-Cas, it is not the only one. The use of these systems in biotechnology for altering metabolic pathways of various prokaryotic and eukaryotic organisms used in industry may be also promising. In addition, CRISPR-Cas can be a tool for bacterial studies, including pathogenic ones. These studies include, but are not limited to: genotyping and epidemiological studies, studying the role of individual genes in the pathogenesis and survival of bacteria (including search for new drugs' biotargets), and for targeted killing of certain bacterial strains, including drug resistant ones.

2. Structure, Classification, and Function of CRISPR-Cas Systems

2.1. Structure

CRISPR-Cas systems are diverse in structure, but include several mandatory elements. They usually consist of two parts: the CRISPR cassette, which consists of a number of unique sequences of about the same length (spacers), separated by short repeating segments (direct repeats, DRs), and a locus of CRISPR-associated genes (*cas*). The spacer sequences in CRISPR cassettes were found in phage genomes, plasmids, and transposons. Spacers are involved in the recognition of foreign DNA. Their length can vary and usually consists of 20–40 nucleotides; the length of direct repeats corresponds approximately to the length of the spacers. The *cas* genes encode proteins with nuclease activity that are involved in the digestion of foreign DNA and its integration into the genome as new spacers. Another obligatory element is a leader sequence [18]. The most important role belongs to the so-called adaptation module. In the majority of CRISPR-Cas systems it consists of two proteins—Cas1 and Cas2—which participate in the integration of new spacers in the CRISPR cassette, complementary to phages' genomes and transposons encountered by the cell and its ancestors during their existence. New spacers are integrated into the cassette next to the leader sequence containing the promoter. The adaptation module is mandatory for the system to function. However the type IV CRISPR-Cas systems are an exception to this rule: they are mostly located on plasmids, prophages, and mobile genetic elements [19]; these systems probably use adaptation modules from type I systems [20]. Cas1 is responsible for the integration of the new spacer, while Cas2 is almost not involved neither in Cas1–Cas2 complex formation, nor in spacer integration. Cas2 represents an mRNA-interferase, which specifically cleaves ribosome-associated mRNA, that appears useless for new spacers integration. There is a hypothesis about the possible origin of Cas2 from the toxin-antitoxin (TA) systems; in this case, it can be assumed that Cas2 retains the ancestral function of endo-RNase, whose activity is reversibly inhibited by interaction with Cas1 and the formation of the Cas1-

Cas2 complex. According to this hypothesis, when CRISPR-Cas systems fail to prevent viral replication in a cell, Cas2 can be activated to stop translation, presumably leading to cell death [21,22].

2.2. Classification

CRISPR-Cas systems are usually classified by the *cas* loci structure. The current classification includes 2 large classes, 6 types, and 33 subtypes [23]. Class 1 (types I, III, and IV, including 16 subtypes) includes CRISPR-Cas systems with multi-subunit effector complexes, while in class 2 systems (types II, V, and VI, including 17 subtypes), all functions of the effector complexes are performed by a single protein, such as Cas9 in type II or Cas12 in type V [23]. Type I, II, and V systems target DNA, type VI systems—RNA, and type III systems target both DNA and RNA [24,25]. Type IV systems are quite unique: as mentioned above, they are mostly located on plasmids, prophages, and mobile genetic elements, and use type I systems' adaptation module for acquiring new spacers. These systems are hypothesized to be involved in competition between plasmids [20]. Most CRISPR-Cas systems can be unambiguously classified into one of six main types. However, there are a number of organisms whose *cas* loci do not fit into the current classification due to their complex modular structure and variability. For example, the target of Cas13d is not DNA, but RNA. Nevertheless, modern approaches that consider many aspects, such as phylogenetic similarity, particularly the phylogeny of *cas1* as an obligatory element of all CRISPR-Cas systems discovered to date, allow them to be classified into the two classes and six types mentioned above. The CRISPR-Cas classification is constantly evolving: back in 2015 it consisted of only 5 classes and 16 subtypes [26]. Considering the exceptional importance of type II CRISPR-Cas systems in biotechnology, it is worth noting that they are quite rare and have been found exclusively in bacterial genomes [23].

The class 2 endonucleases Cas9, Cas12, and Cas13 are widely used as genome editing tools [27]. This finds application in the treatment of genetic diseases, the creation of genetically engineered microorganisms and plants, and the fight against pathogens' drug resistance [28–30]. Currently, these nucleases are among the most used, although they have certain drawbacks. In this regard, there is a constant search for new Cas nucleases.

2.3. Function

The general scheme of the CRISPR-Cas system function includes the following stages: the adaptation phase, during which exogenous nucleic acids enter the organism, are digested and integrated as spacers in the CRISPR cassette; expression phase, when the CRISPR cassette is transcribed to crRNA precursor (pre-crRNA), which is cleaved by RNAses to generate mature crRNA guides; interference phase: mature crRNA is used to target effector complexes that capture and destroy exogenous nucleic acids [18]. There are additional mechanisms, which prevent CRISPR-Cas systems from targeting the cell's own chromosomal DNA. The inviolability of a bacterium's own chromosome is mediated by specific sequences that are present in the bacterial genome with a certain frequency and prevent the use of any parts of the genome as spacers. It has been shown on *Staphylococcus epidermidis* that mismatches between the target and crRNA at certain positions outside the spacer sequence cause digestion of foreign DNA, while the coincidence between crRNA and CRISPR-repeats on the bacterial chromosome prevents its cleavage. This kind of differential complementarity beyond the spacer sequence is characteristic to all the CRISPR-Cas systems [31].

To acquire new spacers for type I, II, and V CRISPR-Cas systems, exogenous DNA must be marked with a special DNA motif (protospacer adjacent motif, PAM), numbering from 3 to 8 nucleotides. Only the region adjacent to the PAM can become a protospacer (a section of exogenous DNA that will be included in the CRISPR cassette in the form of a spacer). PAMs are also important in the interference phase. PAMs are required to prevent the system from targeting the cell's own DNA, which would lead to its destruction [32]. Due to the presence of multiple PAMs in the genome of an exogenous element, the encounter of the same agent by different cells may lead to the acquisition of different spacers. This makes certain evolutionary sense, since a mutation in the viral genome at the protospacer site can make such a spacer useless.

3. Genome Editing

The most important area of application of CRISPR-Cas is genome editing—the targeted modification of DNA, such as deletion, insertion, and translocation of individual genes, and introduction of point mutations (SNPs), including reversed mutations to correct the ones that previously appeared.

Currently, there is a number of other tools also used for this purpose, besides CRISPR-Cas: oligonucleotide directed mutagenesis (ODM), TALEN systems (transcription activator-like effector nucleases), and ZFN (zinc finger nuclease, “zinc fingers”) [33,34].

3.1. Early Genome Editing Systems

TALEN nucleases were first discovered in the pathogenic bacterium *Xanthomonas* [35]. TALE proteins contain about 30 amino acid repeats flanking the DNA-binding region. The region of DNA binding is called RVD and consists of repetitive variable di-residues capable of specific DNA binding. Based on these proteins, chimeric nucleases have been developed, including the TALE and FokI nuclease domains [36].

ZFN are proteins, which include the so-called zinc fingers, which are a structural protein motif comprising two zinc ions linked to two histidines and cysteines (Cys2-His2). ZFNs were also fused to the nonspecific FokI endonuclease domain to obtain zinc finger proteins (ZFPs) [37]. ZFPs require a specific site, located at a 200 bp distance from the cleavage site. The choice of these sites is limited. This technique is suitable for deleting or knocking out a gene, but cannot be used for gene insertion or correction.

TALEN and zinc fingers have several disadvantages (in particular, low transfection efficiency) but are still used. TALEN, which are more specific and less cytotoxic than ZFN, are difficult to pack into viral delivery systems due to their large size [38].

CRISPR-Cas systems, in turn, due to their efficiency, accuracy, and wide range of applications, are recognized as the most promising genome editing technology [39]. Their specificity and targeting are determined only by RNA sequence, the design of which is much simpler than that of a protein. CRISPR-Cas systems also allow targeting multiple genes simultaneously. This is a significant advantage over TALEN and ZFN.

3.2. Mechanism of Genome Editing

Genomic editing systems based on Cas9 nuclease are currently the most widely used, since this system requires only this protein, in contrast to complex multiprotein complexes that are necessary for the functioning of other CRISPR systems. Cas9 from *Streptococcus pyogenes* (Cas9_{spy}) is the most frequently used Cas9 nuclease. However, in some cases, *Streptococcus thermophilus* nuclease is also used. Cas9 nuclease contains two functional domains—HNH and RuvC. The first one cleaves the target DNA strand, while the second domain cuts the complementary strand [40]. The CRISPR-Cas9 is a class 2, type II system. It includes the Cas9 nuclease, the trans-activating CRISPR RNA (tracrRNA), and crRNA. crRNA is formed during the processing of precrRNA, transcribed from the CRISPR cassette. precrRNA is cut into crRNAs, consisting of spacers and their adjacent DRs. crRNA in complex with tracrRNA guides Cas9 (the so-called interference module) to target specific sequences on the exogenous DNA. crRNA and tracrRNA can be fused into a chimeric single guide RNA (sgRNA) [41–43]. The specificity of targeting in this case is determined by both the complementarity of sgRNA and the target DNA, and by PAM motifs. The complex also requires RNase III processing for its function. The ability to use sgRNAs instead of separate crRNAs and tracrRNAs is more convenient for genome editing and represents another advantage of Cas9.

The Cas9/sgRNA complex scans the DNA sequence until PAM is found. Then DNA uncoiling occurs in the so-called priming region (10 bp immediately after the PAM) [44]. If the DNA sequence is complementary to the sgRNA sequence, the Cas9 HNH-domain introduces a break in the target strand, and the RuvC-domain—in the non-target strand [45,46]. Thus, CRISPR-Cas9 is capable of introducing double-stranded breaks (DSBs) at target sites in genomic DNA, resulting in a gap [47].

The introduced gap must be repaired afterwards. In eukaryotic genome editing techniques, this may be done by the non-homologous end-joining (NHEJ). In this case, the cut DNA ligation occurs without a homologous DNA matrix, either directly or with the introduction of an insertion or deletion. This method has a number of drawbacks, primarily consisting in the significant frequency of insertions and deletions leading to frameshifts and a loss of gene functionality [48]. However, this property of NHEJ can be used to target gene knockout. NHEJ can be also used to completely remove a target gene.

Another mechanism is homologous directed repair (HDR), which involves replacing a deleted sequence with a new sequence complementary to a specifically introduced DNA template. Its advantage is high accuracy, while its disadvantage is low frequency (less than 20%, while NHEJ is up to 60%) [49]. The search for ways of increasing HDR frequency is very important for further development of this area. HDR can be used both for introducing and reverting target mutation in the genome, and for inserting whole genes in the genome using a template [50,51].

One of the drawbacks of HDR consists of occasional NHEJ repair that occurs despite the presence of the template. Specialized NHEJ inhibitors are used to override this, but they can have a cytotoxic effect [52,53].

3.3. CRISPR-Cas9 Delivery Systems

CRISPR-Cas9 can be delivered to the organism as a plasmid harboring genes encoding the entire editing system, or as a Cas9 protein in a complex with a guide RNA (gRNA), and in some cases as mRNA [54].

Microinjection is suitable for delivering all three forms and is considered the safest and most effective method [55]. The advantage of the method is the lack of restrictions on the size of the injected component, but it is not suitable for in vivo application, since it requires injection directly into the cell.

Another delivery option, quite versatile like microinjection, is electroporation, which uses an electric pulse to produce pores in the cell membrane. Electroporation is mostly used with bacterial cells, since eukaryotic cells are more sensitive to electric stress [51].

Viral delivery systems based on adenoviruses, lentiviruses, and adeno-associated viruses (AAV), and phage-delivery systems are also quite popular. Phages are capable of specifically infecting bacteria, including drug-resistant strains, and replicating in them. Adenoviral delivery systems have a number of advantages, such as low immunogenicity, almost no risk of integration into the human genome, but their size imposes certain restrictions on the size of the packaged product. The main tool for genome editing, Cas9, is quite large, which creates certain difficulties when packing into a virus or a phage. Cas9 from *Staphylococcus aureus* (Cas9_{Sau}) is significantly smaller (by 1000 bp) than Cas9_{Spy}, thus it is more convenient for packaging into AAV, while its efficiency is comparable to Cas9_{Spy} [56].

Other delivery systems also include various organic and inorganic nanoparticles (lipid and gold nanoparticles), mesoporous silica particles. Delivery using nanoparticles has both advantages and disadvantages. Nanoparticles are safer than viral ones, but their efficiency is much lower. In addition, as with viral delivery systems, there is a limit to the size of the “cargo” that can be delivered with their help [51].

3.4. Application of CRISPR-Cas in Biotechnology

Genome editing using CRISPR-Cas systems has promising prospects for applications in genetic engineering of both pro- and eukaryotes, and in medicine. Currently, they are used in gene surgery, in the production of new drugs, in the study of functional interaction of genes, in the creation of transgenic microorganisms, animals, plants, and cell lines [57].

In agriculture, CRISPR-Cas is widely used for genetic modification of plants and animals, including for imparting medicinal properties to food. Using the pYL/CRISPR-Cas9 multiplex vector system, tomatoes with an increased content of gamma-aminobutyric acid, which is involved in the brain metabolic processes were obtained [58]. Targeted introduction of mutations in the *SBEI* and *SBEIIb* genes in rice allowed the production of grain with increased starch and amylase content, and thus having a greater nutritional value [59]. A breed of transgenic sheep has been also created,

expressing *AANAT* and *ASMT* genes in mammary gland cells, thus producing milk enriched with melatonin—a powerful antioxidant and a regulator of the circadian rhythm [60].

3.5. Development of Animal Models

Animal genome editing is also used to create animal lines that can be models for various human diseases. In this case, a gene may be edited (point mutation introduction), added or knocked out.

Indels in rat's *p53* (a transcription factor that regulates the cell cycle and is a key tumor suppressor) and *lkb1* genes, leading to loss of their functions, and a point G12D mutation in the *kras* gene made it possible to model adenocarcinoma [61]. The HTT gene knockin has resulted in pigs with Huntington's disease clinical manifestation [62]. Currently successful models of atherosclerosis (pig), retinal dystrophy (rat), glioblastoma (mouse), and deafness (mouse) have already been developed [63–65].

As genomic editing using CRISPR-Cas is less time consuming than TALEN and ZFN, it allowed a scientific group from China to obtain over 17 model lines of rabbits, including models of congenital cataract, ectodermal dysplasia, Duchenne muscular dystrophy (DMD), deafness, X-linked hypophosphatemia (XLH), etc., since 2016 [66].

Genome editing in cell lines is also applied for this purpose, since it is even less time-consuming due to higher in vitro editing efficiency as compared to in vivo procedures [67]. In particular, models of colorectal cancer were developed based on human intestinal stem cells (hISCs) by editing *APC*, *P53*, *KRAS*, and *SMAD4* genes [68].

3.6. Application in Gene Therapy

CRISPR-Cas systems are of considerable interest in the treatment of inherited disorders. Gene therapy implies making changes (correcting mutations, adding genes, or altering their expression) in the genome in order to cure diseases or eliminate a predisposition to them. It can be carried out both at the stage of embryo formation (fetal gene therapy) or in somatic cells of an adult patient. Fetal gene therapy has both pros (the introduction of corrective changes at an early stage leads to their transmission to almost all body cells) and cons—even a single mistake, caused by the off-target effect of the currently implied CRISPR-Cas systems, made at this stage can become fatal. Thus, the application of state-of-the-art genome editing techniques for fetal gene therapy raises multiple ethical questions.

However, in 2018, fetal gene therapy was performed to confer HIV resistance by modifying the CCR5 receptor. The two children, born as a result of the experiment, were reported to be healthy and have an innate resistance to HIV, but these results have caused fierce polemics and both their ethical and scientific aspects are still debated [69].

In this respect, somatic cell therapy does not present such an ethical dilemma. As of today, the treatment of inherited disorders such as hemophilia, Duchenne muscular dystrophy, and rhinitis pigmentosa has been carried out on model animals [70–74].

Mutations in the β -globin (*HBB*) gene lead to thalassemia, a type of anemia. It can be cured either by editing *HBB* using CRISPR-Cas9 or by reactivating fetal hemoglobin (*HbF*) expression by knocking out the *BCL11A* gene, which suppresses *HbF* expression [75,76].

The sickle-cell anemia, an inherited blood disease, upon which the normal hemoglobin A due to an SNP (L-glutamic acid in position 6 of the β -chain globin is substituted for L-valine) acquires a special shape, hemoglobin S, prone to crystallization instead of the formation of normal quaternary structure, can also be treated with CRISPR-Cas9 genome editing [75].

The use of CRISPR-Cas is also very promising in cancer therapy. Cancer is currently the second leading cause of death after cardiovascular diseases, and is quite often not responding well to chemotherapy [77].

On the one hand, CRISPR-Cas can be used to overcome acquired drug resistance in tumors; on the other hand, CRISPR-Cas can be used directly for targeted knock-out of oncogenes—genes whose expression can stimulate the formation of a malignant tumor. When proto-oncogene (a common gene

capable of converting into an oncogene) becomes an oncogene as a result of mutations, CRISPR-Cas can precisely target malignant cells [78].

In addition, a number of cancers are associated with viruses, such as the human papillomavirus (HPV), which may cause cervical cancer. The HPV16 genes E6 and E7 are responsible for viral replication in epithelial cells and have oncogenic activity by activating the telomerase and suppressing the antitumor suppressor pRB [79]. Specific sgRNAs targeting the E6 and E7 genes blocks their expression and leads to the destruction of the virus [80].

However, genome-edited cells themselves were reported to be able to undergo malignant transformation. CRISPR-Cas-mediated DSBs were shown to activate the p53 signaling pathway in two independent studies [81,82].

Although there is still no direct evidence to support the link between CRISPR-Cas-mediated genome editing and carcinogenesis, further research is required to eliminate even minimal risks before genomic editing can be applied to humans.

Current successful applications of genome editing in humans include the use of TALEN in the treatment of CD19+ acute lymphoblastic leukemia in an 11-month-old infant, and ZFNs for the treatment of type II mucopolysaccharidosis (Hunter's syndrome) [83,84].

A proper improvement of the CRISPR-Cas editing system, aimed at lowering the risk of off-target action, may open up broad prospects for personalized medicine with application in gene therapy, including fetal therapy, development of highly specific model systems, etc.

4. CRISPR-Cas Implementation to Control Human Infectious Diseases

The mortality rate from infectious diseases in the world is constantly increasing. Therapy for infectious diseases includes both treatment and prevention. Some of them (measles, polio, tetanus, etc.) can be prevented through vaccination, while the main approach in infectious diseases' treatment involves application of drugs specific to the pathogen [85,86]. Besides drugs, targeting the pathogen directly, bacteriophages (phage therapy), blood preparations (hemotherapy), gammaglobulins, or immunoglobulins (serotherapy) are also used [87–89]. In addition, timely diagnosis is of great importance.

Problems associated with drug therapy can be associated with both the patient (side effects and contraindications) and pathogen (drug-resistance) [90]. Another obstacle of drug use is the possible lack of specificity of new drugs to pathogens. Human symbiotic microflora is also often damaged by antibiotic therapy, while part of the pathogen population usually survives due to drug-resistance or persistence. One of the approaches to combat drug resistance and a nonspecific effect of drugs on symbiotic microflora is to search for new potential targets for the development of antibacterial agents [91].

Attempts to establish the relationship between the phenomenon of drug resistance and CRISPR-Cas have been made for a long time. At first glance, it may seem that there is no direct connection between the systems of adaptive immunity and drug resistance in microorganisms. However, CRISPR-Cas is involved in the development of antibiotic resistance. For the first time, an inverse relationship between drug resistance and the CRISPR-Cas system was established in enterococci with two systems: CRISPR1-Cas, CRISPR3-Cas, and additionally a single CRISPR2 cassette lacking *cas* genes. Drug resistance genes in enterococci are often located on transposons, which are actively transmitted in the bacterial population by conjugation and on plasmids, some fragments of which may be included as CRISPR spacers in the cassette. Cleavage of plasmid DNA upon entry into a cell thus prevents the pathogen from acquiring resistance [92]. A similar situation is typical for *Klebsiella pneumoniae* [93]. In addition, in a number of microorganisms, strains lacking CRISPR-Cas systems more actively form biofilms, which provide additional protection against antibiotic penetration. In *Enterococcus faecalis*, which is the cause of infection in dental canals, a gene, encoding a surface aggregation factor cytolysin (a virulence factor, which interacts with host immune system), is often located in prophages and pathogenicity islands (PAI), and is able to be transmitted in the population similarly to the drug resistance determinants. The loss of the CRISPR cassette results in an increased chance of acquiring mobile genetic elements carrying those genes [94].

In 2017, mutations in the genes of the adaptation module (*cas1* and *cas2*) were shown to be associated with drug-resistance emergence in the intestinal pathogen *Shigella*. These data are experimental, and there is currently no explanation of this phenomenon [95]. CRISPR-Cas is involved in the formation of the cell wall in *Francisella novicida* through BLP regulation. This leads to the development of antibiotic tolerance [16].

However, despite the fact that the CRISPR-Cas system could be involved in the development of drug resistance, they do not represent interest as drug targets, because, despite their essential role in maintaining bacterial immunity, they are not essential for cell survival; moreover, CRISPR-Cas can have structural variants within the same species and differ in different strains.

On the other hand, the use of CRISPR-Cas systems seems promising as a tool for combating drug resistance, since the field of their application is very wide.

4.1. Application of CRISPR-Cas in Genotyping

Bacterial genotyping is an important tool for epidemiological research and is indispensable for investigating outbreaks of infectious diseases, tracking their pathways, and identifying sources. Genotyping is also important for evolutionary research.

Even before the description of the immune function of the CRISPR-Cas systems, direct repeats were used for genotyping (spoligotyping) of *Mycobacterium tuberculosis* clinical isolates [96]. This method is based on the analysis of polymorphism of the CRISPR locus (formerly DR): PCR products obtained using primers complementary to DRs are hybridized with a set of synthetic oligonucleotides covalently linked to a nitrocellulose membrane. The hybridization pattern serves as a characteristic (spoligotype) of each strain. Spoligotyping has long been considered the “gold standard” in *M. tuberculosis* genotyping.

Besides *M. tuberculosis*, spoligotyping is successfully used for genotyping *Corynebacterium diphtheriae*, the causative agent of diphtheria [97], *Yersinia pestis*, the causative agent of the plague, *Salmonella* and *Campylobacter jejuni* [98].

There are other genotyping approaches based on CRISPR locus analysis. Real-time PCR is used for identification of certain spacers characteristic to the so-called the Big Six *E. coli* serotypes (O26:H11, O45:H2, O103:H2, O111:H8, O121:H19, O145:H28, and O157:H7), the most clinically significant and common ones in Europe and the USA [99].

Additionally, typing can be done based on the length of CRISPR arrays, which is directly related to the number of spacers. This approach was used for *Yersinia pestis* and *Salmonella* [98].

4.2. Application of CRISPR-Cas in Diagnostics

CRISPR-Cas can also be used to diagnose infectious diseases. Cas13 nuclease, which can cleave not only the target sequence, but also nearby off-target RNAs, was used for this purpose. The SHERLOCK (specific high sensitivity enzymatic reporter unlocking) platform has been developed for in vitro nucleic acid detection, based on this property of Cas13. It includes the Cas13a nuclease, sgRNA targeting specific RNA sequences, and fluorescent RNA reporters. After Cas13a cleaves the target RNA, it cuts off the reporter RNA, releasing a detectable fluorescent label [100,101]. Viruses, pathogens and some specific mutations characteristic of tumor cells can be identified by this system.

The DETECTR (DNA endonuclease-targeted CRISPR trans reporter) platform was created for the detection of nucleic acids, based on Cas12a nuclease, which has similar activity. DETECTR can be used to diagnose the HPV16 and HPV18 subtypes of human papillomaviruses associated with cervical cancer [102].

These detection methods look very promising, however commercial diagnostic kits are currently not available.

4.3. Application of CRISPRi to Study Metabolic Pathways and Search for New Drugs' Biotargets

It has been shown, that the introduction of two point mutations in Cas9 amino acid sequence (D10A and H840A) leads to a complete loss of endonuclease activity, while maintaining its ability to

recognize and bind to target DNA sequence. This mutant is referred to as dCas9, or nuclease dead Cas9. Targeting dCas9 with specific sgRNAs to a gene's promoter region prevents the RNA polymerase from doing the same, thus interfering with the transcription. This approach was named CRISPR interference (CRISPRi) [103]. CRISPRi can be used to conditionally knock-out almost any gene with high specificity. A simultaneous expression of different sgRNAs may be used for a knock-out of multiple genes [104]. Chimeric dCas9, fused to various transcription repressor domains, may be used to increase the efficiency of repression. The use of the Krüppel-associated box domain (KRAB domain) of the Kox1 protein was shown to provide the highest efficiency of transcription repression (up to 99%) [105,106].

CRISPRi can be used not only to downregulate, but also to upregulate a gene's expression. dCas9 can be fused to transcription activator domains to form the so-called CRISPRa. CRISPRa allows activating multiple genes by using multiple sgRNAs. Using multiple transcription activators simultaneously (synergistic activation mediators, SAM), such as VP64 (a tandem of four copies of the viral herpes simplex protein V16), p65AD (transactivation domain of the NF- κ B subunit p65), and Epstein–Barr virus transactivator R (Rta) (VPR) increases CRISPRa efficiency [106,107].

Besides gene expression control, dCas9-based systems can also be used for genome editing. Hybrid dCas9, supplemented with modified adenosine deaminase, is able to point-convert A/T base pair to a G/C pair with a 50% efficiency. In this case, a non-functional nuclease is used for precise targeting, while adenosine deaminase is used directly for base modification without introducing DSBs [108].

CRISPRi and CRISPRa can also be used as genetic screening tools, especially in the development of new drugs, when it is necessary to establish the biotarget and possible factors mediating resistance for the candidate compounds selected during the initial screening [109]. CRISPRi is a powerful tool for discovering new genetic pathways, functional genes' studies, including determining their role in drug resistance development. Cell screening using a random CRISPR library allows positive selection of cells in the population that have a fitness advantage, such as drug resistance. gRNAs from selected cells are analyzed to detect the genes conferring this advantage. In contrast, negative selection can result in finding vital genes–biotargets for further drug development [110].

CRISPRi is an alternative to RNA interference (RNAi) based on double-stranded synthetic small RNA, complementary to a certain gene and designed to reduce the level of expression of this gene, widely used previously. The advantage of CRISPRi is its ability to almost completely knock-down a gene's expression, while RNAi can only reduce it [111].

CRISPRi systems are currently mainly used in prokaryotes to study metabolic pathways for novel biotargets' identification. However, it can also be used directly for disease treatment. In 2020 a doxycycline-induced system was successfully used to treat epilepsy in a rodent model. An increase in the expression of the Kcna1 potassium channel gene resulted in a significant decrease in neuronal excitability and, as a consequence, in relief of epilepsy symptoms [112]. Progress has been made in the immunotherapy of malignant diseases through the activation of several genes involved in an antitumor response (multiplexed activation of endogenous genes as an immunotherapy, MAEGI) [113].

4.4. Application of CRISPR-Cas to Combat Drug-Resistant Microorganisms

CRISPR-Cas has the potential to be used as antimicrobial agents for targeted cleavage of antibiotic resistance genes located on plasmids. When targeting drug resistance genes with plasmid localization, CRISPR-Cas systems cannot be considered a drug themselves. Nevertheless, such an approach, aimed to reduce the spread of drug-resistant strains, rather than to destroy the pathogen itself, makes sense, since an attempt to completely eliminate pathogenic microorganisms from their niches can lead to unpredictable consequences [30]. Drug-sensitive strains are put under the pressure of positive selection, and resistant ones, under negative selection. The main goal of introducing this technique is to remove drug resistance genes from the population and make it susceptible to drugs [114]. However, intended or accidental targeting of the bacterial genome sequence by the CRISPR-Cas system leads to cell death due to the appearance of irreversible chromosomal damage [115].

In 2014 two groups showed that efficiently delivered RNA-guided nucleases can be used either for pathogen drug sensitization, or for its direct killing (depending on their targeting) both in vitro and in vivo [116,117]. Bikard et al. used a CRISPR-Cas9 system delivered by the NM1 phage to destroy 99% of plasmids carrying antibiotic resistance genes, resulting in significant antibiotic sensitization of *Staphylococcus aureus*. Targeting genes on the chromosome had a bactericidal effect, which was also confirmed in vivo in the mouse skin colonization model [116]. The second study, reported by Citorik et al. implied two approaches to deliver the Cas9 nuclease targeting blaNDM-1 and blaSHV-18 (provide resistance to beta-lactam antibiotics): an M13-based phasmid and a conjugative plasmid. The use of a phage delivery system resulted in a 2–3-fold decrease in the number of viable enterohemorrhagic *E. coli* cells. It was additionally shown that the CRISPR-Cas system could selectively target drug-resistant strains. A phasmid, targeting the *gyrA* gene (responsible for fluoroquinolones resistance), was cytotoxic only for quinolone-resistant cells with a mutation in the *gyrA* gene while being inactive against cells with the wild-type *gyrA*. This system worked as well in vivo in a *Galleria mellonella* infection model [117].

CRISPR-Cas may be considered an alternative to conventional antituberculosis chemotherapy. Currently administered drugs are effective against drug-susceptible *Mycobacterium tuberculosis* strains; however, the number of drug-resistant tuberculosis cases is constantly growing. *M. tuberculosis* lacks horizontal gene transfer and its drug resistance is mediated by mutations in genes encoding drug biotargets, activators of prodrugs, and efflux pumps' transcriptional regulators [118]. *M. tuberculosis* possesses a subtype III-A CRISPR-Cas system (comprising the *cas1*–*cas2* adaptation module, six *csn* genes, *cas6* gene responsible for crRNA processing, and two CRISPR cassettes), allowing an easy implementation of exogenous type II (Cas9) and type V (Cas12a) systems [119–121], furthermore a large number of known mycobacteriophages provides a good source for selecting specific and effective delivery systems [122].

While RNA-guided antituberculosis agents have not yet been reported, several CRISPRi systems were developed for mycobacteria, with the one based on *Streptococcus thermophilus* dCas9 (dCas9^{StH1}) being the most efficient one [104]. These systems are mainly used for search of novel drug targets [123].

Thus, CRISPR-Cas systems are emerging as powerful tools for combating the emergence of drug resistance. Their key features are high specificity, which allows them to be used to combat only a specific pathogen, without affecting the host microbiome, and the low chance of developing resistance to themselves due to the possibility of aiming several targets simultaneously.

5. Alternative Cas Nucleases for Genome Editing

Cas9 precision and efficiency depend upon many factors, such as: the guide and target sequence [124], DSB repair mechanism (NHEJ or HDR template DNA insertion, see Section 3.2) and delivery system (see Section 3.3). Moreover, cytotoxicity is also case-specific for Cas9-based systems. For example, overexpression of Cas9^{Sp} and its catalytically inactive modification dCas9 in *Corynebacterium glutamicum* lead to cells death even without gRNA expression, which creates limitations for its use as a tool for bacterial genome editing [125].

A number of CRISPR-Cas9 modifications have been lately developed. As mentioned above, dCas9 can be fused to FokI nuclease domain to increase its specificity [126], or even fused to ZFPs or TALENs [127]. Besides engineering Cas9 to improve its efficiency and specificity, another major area of research focuses on the search for alternative Cas nucleases.

One of these is the RuvC-like nuclease Cpf1 found in *Prevotella* and *Francisella*. Cpf1 is a type V CRISPR-Cas nuclease, being the distinctive feature of this class. The main difference of Cpf1 from Cas9 is its ability to cut target DNA forming sticky ends. This increases the efficiency of DNA repair with a homologous template [128]. Cpf1, also known as Cas12a, is currently the most promising tool for mycobacteria genome editing [129].

Since the specificity of RNA-guided CRISPR nucleases is determined by both the protospacer sequence and the PAM motif, this imposes certain limitations, important for genomic editing using CRISPR-Cas [32,43]. The effectiveness mostly depends on the positioning accuracy of the double-

stranded DNA break. In the case of CRISPR-Cas9, PAM consists of three nucleotides 5'-NGG-3' (or 5'-NAG-3') [32]. Therefore, in order to increase the number of possible target sites for DSB introduction, it is desirable to have additional CRISPR nucleases with different PAMs in the arsenal. The Cpf1 PAM is 5'-TTTN-3', which expands the range of protospacers that can be used for CRISPR-mediated gene editing. Cpf1 crRNA does not require a tracrRNA and directly functions as a gRNA, which simplifies its use [130]. Cpf1 (Cas12a) also lacks cytotoxicity in several bacteria, and was shown to be effective for genome editing not only in mycobacteria, but in *C. glutamicum* as well [131].

Another alternative is the Cas13 effector protein, which is characteristic of class 2 type VI systems, which has properties that are unique among all CRISPR proteins [25]. Among Cas13, four protein families have been identified: Cas13a, Cas13b, Cas13c, and Cas13d [132]. Instead of the usual Cas domains, Cas13 has two domains, one of which is homologous to the eukaryotic, and the other to the prokaryotic nucleotide-binding domain (HEPN). Together they form ribonuclease site [133]. The CRISPR-Cas13 system includes the Cas13 RNA-guided RNase and a short crRNA that recognizes the target RNA sequence.

6. Potential Challenges in Using CRISPR-Cas Systems

Despite the relatively high efficiency of CRISPR-Cas as a tool for genome editing, it has a number of potential disadvantages and risks. These include, in particular, the use of the NHEJ, leading to a high probability of mutations (see above), and the off-target effect also resulting in editing mistakes [57,134,135]. Though the frequency of such off-target mutations is quite low, allowing to use CRISPR-Cas systems for plants and microorganisms genome editing, they nevertheless represent the most serious problem in gene therapy, since they can lead to unpredictable short-term and long-term consequences [136]. These limitations can be overcome by ex vivo gene therapy approaches [137], while in vivo genome editing in humans, especially fetal gene therapy and manipulations with germ line cells, may still raise ethical questions [138].

The Cas9 nuclease, as mentioned earlier, possesses its own cytotoxic effect on certain microorganisms [139]. These difficulties can be overcome by using endogenous nucleases, when working with those microorganisms, which possess them. Endogenous nucleases are also used when working with genomes of extremophilic bacteria or archaea, since mesophilic bacterial nucleases do not work well in these cells [140].

Another problem of using CRISPR-Cas, especially when using them as antibacterial agents, is their delivery. RNA-guided Cas nucleases targeting plasmid-located virulence and drug-resistance genes can be delivered to microbial populations using bacteriophages or bacteria carrying conjugative plasmids.

Phage delivery systems are a promising tool in the fight against drug resistance, but they have several disadvantages. Despite the difficulties encountered by bacteria in developing resistance to the injected CRISPR-Cas system itself, they can easily develop phage resistance. A narrow species-specificity of a phage, that on the one hand makes it a convenient tool for CRISPR-Cas delivery to a specific microorganism, on the other hand limits its use for other bacteria, thus a search for various phage-delivery systems is required.

As phages themselves are the natural targets of CRISPR-Cas systems, some of them have anti-CRISPR proteins (Acrs) that target various components of the effector complexes. They are usually highly-specific to certain CRISPR-Cas systems [141–143]. The anti-CRISPR proteins are relatively small (not exceeding 150 amino acid residues). The expression of genes encoding these proteins is regulated by the Aca1 transcription factor, the presence of which is mandatory for all anti-CRISPR systems. The mechanism of action of this system has not yet been fully elucidated, and only six anti-CRISPR proteins have been studied. AcrF1 and AcrF2 form a complex with Cas-proteins and RNA, preventing its binding to phage DNA. AcrF3 inhibits Cas3 activity, also by forming a non-functional complex. AcrIIC1 inhibits the Cas9_{spy} nuclease, which can be implied in gene surgery and engineering to regulate CRISPR-Cas activity in certain tissues or at certain stages of the organism's life cycle [143].

7. Conclusions

CRISPR-Cas systems are currently the most promising tool for genome editing, but their application is far from being limited only to genome editing.

CRISPRi systems, based on dCas9 protein depleted of nuclease activity are currently widely used for prokaryotic genes' functional studies and identification of novel metabolic pathways for new biotargets identification. This system is promising as well for treating many human diseases, including autoimmune, cardiovascular, and oncological diseases, which are caused by incorrect expression of a number of genes. In this respect, the use of dCas9, devoid of the risk of introducing off-target mutations, to regulate target genes' expression may be an alternative to genome editing by Cas9_{spy}.

The evolution of microorganisms is rapidly moving towards the acquisition of mutations in genes encoding drug targets, and the genes of drug resistance themselves, through horizontal gene transfer, nullifying all the efforts of developing novel antimicrobial agents. CRISPR-Cas represents an alternative to traditional drug therapy. These systems can soon become the basis for the development of "smart" antibiotics, which may be species- or even strain-specific. This approach may be also of interest for the correction of human gut microbiota. However, it should be noted that CRISPR-Cas systems need much improvement to minimize the off-target effect and increase their efficiency. Search for new Cas-nucleases, new PAMs, and enhanced delivery systems is of great importance.

Author Contributions: Conceptualization, D.A.M.; writing—original draft preparation, M.V.Z. and D.A.M.; writing—review and editing, D.A.M. and V.N.D.; supervision, V.N.D.; project administration, D.A.M.; funding acquisition, D.A.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Russian Foundation for Basic Research (RFBR), grant number 20-315-70025.

Acknowledgments: We would like to thank Olesya Maslova for carefully reading the manuscript and providing useful comments.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Jackson, M.; Marks, L.; May, G.H.W.; Wilson, J.B. The genetic basis of disease. *Essays Biochem.* **2018**, *62*, 643–723.
2. Grissa, I.; Vergnaud, G.; Pourcel, C. The CRISPRdb database and tools to display CRISPRs and to generate dictionaries of spacers and repeats. *BMC Bioinform.* **2007**, *8*, 172.
3. Marraffini, L.A.; Sontheimer, E.J. CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. *Nat. Rev. Genet.* **2010**, *11*, 181–190.
4. Ishino, Y.; Shinagawa, H.; Makino, K.; Amemura, M.; Nakata, A. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*, and identification of the gene product. *J. Bacteriol.* **1987**, *169*, 5429–5433.
5. Bolotin, A.; Quinquis, B.; Sorokin, A.; Ehrlich, S.D. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology* **2005**, *151*, 2551–2561.
6. Barrangou, R.; Fremaux, C.; Deveau, H.; Richards, M.; Boyaval, P.; Moineau, S.; Romero, D.A.; Horvath, P. CRISPR provides acquired resistance against viruses in prokaryotes. *Science* **2007**, *315*, 1709–1712.
7. Westra, E.R.; Buckling, A.; Fineran, P.C. CRISPR-Cas systems: Beyond adaptive immunity. *Nat. Rev. Microbiol.* **2014**, *12*, 317–326.
8. Babu, M.; Beloglazova, N.; Flick, R.; Graham, C.; Skarina, T.; Nocek, B.; Gagarinova, A.; Pogoutse, O.; Brown, G.; Binkowski, A.; et al. A dual function of the CRISPR-Cas system in bacterial antiviral immunity and DNA repair. *Mol. Microbiol.* **2011**, *79*, 484–502.
9. Heussler, G.E.; Cady, K.C.; Koeppen, K.; Bhujju, S.; Stanton, B.A.; O'Toole, G.A. Clustered Regularly Interspaced Short Palindromic Repeat-Dependent, Biofilm-Specific Death of *Pseudomonas aeruginosa* Mediated by Increased Expression of Phage-Related Genes. *MBio* **2015**, *6*, e00129.

10. Gong, T.; Zeng, J.; Tang, B.; Zhou, X.; Li, Y. CRISPR-Cas systems in oral microbiome: From immune defense to physiological regulation. *Mol. Oral Microbiol.* **2020**, *35*, 41–48.
11. Tang, B.; Gong, T.; Zhou, X.; Lu, M.; Zeng, J.; Peng, X.; Wang, S.; Li, Y. Deletion of cas3 gene in *Streptococcus mutans* affects biofilm formation and increases fluoride sensitivity. *Arch. Oral Biol.* **2019**, *99*, 190–197.
12. Wimmer, F.; Beisel, C.L. CRISPR-Cas Systems and the Paradox of Self-Targeting Spacers. *Front. Microbiol.* **2019**, *10*, 3078.
13. Touchon, M.; Charpentier, S.; Clermont, O.; Rocha, E.P.C.; Denamur, E.; Branger, C. CRISPR distribution within the *Escherichia coli* species is not suggestive of immunity-associated diversifying selection. *J. Bacteriol.* **2011**, *193*, 2460–2467.
14. Vasquez-Rifo, A.; Veksler-Lublinsky, I.; Cheng, Z.; Ausubel, F.M.; Ambros, V. The *Pseudomonas aeruginosa* accessory genome elements influence virulence towards *Caenorhabditis elegans*. *Genome Biol.* **2019**, *20*, 270–22.
15. Viswanathan, P.; Murphy, K.; Julien, B.; Garza, A.G.; Kroos, L. Regulation of dev, an operon that includes genes essential for *Myxococcus xanthus* development and CRISPR-associated genes and repeats. *J. Bacteriol.* **2007**, *189*, 3738–3750.
16. Sampson, T.R.; Saroj, S.D.; Llewellyn, A.C.; Tzeng, Y.-L.; Weiss, D.S. A CRISPR/Cas system mediates bacterial innate immune evasion and virulence. *Nature* **2013**, *497*, 254–257.
17. Louwen, R.; Horst-Kreft, D.; de Boer, A.G.; van der Graaf, L.; de Knecht, G.; Hamersma, M.; Heikema, A.P.; Timms, A.R.; Jacobs, B.C.; Wagenaar, J.A.; et al. A novel link between *Campylobacter jejuni* bacteriophage defence, virulence and Guillain-Barré syndrome. *Eur. J. Clin. Microbiol. Infect. Dis.* **2013**, *32*, 207–226.
18. Wang, F.; Wang, L.; Zou, X.; Duan, S.; Li, Z.; Deng, Z.; Luo, J.; Lee, S.Y.; Chen, S. Advances in CRISPR-Cas systems for RNA targeting, tracking and editing. *Biotechnol. Adv.* **2019**, *37*, 708–729.
19. Faure, G.; Shmakov, S.A.; Yan, W.X.; Cheng, D.R.; Scott, D.A.; Peters, J.E.; Makarova, K.S.; Koonin, E.V. CRISPR-Cas in mobile genetic elements: Counter-defence and beyond. *Nat. Rev. Microbiol.* **2019**, *17*, 513–525.
20. Pinilla-Redondo, R.; Mayo-Muñoz, D.; Russel, J.; Garrett, R.A.; Randau, L.; Sørensen, S.J.; Shah, S.A. Type IV CRISPR-Cas systems are highly diverse and involved in competition between plasmids. *Nucleic Acids Res.* **2019**, *48*, 2000–2012.
21. Makarova, K.S.; Zhang, F.; Koonin, E.V. SnapShot: Class 1 CRISPR-Cas Systems. *Cell* **2017**, *168*, 946–946.e1.
22. Goeders, N.; Van Melder, L. Toxin-antitoxin systems as multilevel interaction systems. *Toxins (Basel)* **2014**, *6*, 304–324.
23. Makarova, K.S.; Wolf, Y.I.; Iranzo, J.; Shmakov, S.A.; Alkhnbashi, O.S.; Brouns, S.J.J.; Charpentier, E.; Cheng, D.; Haft, D.H.; Horvath, P.; et al. Evolutionary classification of CRISPR-Cas systems: A burst of class 2 and derived variants. *Nat. Rev. Microbiol.* **2020**, *18*, 67–83.
24. Samai, P.; Pyenson, N.; Jiang, W.; Goldberg, G.W.; Hatoum-Aslan, A.; Marraffini, L.A. Co-transcriptional DNA and RNA Cleavage during Type III CRISPR-Cas Immunity. *Cell* **2015**, *161*, 1164–1174.
25. Koonin, E.V.; Makarova, K.S.; Zhang, F. Diversity, classification and evolution of CRISPR-Cas systems. *Curr. Opin. Microbiol.* **2017**, *37*, 67–78.
26. Makarova, K.S.; Wolf, Y.I.; Alkhnbashi, O.S.; Costa, F.; Shah, S.A.; Saunders, S.J.; Barrangou, R.; Brouns, S.J.J.; Charpentier, E.; Haft, D.H.; et al. An updated evolutionary classification of CRISPR-Cas systems. *Nat. Rev. Microbiol.* **2015**, *13*, 722–736.
27. Wu, W.Y.; Lebbink, J.H.G.; Kanaar, R.; Geijsen, N.; van der Oost, J. Genome editing by natural and engineered CRISPR-associated nucleases. *Nat. Chem. Biol.* **2018**, *14*, 642–651.
28. Hussain, W.; Mahmood, T.; Hussain, J.; Ali, N.; Shah, T.; Qayyum, S.; Khan, I. CRISPR/Cas system: A game changing genome editing technology, to treat human genetic diseases. *Gene* **2019**, *685*, 70–75.
29. Jiang, W.; Bikard, D.; Cox, D.; Zhang, F.; Marraffini, L.A. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nat. Biotechnol.* **2013**, *31*, 233–239.
30. Goren, M.; Yosef, I.; Qimron, U. Sensitizing pathogens to antibiotics using the CRISPR-Cas system. *Drug Resist. Updat.* **2017**, *30*, 1–6.
31. Marraffini, L.A.; Sontheimer, E.J. Self versus non-self discrimination during CRISPR RNA-directed immunity. *Nature* **2010**, *463*, 568–571.
32. Mojica, F.J.M.; Díez-Villaseñor, C.; García-Martínez, J.; Almendros, C. Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology* **2009**, *155*, 733–740.

33. Sauer, N.J.; Mozoruk, J.; Miller, R.B.; Warburg, Z.J.; Walker, K.A.; Beetham, P.R.; Schöpke, C.R.; Gocal, G.F.W. Oligonucleotide-directed mutagenesis for precision gene editing. *Plant. Biotechnol. J.* **2016**, *14*, 496–502.
34. Carroll, D. Genome engineering with targetable nucleases. *Annu. Rev. Biochem.* **2014**, *83*, 409–439.
35. Zhang, Z.; Zhang, S.; Huang, X.; Orwig, K.E.; Sheng, Y. Rapid assembly of customized TALENs into multiple delivery systems. *PLoS ONE* **2013**, *8*, e80281.
36. Joung, J.K.; Sander, J.D. TALENs: A widely applicable technology for targeted genome editing. *Nat. Rev. Mol. Cell Biol.* **2013**, *14*, 49–55.
37. Urnov, F.D.; Rebar, E.J.; Holmes, M.C.; Zhang, H.S.; Gregory, P.D. Genome editing with engineered zinc finger nucleases. *Nat. Rev. Genet.* **2010**, *11*, 636–646.
38. Doudna, J.A.; Charpentier, E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science* **2014**, *346*, 1258096.
39. Ledford, H. CRISPR, the disruptor. *Nature* **2015**, *522*, 20–24.
40. Ishino, Y.; Krupovic, M.; Forterre, P. History of CRISPR-Cas from Encounter with a Mysterious Repeated Sequence to Genome Editing Technology. *J. Bacteriol.* **2018**, *200*, 5429.
41. Hsu, P.D.; Lander, E.S.; Zhang, F. Development and applications of CRISPR-Cas9 for genome engineering. *Cell* **2014**, *157*, 1262–1278.
42. Deltcheva, E.; Chylinski, K.; Sharma, C.M.; Gonzales, K.; Chao, Y.; Pirzada, Z.A.; Eckert, M.R.; Vogel, J.; Charpentier, E. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* **2011**, *471*, 602–607.
43. Jinek, M.; Chylinski, K.; Fonfara, I.; Hauer, M.; Doudna, J.A.; Charpentier, E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **2012**, *337*, 816–821.
44. Szczelkun, M.D.; Tikhomirova, M.S.; Sinkunas, T.; Gasiunas, G.; Karvelis, T.; Pschera, P.; Siksnys, V.; Seidel, R. Direct observation of R-loop formation by single RNA-guided Cas9 and Cascade effector complexes. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 9798–9803.
45. Anders, C.; Niewoehner, O.; Duerst, A.; Jinek, M. Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. *Nature* **2014**, *513*, 569–573.
46. Nishimasu, H.; Cong, L.; Yan, W.X.; Ran, F.A.; Zetsche, B.; Li, Y.; Kurabayashi, A.; Ishitani, R.; Zhang, F.; Nureki, O. Crystal Structure of *Staphylococcus aureus* Cas9. *Cell* **2015**, *162*, 1113–1126.
47. Chneiweiss, H.; Hirsch, F.; Montoliu, L.; Müller, A.M.; Fenet, S.; Abecassis, M.; Merchant, J.; Baertschi, B.; Botbol-Baum, M.; Houghton, J.A.; et al. Fostering responsible research with genome editing technologies: A European perspective. *Transgenic Res.* **2017**, *26*, 709–713.
48. Kosicki, M.; Tomberg, K.; Bradley, A. Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements. *Nat. Biotechnol.* **2018**, *36*, 765–771.
49. Maruyama, T.; Dougan, S.K.; Truttmann, M.C.; Bilate, A.M.; Ingram, J.R.; Ploegh, H.L. Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. *Nat. Biotechnol.* **2015**, *33*, 538–542.
50. Roy, B.; Zhao, J.; Yang, C.; Luo, W.; Xiong, T.; Li, Y.; Fang, X.; Gao, G.; Singh, C.O.; Madsen, L.; et al. CRISPR/Cascade 9-Mediated Genome Editing-Challenges and Opportunities. *Front. Genet.* **2018**, *9*, 240.
51. Lino, C.A.; Harper, J.C.; Carney, J.P.; Timlin, J.A. Delivering CRISPR: A review of the challenges and approaches. *Drug Deliv.* **2018**, *25*, 1234–1257.
52. Tomkinson, A.E.; Howes, T.R.L.; Wiest, N.E. DNA ligases as therapeutic targets. *Transl. Cancer Res.* **2013**, *2*.
53. Yu, C.; Liu, Y.; Ma, T.; Liu, K.; Xu, S.; Zhang, Y.; Liu, H.; La Russa, M.; Xie, M.; Ding, S.; et al. Small molecules enhance CRISPR genome editing in pluripotent stem cells. *Cell Stem Cell* **2015**, *16*, 142–147.
54. Sun, W.; Ji, W.; Hall, J.M.; Hu, Q.; Wang, C.; Beisel, C.L.; Gu, Z. Self-assembled DNA nanoclews for the efficient delivery of CRISPR-Cas9 for genome editing. *Angew. Chem. Int. Ed. Engl.* **2015**, *54*, 12029–12033.
55. Horii, T.; Arai, Y.; Yamazaki, M.; Morita, S.; Kimura, M.; Itoh, M.; Abe, Y.; Hatada, I. Validation of microinjection methods for generating knockout mice by CRISPR/Cas-mediated genome engineering. *Sci. Rep.* **2014**, *4*, 4513–4516.
56. Ran, F.A.; Cong, L.; Yan, W.X.; Scott, D.A.; Gootenberg, J.S.; Kriz, A.J.; Zetsche, B.; Shalem, O.; Wu, X.; Makarova, K.S.; et al. In vivo genome editing using *Staphylococcus aureus* Cas9. *Nature* **2015**, *520*, 186–191.

57. Saha, S.K.; Saikot, F.K.; Rahman, M.S.; Jamal, M.A.H.M.; Rahman, S.M.K.; Islam, S.M.R.; Kim, K.-H. Programmable Molecular Scissors: Applications of a New Tool for Genome Editing in Biotech. *Mol. Ther. Nucleic Acids* **2019**, *14*, 212–238.
58. Li, R.; Li, R.; Li, X.; Fu, D.; Zhu, B.; Tian, H.; Luo, Y.; Zhu, H. Multiplexed CRISPR/Cas9-mediated metabolic engineering of γ -aminobutyric acid levels in *Solanum lycopersicum*. *Plant. Biotechnol. J.* **2018**, *16*, 415–427.
59. Sun, Y.; Jiao, G.; Liu, Z.; Zhang, X.; Li, J.; Guo, X.; Du, W.; Du, J.; Francis, F.; Zhao, Y.; et al. Generation of High-Amylose Rice through CRISPR/Cas9-Mediated Targeted Mutagenesis of Starch Branching Enzymes. *Front. Plant. Sci.* **2017**, *8*, 298.
60. Ma, T.; Tao, J.; Yang, M.; He, C.; Tian, X.; Zhang, X.; Zhang, J.; Deng, S.; Feng, J.; Zhang, Z.; et al. An AANAT/ASMT transgenic animal model constructed with CRISPR/Cas9 system serving as the mammary gland bioreactor to produce melatonin-enriched milk in sheep. *J. Pineal Res.* **2017**, *63*, e12406.
61. Platt, R.J.; Chen, S.; Zhou, Y.; Yim, M.J.; Swiech, L.; Kempton, H.R.; Dahlman, J.E.; Parnas, O.; Eisenhaure, T.M.; Jovanovic, M.; et al. CRISPR-Cas9 knockin mice for genome editing and cancer modeling. *Cell* **2014**, *159*, 440–455.
62. Yan, S.; Tu, Z.; Liu, Z.; Fan, N.; Yang, H.; Yang, S.; Yang, W.; Zhao, Y.; Ouyang, Z.; Lai, C.; et al. A Huntingtin Knockin Pig Model Recapitulates Features of Selective Neurodegeneration in Huntington's Disease. *Cell* **2018**, *173*, 989–1002.e13.
63. Fang, B.; Ren, X.; Wang, Y.; Li, Z.; Zhao, L.; Zhang, M.; Li, C.; Zhang, Z.; Chen, L.; Li, X.; et al. Apolipoprotein E deficiency accelerates atherosclerosis development in miniature pigs. *Dis. Model. Mech.* **2018**, *11*, dmm036632.
64. Bakondi, B.; Lv, W.; Lu, B.; Jones, M.K.; Tsai, Y.; Kim, K.J.; Levy, R.; Akhtar, A.A.; Breunig, J.J.; Svendsen, C.N.; et al. In Vivo CRISPR/Cas9 Gene Editing Corrects Retinal Dystrophy in the S334ter-3 Rat Model of Autosomal Dominant Retinitis Pigmentosa. *Mol. Ther.* **2016**, *24*, 556–563.
65. Zuckermann, M.; Hovestadt, V.; Knobbe-Thomsen, C.B.; Zapatka, M.; Northcott, P.A.; Schramm, K.; Belic, J.; Jones, D.T.W.; Tschida, B.; Moriarity, B.; et al. Somatic CRISPR/Cas9-mediated tumour suppressor disruption enables versatile brain tumour modelling. *Nat. Commun.* **2015**, *6*, 7391–7399.
66. Xu, Y.; Li, Z. CRISPR-Cas systems: Overview, innovations and applications in human disease research and gene therapy. *Comput. Struct. Biotechnol. J.* **2020**, *18*, 2401–2415.
67. Ding, Q.; Regan, S.N.; Xia, Y.; Ostrom, L.A.; Cowan, C.A.; Musunuru, K. Enhanced efficiency of human pluripotent stem cell genome editing through replacing TALENs with CRISPRs. *Cell Stem Cell* **2013**, *12*, 393–394.
68. Drost, J.; van Jaarsveld, R.H.; Ponsioen, B.; Zimmerlin, C.; van Boxtel, R.; Buijs, A.; Sachs, N.; Overmeer, R.M.; Offerhaus, G.J.; Begthel, H.; et al. Sequential cancer mutations in cultured human intestinal stem cells. *Nature* **2015**, *521*, 43–47.
69. Ormond, K.E.; Bombard, Y.; Bonham, V.L.; Hoffman-Andrews, L.; Howard, H.; Isasi, R.; Musunuru, K.; Riggan, K.A.; Michie, M.; Allyse, M. The clinical application of gene editing: Ethical and social issues. *Per. Med.* **2019**, *16*, 337–350.
70. Long, C.; McAnally, J.R.; Shelton, J.M.; Mireault, A.A.; Bassel-Duby, R.; Olson, E.N. Prevention of muscular dystrophy in mice by CRISPR/Cas9-mediated editing of germline DNA. *Science* **2014**, *345*, 1184–1188.
71. Sürün, D.; Schwäble, J.; Tomasovic, A.; Ehling, R.; Stein, S.; Kurrle, N.; Melchner, von, H.; Schnütgen, F. High Efficiency Gene Correction in Hematopoietic Cells by Donor-Template-Free CRISPR/Cas9 Genome Editing. *Mol. Ther. Nucleic Acids* **2018**, *10*, 1–8.
72. Hartong, D.T.; Berson, E.L.; Dryja, T.P. Retinitis pigmentosa. *Lancet* **2006**, *368*, 1795–1809.
73. Guan, Y.; Ma, Y.; Li, Q.; Sun, Z.; Ma, L.; Wu, L.; Wang, L.; Zeng, L.; Shao, Y.; Chen, Y.; et al. CRISPR/Cas9-mediated somatic correction of a novel coagulator factor IX gene mutation ameliorates hemophilia in mouse. *EMBO Mol. Med.* **2016**, *8*, 477–488.
74. Wong, T.W.Y.; Cohn, R.D. Therapeutic Applications of CRISPR/Cas for Duchenne Muscular Dystrophy. *Curr. Gene Ther.* **2017**, *17*, 301–308.
75. Dever, D.P.; Bak, R.O.; Reinisch, A.; Camarena, J.; Washington, G.; Nicolas, C.E.; Pavel-Dinu, M.; Saxena, N.; Wilkens, A.B.; Mantri, S.; et al. CRISPR/Cas9 β -globin gene targeting in human haematopoietic stem cells. *Nature* **2016**, *539*, 384–389.
76. Xie, F.; Ye, L.; Chang, J.C.; Beyer, A.I.; Wang, J.; Muench, M.O.; Kan, Y.W. Seamless gene correction of β -thalassemia mutations in patient-specific iPSCs using CRISPR/Cas9 and piggyBac. *Genome Res.* **2014**, *24*, 1526–1533.

77. Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R.L.; Torre, L.A.; Jemal, A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* **2018**, *68*, 394–424.
78. Liu, T.; Shen, J.K.; Li, Z.; Choy, E.; Hornicek, F.J.; Duan, Z. Development and potential applications of CRISPR-Cas9 genome editing technology in sarcoma. *Cancer Lett.* **2016**, *373*, 109–118.
79. Harden, M.E.; Munger, K. Human papillomavirus molecular biology. *Mutat. Res. Rev. Mutat. Res.* **2017**, *772*, 3–12.
80. Kennedy, E.M.; Kornepati, A.V.R.; Goldstein, M.; Bogerd, H.P.; Poling, B.C.; Whisnant, A.W.; Kastan, M.B.; Cullen, B.R. Inactivation of the human papillomavirus E6 or E7 gene in cervical carcinoma cells by using a bacterial CRISPR/Cas RNA-guided endonuclease. *J. Virol.* **2014**, *88*, 11965–11972.
81. Haapaniemi, E.; Botla, S.; Persson, J.; Schmierer, B.; Taipale, J. CRISPR-Cas9 genome editing induces a p53-mediated DNA damage response. *Nat. Med.* **2018**, *24*, 927–930.
82. Ihry, R.J.; Worringer, K.A.; Salick, M.R.; Frias, E.; Ho, D.; Theriault, K.; Kommineni, S.; Chen, J.; Sondey, M.; Ye, C.; et al. p53 inhibits CRISPR-Cas9 engineering in human pluripotent stem cells. *Nat. Med.* **2018**, *24*, 939–946.
83. Sestito, S.; Falvo, F.; Scozzafava, C.; Apa, R.; Pensabene, L.; Bonapace, G.; Moricca, M.T.; Concolino, D. Genetics and Gene Therapy in Hunter Disease. *Curr. Gene Ther.* **2018**, *18*, 90–95.
84. Couzin-Frankel, J. Cancer Immunotherapy. Baby's leukemia recedes after novel cell therapy. *Science* **2015**, *350*, 731–731.
85. Troisi, M.; Andreano, E.; Sala, C.; Kabanova, A.; Rappuoli, R. Vaccines as remedy for antimicrobial resistance and emerging infections. *Curr. Opin. Immunol.* **2020**, *65*, 102–106.
86. Esposito, S. Infectious Diseases: Pathophysiology, Diagnostics and Prevention. *Int. J. Mol. Sci.* **2016**, *17*, 1464.
87. Vlassov, V.V.; Tikunova, N.V.; Morozova, V.V. Bacteriophages as Therapeutic Preparations: What Restricts Their Application in Medicine. *Biochem. Mosc.* **2020**, *85*, 1350–1361.
88. Dogan, L.; Kaya, D.; Sarikaya, T.; Zengin, R.; Dincer, A.; Akinci, I.O.; Afsar, N. Plasmapheresis treatment in COVID-19-related autoimmune meningoencephalitis: Case series. *Brain Behav. Immun.* **2020**, *87*, 155–158.
89. Norgan, A.P.; Juskewitch, J.E.; Pritt, B.S.; Winters, J.L. The use of cytapheresis in the treatment of infectious diseases. *J. Clin. Apher.* **2018**, *33*, 529–537.
90. Dodds, D.R. Antibiotic resistance: A current epilogue. *Biochem. Pharmacol.* **2017**, *134*, 139–146.
91. Becattini, S.; Taur, Y.; Pamer, E.G. Antibiotic-Induced Changes in the Intestinal Microbiota and Disease. *Trends Mol. Med.* **2016**, *22*, 458–478.
92. Palmer, K.L.; Gilmore, M.S. Multidrug-resistant enterococci lack CRISPR-cas. *MBio* **2010**, *1*, 297.
93. Wang, G.; Song, G.; Xu, Y. Association of CRISPR/Cas System with the Drug Resistance in *Klebsiella pneumoniae*. *Infect. Drug Resist.* **2020**, *13*, 1929–1935.
94. Tong, Z.; Du, Y.; Ling, J.; Huang, L.; Ma, J. Relevance of the clustered regularly interspaced short palindromic repeats of *Enterococcus faecalis* strains isolated from retreatment root canals on periapical lesions, resistance to irrigants and biofilms. *Exp. Ther. Med.* **2017**, *14*, 5491–5496.
95. Ren, L.; Deng, L.-H.; Zhang, R.-P.; Wang, C.-D.; Li, D.-S.; Xi, L.-X.; Chen, Z.-R.; Yang, R.; Huang, J.; Zeng, Y.-R.; et al. Relationship between drug resistance and the clustered, regularly interspaced, short, palindromic repeat-associated protein genes cas1 and cas2 in *Shigella* from giant panda dung. *Medicine (Baltimore)* **2017**, *96*, e5922.
96. Kamerbeek, J.; Schouls, L.; Kolk, A.; van Agterveld, M.; van Soolingen, D.; Kuijper, S.; Bunschoten, A.; Molhuizen, H.; Shaw, R.; Goyal, M.; et al. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *J. Clin. Microbiol.* **1997**, *35*, 907–914.
97. Mokrousov, I.; Narvskaya, O.; Limeschenko, E.; Vyazovaya, A. Efficient discrimination within a *Corynebacterium diphtheriae* epidemic clonal group by a novel macroarray-based method. *J. Clin. Microbiol.* **2005**, *43*, 1662–1668.
98. Shariat, N.; Dudley, E.G. CRISPRs: Molecular signatures used for pathogen subtyping. *Appl. Environ. Microbiol.* **2014**, *80*, 430–439.
99. Delannoy, S.; Beutin, L.; Fach, P. Use of clustered regularly interspaced short palindromic repeat sequence polymorphisms for specific detection of enterohemorrhagic *Escherichia coli* strains of serotypes O26:H11, O45:H2, O103:H2, O111:H8, O121:H19, O145:H28, and O157:H7 by real-time PCR. *J. Clin. Microbiol.* **2012**, *50*, 4035–4040.

100. Gootenberg, J.S.; Abudayyeh, O.O.; Lee, J.W.; Essletzbichler, P.; Dy, A.J.; Joung, J.; Verdine, V.; Donghia, N.; Daringer, N.M.; Freije, C.A.; et al. Nucleic acid detection with CRISPR-Cas13a/C2c2. *Science* **2017**, *356*, 438–442.
101. Kellner, M.J.; Koob, J.G.; Gootenberg, J.S.; Abudayyeh, O.O.; Zhang, F. SHERLOCK: Nucleic acid detection with CRISPR nucleases. *Nat. Protoc.* **2019**, *14*, 2986–3012.
102. Chen, J.S.; Ma, E.; Harrington, L.B.; Da Costa, M.; Tian, X.; Palefsky, J.M.; Doudna, J.A. CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity. *Science* **2018**, *360*, 436–439.
103. Qi, L.S.; Larson, M.H.; Gilbert, L.A.; Doudna, J.A.; Weissman, J.S.; Arkin, A.P.; Lim, W.A. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* **2013**, *152*, 1173–1183.
104. Choudhary, E.; Thakur, P.; Pareek, M.; Agarwal, N. Gene silencing by CRISPR interference in mycobacteria. *Nat. Commun.* **2015**, *6*, 6267.
105. Groner, A.C.; Meylan, S.; Ciuffi, A.; Zangger, N.; Ambrosini, G.; Dénervaud, N.; Bucher, P.; Trono, D. KRAB-zinc finger proteins and KAP1 can mediate long-range transcriptional repression through heterochromatin spreading. *PLoS Genet.* **2010**, *6*, e1000869.
106. Lo, A.; Qi, L. Genetic and epigenetic control of gene expression by CRISPR-Cas systems. *F1000Res* **2017**, *6*, 747.
107. Chavez, A.; Scheiman, J.; Vora, S.; Pruitt, B.W.; Tuttle, M.; Iyer, E.; Lin, S.; Kiani, S.; Guzman, C.D.; Wiegand, D.J.; et al. Highly efficient Cas9-mediated transcriptional programming. *Nat. Methods* **2015**, *12*, 326–328.
108. Gaudelli, N.M.; Komor, A.C.; Rees, H.A.; Packer, M.S.; Badran, A.H.; Bryson, D.I.; Liu, D.R. Programmable base editing of A•T to G•C in genomic DNA without DNA cleavage. *Nature* **2017**, *551*, 464–471.
109. Kampmann, M. Elucidating drug targets and mechanisms of action by genetic screens in mammalian cells. *Chem. Commun. (Camb.)* **2017**, *53*, 7162–7167.
110. Kurata, M.; Yamamoto, K.; Moriarity, B.S.; Kitagawa, M.; Largaespada, D.A. CRISPR/Cas9 library screening for drug target discovery. *J. Hum. Genet.* **2018**, *63*, 179–186.
111. Unniyampurath, U.; Pilankatta, R.; Krishnan, M.N. RNA Interference in the Age of CRISPR: Will CRISPR Interfere with RNAi? *Int. J. Mol. Sci.* **2016**, *17*, 291.
112. Colasante, G.; Qiu, Y.; Massimino, L.; Di Berardino, C.; Cornford, J.H.; Snowball, A.; Weston, M.; Jones, S.P.; Giannelli, S.; Lieb, A.; et al. In vivo CRISPRa decreases seizures and rescues cognitive deficits in a rodent model of epilepsy. *Brain* **2020**, *143*, 891–905.
113. Wang, G.; Chow, R.D.; Bai, Z.; Zhu, L.; Errami, Y.; Dai, X.; Dong, M.B.; Ye, L.; Zhang, X.; Renauer, P.A.; et al. Multiplexed activation of endogenous genes by CRISPRa elicits potent antitumor immunity. *Nat. Immunol.* **2019**, *20*, 1494–1505.
114. Esvelt, K.M.; Smidler, A.L.; Catteruccia, F.; Church, G.M. Concerning RNA-guided gene drives for the alteration of wild populations. *Elife* **2014**, *3*, 20131071.
115. Cui, L.; Bikard, D. Consequences of Cas9 cleavage in the chromosome of Escherichia coli. *Nucleic Acids Res.* **2016**, *44*, 4243–4251.
116. Bikard, D.; Euler, C.W.; Jiang, W.; Nussenzweig, P.M.; Goldberg, G.W.; Duportet, X.; Fischetti, V.A.; Marraffini, L.A. Exploiting CRISPR-Cas nucleases to produce sequence-specific antimicrobials. *Nat. Biotechnol.* **2014**, *32*, 1146–1150.
117. Citorik, R.J.; Mimee, M.; Lu, T.K. Sequence-specific antimicrobials using efficiently delivered RNA-guided nucleases. *Nat. Biotechnol.* **2014**, *32*, 1141–1145.
118. Hameed, H.M.A.; Islam, M.M.; Chhotaray, C.; Wang, C.; Liu, Y.; Tan, Y.; Li, X.; Tan, S.; Delorme, V.; Yew, W.W.; et al. Molecular Targets Related Drug Resistance Mechanisms in MDR-, XDR-, and TDR- Mycobacterium tuberculosis Strains. *Front. Cell. Infect. Microbiol.* **2018**, *8*, 114.
119. He, L.; Fan, X.; Xie, J. Comparative genomic structures of Mycobacterium CRISPR-Cas. *J. Cell Biochem.* **2012**, *113*, 2464–2473.
120. Brosch, R.; Pym, A.S.; Gordon, S.V.; Cole, S.T. The evolution of mycobacterial pathogenicity: Clues from comparative genomics. *Trends Microbiol.* **2001**, *9*, 452–458.
121. Zaychikova, M.V.; Zakharevich, N.V.; Chekalina, M.S.; Danilenko, V.N. CRISPR-Cas systems of *Mycobacterium tuberculosis*: The structure, transformation in different lineages in the process of evolution and a possible role in the formation of virulence and drug resistance. *BRSMU* **2018**, 5–13.
122. Sarkis, G.J.; Hatfull, G.F. Mycobacteriophages. *Methods Mol. Biol.* **1998**, *101*, 145–173.
123. Rock, J. Tuberculosis drug discovery in the CRISPR era. *PLoS Pathog.* **2019**, *15*, e1007975.

124. Wu, X.; Kriz, A.J.; Sharp, P.A. Target specificity of the CRISPR-Cas9 system. *Quant. Biol.* **2014**, *2*, 59–70.
125. Cho, S.; Choe, D.; Lee, E.; Kim, S.C.; Palsson, B.; Cho, B.-K. High-Level dCas9 Expression Induces Abnormal Cell Morphology in *Escherichia coli*. *ACS Synth. Biol.* **2018**, *7*, 1085–1094.
126. Guilinger, J.P.; Thompson, D.B.; Liu, D.R. Fusion of catalytically inactive Cas9 to FokI nuclease improves the specificity of genome modification. *Nat. Biotechnol.* **2014**, *32*, 577–582.
127. Bolukbasi, M.F.; Gupta, A.; Oikemus, S.; Derr, A.G.; Garber, M.; Brodsky, M.H.; Zhu, L.J.; Wolfe, S.A. DNA-binding-domain fusions enhance the targeting range and precision of Cas9. *Nat. Methods* **2015**, *12*, 1150–1156.
128. Zaidi, S.S.-E.-A.; Mahfouz, M.M.; Mansoor, S. CRISPR-Cpf1: A New Tool for Plant Genome Editing. *Trends Plant. Sci.* **2017**, *22*, 550–553.
129. Yan, M.-Y.; Yan, H.-Q.; Ren, G.-X.; Zhao, J.-P.; Guo, X.-P.; Sun, Y.-C. CRISPR-Cas12a-Assisted Recombineering in Bacteria. *Appl. Environ. Microbiol.* **2017**, *83*, 1.
130. Zetsche, B.; Gootenberg, J.S.; Abudayyeh, O.O.; Slaymaker, I.M.; Makarova, K.S.; Essletzbichler, P.; Volz, S.E.; Joung, J.; van der Oost, J.; Regev, A.; et al. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* **2015**, *163*, 759–771.
131. Jiang, Y.; Qian, F.; Yang, J.; Liu, Y.; Dong, F.; Xu, C.; Sun, B.; Chen, B.; Xu, X.; Li, Y.; et al. CRISPR-Cpf1 assisted genome editing of *Corynebacterium glutamicum*. *Nat. Commun.* **2017**, *8*, 15179.
132. Smargon, A.A.; Cox, D.B.T.; Pyzocha, N.K.; Zheng, K.; Slaymaker, I.M.; Gootenberg, J.S.; Abudayyeh, O.A.; Essletzbichler, P.; Shmakov, S.; Makarova, K.S.; et al. Cas13b Is a Type VI-B CRISPR-Associated RNA-Guided RNase Differentially Regulated by Accessory Proteins Csx27 and Csx28. *Mol. Cell* **2017**, *65*, 618–630.
133. Shmakov, S.; Abudayyeh, O.O.; Makarova, K.S.; Wolf, Y.I.; Gootenberg, J.S.; Semenova, E.; Minakhin, L.; Joung, J.; Konermann, S.; Severinov, K.; et al. Discovery and Functional Characterization of Diverse Class 2 CRISPR-Cas Systems. *Mol. Cell* **2015**, *60*, 385–397.
134. Ma, Y.; Zhang, L.; Qin, C. The first genetically gene-edited babies: It's “irresponsible and too early”. *Animal Model. Exp. Med.* **2019**, *2*, 1–4.
135. Anderson, J.E.; Michno, J.-M.; Kono, T.J.Y.; Stec, A.O.; Campbell, B.W.; Curtin, S.J.; Stupar, R.M. Genomic variation and DNA repair associated with soybean transgenesis: A comparison to cultivars and mutagenized plants. *BMC Biotechnol.* **2016**, *16*, 41–13.
136. Manghwar, H.; Li, B.; Ding, X.; Hussain, A.; Lindsey, K.; Zhang, X.; Jin, S. CRISPR/Cas Systems in Genome Editing: Methodologies and Tools for sgRNA Design, Off-Target Evaluation, and Strategies to Mitigate Off-Target Effects. *Adv. Sci. (Weinh.)* **2020**, *7*, 1902312.
137. Hirakawa, M.P.; Krishnakumar, R.; Timlin, J.A.; Carney, J.P.; Butler, K.S. Gene editing and CRISPR in the clinic: Current and future perspectives. *Biosci. Rep.* **2020**, *40*, 860.
138. Caplan, A.L.; Parent, B.; Shen, M.; Plunkett, C. No time to waste—The ethical challenges created by CRISPR. *EMBO Rep.* **2015**, *16*, 1421–1426.
139. Zhang, J.; Zong, W.; Hong, W.; Zhang, Z.-T.; Wang, Y. Exploiting endogenous CRISPR-Cas system for multiplex genome editing in *Clostridium tyrobutyricum* and engineer the strain for high-level butanol production. *Metab. Eng.* **2018**, *47*, 49–59.
140. Li, Y.; Peng, N. Endogenous CRISPR-Cas System-Based Genome Editing and Antimicrobials: Review and Prospects. *Front. Microbiol.* **2019**, *10*, 2471.
141. Bondy-Denomy, J.; Pawluk, A.; Maxwell, K.L.; Davidson, A.R. Bacteriophage genes that inactivate the CRISPR/Cas bacterial immune system. *Nature* **2013**, *493*, 429–432.
142. Pawluk, A.; Davidson, A.R.; Maxwell, K.L. Anti-CRISPR: Discovery, mechanism and function. *Nat. Rev. Microbiol.* **2018**, *16*, 12–17.
143. Maxwell, K.L. The Anti-CRISPR Story: A Battle for Survival. *Mol. Cell* **2017**, *68*, 8–14.

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).