Genome editing technologies to fight infectious diseases

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Abstract

Introduction: Genome editing by programmable nucleases represents a promising tool that could be exploited to develop new therapeutic strategies to fight infectious diseases. These nucleases, such as zinc-finger nucleases, transcription activator-like effector nucleases, clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR-associated protein 9 (Cas9) and homing endonucleases, are molecular scissors that can be targeted at predetermined loci in order to modify the genome sequence of an organism.

Areas covered: By perturbing genomic DNA at predetermined loci, programmable nucleases can be used as antiviral and antimicrobial treatment. This approach includes targeting of essential viral genes or viral sequences able, once mutated, to inhibit viral replication; repurposing of CRISPR-Cas9 system for lethal self-targeting of bacteria; targeting antibiotic-resistance and virulence genes in bacteria, fungi, and parasites; engineering arthropod vectors to prevent vector-borne infections.

Expert commentary: While progress has been done in demonstrating the feasibility of using genome editing as antimicrobial strategy, there are still many hurdles to overcome, such as the risk of off-target mutations, the raising of escape mutants, and the inefficiency of delivery methods, before translating results from preclinical studies into clinical applications.

Keywords

Zinc-finger nuclease; transcription activator-like effector nuclease; CRISPR-Cas9; homing endonucleases; viral infections; bacterial infections; fungal infections; parasite infections; vector-borne infections.

1. Introduction

Advancements in basic research have allowed dissecting pathogen structure, replication mechanisms, and virulence factors at molecular level, leading to the discovery of several new antimicrobial agents. However, notwithstanding these advances, infectious diseases still represent the third cause of mortality worldwide and the increasing emergence of multi-drug resistant pathogens is a matter of concern. Next generation technologies are now helping moving forward scientific knowledge and its translational applications at an unprecedented speed. Among these technologies, genome editing by programmable nucleases represents a promising tool that could be exploited to develop new therapeutic strategies to fight infectious diseases. These nucleases, such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR-associated protein 9 (Cas9) and homing endonucleases (HEs), are molecular scissors that can be targeted at predetermined loci in order to modify the genome sequence of an organism. In this review, we will discuss the main applications of these gene editing tools with particular focus on their use as antiviral and antimicrobial agents and on the critical issues that need to be faced in order to translate promising results from basic research into new treatment modalities for evaluation in clinical trials.

2. Genome editing tools

Despite their architectural and origin differences, all four classes of engineered nucleases mostly used so far, ZNFs, TALENs, CRISPR-Cas, and HEs have one common final aim: to exert DNA double strand brakes (DSBs) into preselected loci of a genome. The first three are composed of two basic units: one is responsible for DNA sequence recognition and bond and the other one, represented by the molecular scissors, for the DNA DSB. Once a DNA DSB occurs, the physiological cell machinery works in order to repair this rupture throughout two types of mechanisms: non-homologous end joining pathways (NHEJ) or homologous recombination (HR) [1]. NHEJ is an error prone mechanism that can introduce insertions or deletions (the so called indels) in the process of reuniting the DNA ends. These indels may be advantageous when they cause frameshifts resulting in gene knock-out due to mRNA degradation or production of non-functional proteins [2]. When a high copy number of an homologous sequence is introduced exogenously into a cells in the form of plasmids or single stranded oligonucleotides, HR can instead take place, allowing the new sequence to be introduced into the cell genome [3,4]. ZNFs are the first class of chimeric programmable nucleases that was applied for genome editing. Their first use dates back to 2001, when ZNFs were shown to promote HR at specific genomic sequences in *Xenopus* embryos [5]. Few years later, ZFNs were shown to allow the genetic correction of the SCID mutation in human cells by HR at high efficiency [6] and brought to the production of the first knockout rats via embryo microinjections [7]. Then, TALENs were applied as genome editing tools in human cells [8]. In 2013, two independent groups demonstrated that bacterial CRISPR-Cas9 system could be adapted to become programmable nuclease for genome editing very efficiently [9,10]. The DNA binding

domain of ZFNs is composed by a tandem array of C_2H_2 zinc fingers with each one recognizing a 3-bp DNA sequence [11] and usually 3–6 zinc-fingers are used to generate a single ZFN subunit that binds to DNA sequences of 9–18 bp. The element responsible for DSBs of ZFNs is represented by the cleavage domain of bacterial restriction enzyme *Fok*I that must dimerize in order to cleave DNA [12]. Consequently, binding of two ZFN monomers to two specific sites is required for effective double-strand DNA cleavage [13]. TALENs are similar to ZFNs as they rely on the same cleavage unit, the restriction enzyme *Fok*I, and are so fore required to bind the DNA grove as dimers [8]. The DNA binding domain of TALENs is composed by TALE proteins, derived from the plant pathogenic *Xanthomonas spp*. bacteria [14], that comprise tandem arrays of 33–35 amino acid repeats, each recognizing a single base-pair of the major groove [15]. Two amino acids at positions 12 and 13 of each repeat domain [16], called repeat variable diresidues (RVDs), are responsible for the nucleotide specificity and four different RVDs are most widely used to recognize the four bases of the DNA. While both ZFNs and TALENs rely on a protein/DNA based interaction, CRISPR-Cas9 system is based on RNA/DNA interaction. In bacteria and archaea, the CRISPR-Cas system naturally provides adaptive immunity against invading phages or plasmids [17]. Briefly, CRISPR regions are incorporated into the bacterial genome, upon capture of a ~20 bp DNA fragments (called protospacer), from the foreign DNA of invading phages or plasmids. In type II CRISPR systems, the CRISPR regions together with an invariable target-independent trans-activating crRNA (tracrRNA) region are transcribed as pre-CRISPR RNA (pre-crRNA) and processed to give rise to target-specific crRNA [18]. Both crRNA and tracrRNA are complexed with the Cas9 protein to form an active DNA endonuclease, able to cleave a target DNA sequence composed of the 20-bp guide sequence, represented by the protospacer and the 5′-NGG-3′ sequence known as PAM (protospacer adjacent motif) [19]. Unlike the other classes of nucleases, in HEs the same domain mediates both DNA recognition and cleavage functions. In microbial organisms, phages, and viruses, HEs are small proteins naturally encoded by elements found within introns of the genome. HEs function by introducing DSBs within homologous alleles that lack the corresponding intron, which are subsequently repaired via HR using the allele containing the HN gene. HEs have been adapted for gene editing purposes by engineering the protein structure to facilitate DNA binding without modifying the cleavage activity [20].

3. Viral infections

When considering genome editing as strategies for antiviral therapy, error-prone NHEJ is the main mechanism by which programmable nucleases operate, by leading to the formation of indels able to perturb the DNA sequence of a virus present in infected cells or by targeting cellular key factors that are essential for virus entry, replication, or reactivation from latency. Since the target of programmable nucleases is a DNA sequence, this constrains the possibility to use these tools only against viruses that have a DNA-based genome or that passes through a viral-DNA intermediate easily accessible by the programmable nucleases, such as in the case of

human immunodeficiency virus (HIV). The CRISPR-Cas9 system has been recently modified to generate gRNAs that were complementary to the genome of hepatitis C virus (HCV), thus demonstrating the feasibility of targeting viruses with a RNA genome [21]. Finally, antiviral strategies that employ programmable nucleases seem to be more suited for chronic or persistent infections, characterized by the persistence of the viral genome as an episome in the nucleus or integrated into host chromosomes, than for acute infections, for which most of antiviral interventions are inefficacious after the onset of symptoms. Genome editing-based antiviral strategies are illustrated in Figure 1.

3.1. Targeting the viral genome

HIV has been a focus for the application of genome editing as antiviral therapy. HIV is a lentivirus that randomly integrates into the host cell genome and persists in CD4+ T cells. The integrated proviral genome serves both as template for gene transcription in the infected cells and for the latency of the virus [22]. By eliminating the integrated provirus, HIV infection could be eradicated. One of the first attempts to disrupt an integrated lentiviral provirus exploited HEs targeting a green-fluorescent protein (GFP) open reading frame, in which the wild-type recognition site for the HE, Y2 I-AniI, was inserted resulting in loss of GFP signal [23]. The two long terminal repeats (LTR) of HIV-1 and in particular the extremely conserved U3 and the transactivation response element (TAR) regions, which are critical for viral replication, have been targeted with engineered ZFNs [24] and TALENs [25], respectively. The ZFNs-U3 system could target and excise the full-length HIV-1 proviral DNA in infected human cell lines with an efficiency of about 30% without affecting cell cycle progression and cell proliferation [24], while TALENs demonstrated an editing efficiency of 55–60% and could eliminate the production of viral particles in HIV-infected in cells [25]. Ebina *et al.* were the first to demonstrate the potential of the CRISPR-Cas9 system to edit the HIV-1 genome by blocking its expression via targeting the LTR promoter region and by showing disruption and excision of the latent provirus from human cells [26]. Then, several other studies showed that CRISPR-Cas9 targeting viral LTR or essential viral genes in the integrated provirus or in its pre-integration form (when the cDNA of the virus is free and readily targetable by nucleases) resulted in profound suppression of HIV-1 production and infection in CD4+ T cells [27-32]. Recently, Kaminski *et al.* [33], in their proof of concept work, demonstrated the feasibility of HIV genome editing *in vivo* by using a recombinant adeno-associated virus 9 (rAAV9) vector injected through the tail-vein in transgenic mice to deliver the gRNAs/Cas9. Treatment led to the excision of a 978 bp DNA fragment spanning between the LTR and Gag gene of integrated HIV-1 DNA in multiple organs such the spleen, liver, heart, lung and kidney as well as in the circulating lymphocytes [33]. In other studies, ZFNs were able to act as repressors of HIV [34,35] and to interfere with integration 36,37].

An alternative genome editing strategy to interfere with HIV infection is the so-called “shock and kill” approach, based on the reactivation of latent virus in infected cells, which, so far, has been attempted with chemical agents, such as a histone deacetylase inhibitors [38]. A particular, a mutant form of Cas9 that has lost its DNA cleavage activity, named CRISPR activation (CRISPRa), can be directed to induce HIV reactivation in latently infected cells, resulting in their recognition by host immune surveillance mechanisms [39].

CRISPRa has been also fused to transcriptional activator domains directed to LTR sequences to drive virus replication and to induce HIV-1 reactivation [40-43].

Hepatitis B virus (HBV) is a hepadnavirus whose genome is composed by a partially double-stranded circular DNA that, upon infection of hepatocytes, is transported to the nucleus and converted to a covalently closed, circular, double-stranded DNA (cccDNA), which remains episomal. The cccDNA is the template for transcription of the three main subgenomic RNAs and of the pregenomic RNA that is reverse transcribed into the negative-strand DNA, converted into partially dsDNAs, and used in part for the packaging of new viral particles and in part shipped to the nucleus where it becomes cccDNA [44]. The first examples of gene editing strategies applied as antiviral therapy for HBV infection include the use of ZNFs designed to bind HBV viral sequences to inactivate the episomal viral genome [45] and the use of self-complementary AAV (sc-AAV) vectors to deliver HBV-specific ZFNs to infected hepatocytes, resulting in decreased HBV DNA synthesis to near-baseline levels without significant toxicity [46]. The efficacy of anti-HBV TALENs was demonstrated by Bloom *et al.*, who designed anti-HBV TALENs to target three HBV open reading frames (S, P and C) [47]. The S and C TALENs were able to cause intended mutations in the viral cccDNA, with an editing rate of 35% and 12%, respectively [47]. Hydrodynamic injection (HDI) of plasmids encoding HBV-targeted TALENs was used for *in vivo* delivery, which led to reduction of HBsAg levels by 90% and circulating viral particle equivalents by 70%, without relevant toxicity in treated mice [47]. The HBV model of HDI was also employed to suppress HBsAg expression by CRISPR-Cas9 system [48] and efficient targeting of the cccDNA and inhibition of HBV replication was also demonstrated in *in vitro* [49, 50] and *in vivo* models [51]. These *in vivo* experiments were performed in HBV-transgenic mice and other limited models of HBV infection, rather than in HBV infection in humanized mice or other physiologically relevant models, so further studies are needed before generalization of these results to human infection.

Human papillomavirus (HPV), a DNA virus belonging to the *Papillomaviridae* family, is the leading cause of infection-related cancer in humans. The virus infects the cells in the basal epithelial layer of the skin and mucosa, where its genome persists in the nucleus as an episome, without active replication. When these basal cells undergo differentiation into keratinocytes, migrating towards the surface of the epidermis, virus replication cycle is triggered, leading to the release of infectious viral particles [52]. The genome of oncogenic HPV types may integrate into cellular chromosomes and this causes overexpression of viral E6 and E7 oncoproteins, which are responsible for cell transformation, mainly through inactivation of p53 and Rb, respectively [53]. Persistent overexpression of HPV E6/E7 is necessary for cell transformation and E6/E7 inactivation leads to tumor regression [53]. The key role of E6 and E7 in HPV-driven tumors supports the use of genome editing tools targeting E6/E7 as anticancer therapy. Studies demonstrated that ZFNs, TALENs, and CRISPR-Cas9 directed against E6 and E7 efficiently cleaved both episomal and in integrated forms of the HPV genome, resulting in the disruption of target oncogenes [54-60]. In particular, ZFNs directed against E7 of oncogenic HPV16 and HPV18 specifically inhibited growth, induced apoptosis and repressed xenograft formation in vivo of corresponding HPV16- and HPV18-positive cervical cancer cell lines [54]. Targeting

E6/E7 with TALENs induced apoptosis, inhibited growth, reduced tumorigenicity in nude mice and restored p53 and Rb in HPV-positive cell lines [60]. In a HPV16 transgenic mouse model of HPV-driven cancer, direct cervical application of polymer-complexed plasmids carrying HPV16-E7–targeted TALENs effectively mutated the E7 oncogene, reduced viral DNA load, and reversed the malignant phenotype [60]. Efficient cleavage of the HPV genome with the introduction inactivating mutations of the E6 and E7 genes was also achieved by using CRISPR-Cas9 with sgRNAs specific for HPV18 E6 or E7 [57]. Treatment resulted in the induction of p53 or Rb, respectively, cell cycle arrest and cell death [57]. These studies paved the way to the initiation of phase I clinical trials with the main objective to evaluate the safety of ZFNs (NCT02800369), TALENs, and CRISPR-Cas9 (NCT03057912) systems. In the NCT02800369 study, patients were treated with suppositories containing 500 μg of ZFN-603 or ZFN-758, which target the HPV16 E7 or HPV18 E7 oncogene, respectively. The primary outcome was the evaluation of safety by reporting treatment-related adverse events of ZFN-603 and ZFN-758 in HPV16-positive and HPV18-positive subjects, respectively. The secondary outcome was the evaluation of efficacy in a time frame of 6 months, by detection of HPV16 and HPV18 persistence, changes in the number of dysplastic cells as measured by Pap test, and the number of patients without disease progression [61]. The primary objective of the NCT03057912 study was to evaluate the safety of therapeutic doses and the dosing regimen of TALENs and CRISPR/Cas9 plasmids targeting E6/E7 of HPV16 or HPV18. Both TALENs and CRISPR-Cas9 were formulated as plasmids in gel. The secondary objective of this study was to evaluate efficacy by measurement of HPV DNA load and cytological and histological regression at 3 and 6 months [62]. No results from these studies have been published so far.

Several studies on other clinically relevant DNA viruses demonstrated the feasibility of viral genome targeting by programmable nucleases. Among these, Epstein-Barr virus (EBV) episomes have been disrupted by specific Cas9/sgRNAs combinations in a latent EBV infection model [63]; a three gRNAs- CRISPR-Cas9 system could suppress JCV T-antigen expression and inhibit viral replication in JCV transformed and in permissive cell lines [64]; delivery of HEs or CRISPR-Cas9 system targeting essential herpes simplex virus type 1 (HSV-1) genes could disrupt latent HSV genome or inhibit viral lytic replication *in vitro* [65-67].

3.2. Targeting host factors required for viral infection and replication

Host factors required for virus entry, replication, and pathogenesis are potential targets for genome editing interventions. This is the case for HIV-1 entry, which is mediated through interaction of gp120 and host CD4 with the help of a coreceptor that, for nearly almost all primary isolates of HIV-1, is represented by chemokine receptor 5 (*CCR5*) [68]. The idea that this coreceptor could be used as a target for HIV-1-entry inhibition comes from the observation that individuals who carry a deletion of 32bp in the *CCR5* gene, known as *CCR5*-Δ32, are protected against R5 strains of HIV-1 [69]. Several preclinical studies showed that ZFNs directed to *CCR5* were able to disrupt the gene in human CD4+ T cells and in CD34+ hematopoietic stem cells (HSCs), able to give rise to multi-lineage progeny with stably disrupted *CCR5*. The efficiency of gene disruption ranged from 17% to 25% when engrafted in immune-deficient or humanized mice, thus reducing HIV-1 levels and conferring resistance to HIV-1 infection [70-74]. Moving from bench to bedside, so far a few clinical trials

involving ZFN-mediated *CCR5* disruption have been completed, with promising results. In these trials, CD4+ T cells or HSCs from HIV-1 infected patients were treated *ex-vivo* and then the corrected cells were reinfused into patients (NCT00842634, NCT01044654, NCT01252641 and NCT02500849). Safety of this protocol was evaluated in an open-label, nonrandomized, uncontrolled study of a single dose of ZFN-modified autologous CD4 T cells, in which the *CCR5* gene was inactivated (NCT00842634) [75]. The study enrolled 12 HIV-positive aviremic patients who were receiving highly active antiretroviral therapy. In six patients, antiretroviral treatment was interrupted 4 weeks after infusion of CD4 T cells, 11%- 28% of which were genetically modified. Treatment was well tolerated and only one serious adverse event occurred, attributed to a transfusion reaction. The count CD4 T-cells significantly increased after transfusion and, in patients who interrupted antiretroviral therapy, the decline in circulating *CCR5*-modified cells was significantly lower than the decline in unmodified cells, suggesting engrafting of the cells and resistance to HIV infection [75].

The aforementioned CRISPRa system can also be exploited to influence the expression of key cellular host factors representing anti-viral defense in human cells, such as antiviral restriction factors. This approach was applied in an HIV-1 model of infection of human CD4+ T cells to enhance the expression of the antiviral genes *APOBEC3G* and *APOBEC3B*, members of the cytidine deaminase gene family that can edit newly synthetized HIV provirus intermediates. As *APOBEC3G* is susceptible to degradation by the HIV-1 Vif protein, whereas *APOBEC3B* is resistant, only the induced *APOBEC3B* inhibited wild-type HIV-1 infectivity whereas *APOBEC3G* inhibited replication of a Vif-deficient (ΔVif) HIV-1 provirus [76]. Such strategy could also be applied to other viruses such as HBV, in which stimulation of IFN by *APOBEC3A* and *3B* [77] or Toll-like receptor 7 [78] has been reported to induce degradation of HBV cccDNA.

From a virological perspective, targeting host factors required for viral infection and replication with programmable nucleases will open up a number of possibilities for basic research. Genome-scale CRISPR–Cas9 screens are already employed to globally investigate the effects of individual human gene products on the biological cycle of clinically relevant viruses, including HCV, HIV, dengue virus, Zika virus, and West Nile virus, leading to new insights into viral molecular biology and giving the opportunity to provide new potential targets for antiviral drug development [79].

4. Bacterial infections

The CRISPR–Cas9 system has evolved in bacteria and archea as an acquired immune system to protect against invading phages and plasmids. These systems can be repurposed for lethal self-targeting, leading to selective bacterial elimination. Due to the lack of efficient DNA repair pathways, bacteria are particularly susceptible to genomic DNA damage and cell death as compared to eukaryotic cells. Furthermore, nuclease-deactivated Cas9 can be used to control bacterial gene expression, rather than to cleave DNA sequences [80]. Genome editing-based antimicrobial strategies are illustrated in Figure 2.

4.1. Lethal self-targeting of bacterial genomes

As mentioned above, an obvious application of CRISPR–Cas systems is their repurposing for self-targeting and damaging bacterial genomes, leading to cell death [81-83]. The proof-of-principle of this approach was demonstrated in the *Escherichia coli* model by engineering potent type I CRISPR–Cas systems, which cleave and degrade DNA through the action of the 3'-to-5' exonuclease Cas3 [84]. In this model, genome targeting was highly sequence-specific and allowed the selective elimination of targeted bacterial strains within mixed cultures. Cell killing was the result of chromosomal injury, since it was similarly potent regardless of the targeted locus, its genomic location, and transcriptional activity. Similar results were achieved by engineering type II CRISPR–Cas systems, in which the Cas9 endonuclease causes double-strand cleavage of target DNA sequences [85,86]. The type I CRISPR–Cas system is expected to be more efficient in inducing DNA damage and cell killing than type II systems, because its exonuclease activity results in large-scale genomic alterations [86,87], thus enabling more potent eradication of a bacterial population. In addition, the type I CRISPR–Cas systems is widespread in many pathogenic bacteria, such as *E. coli* or *Clostridium difficile* [88], and could be repurposed to their self-targeting.

Treatment with CRISPR-Cas-based antimicrobials may lead to uncomplete killing of bacteria populations, mainly due to inefficient delivery of the CRISPR-Cas system and to the emergence of defective CRISPR-Cas [84,85,89]. However, when applied to a mixed bacterial population, sequence-specific antimicrobials allow non-targeted cells to keep growing, which may in turn compete with the small proportion of targeted cells that survive the treatment.

4.2. Targeting bacterial antibiotic resistance and virulence genes.

Antibiotic-resistance and virulence genes represent elective targets for sequence-based antimicrobial strategies. These genes often reside in mobile elements, such as conjugative plasmids, phages, and transposons, and may be horizontally transferred and disseminated. Targeting antibiotic-resistance and virulence genes is feasible because the number of relevant pathogens responsible for the majority of antibiotic-resistant infections in humans is relatively small (e.g., *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Enterococcus faecium*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*), thus requiring the development of only a limited number of tools. Actually, the emergence and worldwide spread of these multidrug-resistant bacteria is a serious threat to public health, leading to significant morbidity, mortality, and increase of healthcare costs.

Targeting bacterial antibiotic resistance and virulence genes was pursued by Citorik *et al*. [85] and Bikard *et al*. [89], who also addressed the difficult task of developing efficient methods to deliver genome editing tools into target bacterial cells. Citorik *et al*. [85] exploited two different horizontal genetic transfer systems of bacteria to deliver molecular constructs to bacterial cells, i.e. plasmid conjugation and viral transduction. The first approach, based on a broad-host-range conjugative plasmid carried by an *E. coli* strain, was hampered by low conjugation efficiency. The second delivery system was based on a M13-phagemid vector, i.e., a plasmid encoding specific genes packaged into a phage capsid. Phagemids were engineered to carry CRISPR-Cas9-

based RNA-guided nucleases targeting the bla_{SHV-18} or bla_{NDM-1} genes, which confer extended-spectrum and pan-resistance to β-lactam antibiotics, respectively. Treatment with phagemids of *E. coli* strains carrying the resistance genes resulted in a significant reduction of viable cells, while no toxicity was observed in wild-type *E. coli* strains. Interestingly, the mechanisms of cell killing after targeted cleavage of endogenous plasmids were shown to result from the activation of plasmid-borne toxin-antitoxin systems. The specificity of the CRISPR-Cas system was challenged by targeting a single-nucleotide mutation in the chromosomal DNA gyrase gene ($gyrA_{D87G}$), which confers resistance to quinolone antibiotics. Experiments showed that a targeted phagemid vector selectively killed only *E. coli* cells harboring the $gyrA_{D87G}$ mutation, but not other *E. coli* strains with the wild-type *gyrA* gene [85]. To demonstrate the versatility of the CRISPR-Cas system to combat pathogens, this was engineered to target the chromosomal *eae* gene, which encodes intimin, a cell-surface adhesin in enterohemorrhagic *E. coli* O157:H7 (EHEC) that is necessary for intestinal colonization and tissue damage. However, *eae* targeting led to only a 20-fold reduction of EHEC cell viability, probably because of inefficient phagemid delivery to this bacterial strain. Anyway, treatment significantly increased the survival of EHEC-infected *G. mellonella* larvae, an *in vivo* infection model to assess the efficacy of antimicrobials [85].

Bikard *et al.* [89] used a similar CRISPR-Cas9-based approach to target antibiotic resistance and virulence genes in bacteria, but exploited the ΦNM1 phage for phagemid encapsidation and *S. aureus* as the model pathogen. A CRISPR-Cas9 antimicrobial that targeted the methicillin resistance gene *mecA* led to a marked decrease of *mecA*-carrying *S. aureus* in mixed cultures, while CRISPR-Cas9 that targeted a plasmid conferring tetracycline resistance in *S. aureus* did not result in cell death, but in selective degradation and loss of the plasmid in a bacterial population. In addition, this CRISPR-Cas9 antimicrobial could immunize nonpathogenic *S. aureus* strains against the transfer of antibiotic-resistant plasmids. *In vivo* tests in a mouse model of skin colonization showed that CRISPR-Cas9 phagemids selectively reduced the proportion targeted *S. aureus* strains, more efficiently than standard treatment with the topical antibiotic mupirocin [89].

An improvement to CRISPR-Cas9 antimicrobial strategies was achieved by combining spacers targeting antibiotic resistance with spacers conferring a selective advantage to antibiotic-sensitive bacteria by protection from a lytic phage [90]. The engineered CRISPR-Cas system was introduced into a λ prophage, and thus made transferable to bacteria by lysogenization. When treated with lytic phages, lysogenized bacterial were protected, enabling the positive selection of a population of antibiotic-sensitized bacteria [90].

4.3. Driving bacterial gene expression

Nuclease-deactivated Cas9, termed dCas9, in which the RuvC and HNH nickase domains have been mutated, is unable to cleave DNA but retains its ability to bind specific DNA sequences when guided by a sgRNA [80,91,92]. Regulation of gene expression by dCas9 was first demonstrated in *E. coli*, where delivery of dCas9 with a sequence-specific sgRNA led to inhibition of gene expression (CRISPR interference, CRISPRi) [93]. The dCas9-sgRNA complex interferes with gene expression probably by blocking RNA polymerase or by hindering the binding of transcription activators [93]. Like the CRISPR-Cas9 nuclease, CRISPRi is highly

efficient, has minimal off-target effects, and is multiplexable, allowing the simultaneous repression of multiple genes. However, in bacteria, CRISPRi is polar, since any operon gene downstream or upstream of the dCas9 binding site is silenced in addition to the targeted gene [94,95]. As mentioned above, mutant dCas9 can be also used as a fusion protein to activate gene expression (CRISPRa). For example, dCas9 fused with the ω-subunit of *E. coli* RNA polymerase enhanced gene expression by facilitating assembly of the enzyme to the targeted promoter [96]. Applications of the CRISPRi and CRISPRa technology to regulate gene expression in bacteria are still limited [93, 95-98]. In *Mycobacterium tuberculosis*, CRISPRi mediated by dCas9 from *Streptococcus thermophilus* could efficiently and specifically knockdown gene expression and inhibit cell growth after targeting essential genes [94]. In addition, CRISPRi proved its usefulness in dissecting the mycobacterial folate biosynthesis pathway, with potential utility in drug target discovery [94].

5. Fungal infections

Antimicrobial strategies based on targeting drug-resistance and virulence genes, which have been developed against bacteria, could potentially be applied to eukaryotic pathogens, such as yeast, protozoa, and nematodes. However, these organisms have a larger size and diploid genome and more efficient DNA repair mechanisms than prokaryotes, making them less susceptible to the lethal effects of gene targeting. At variance, their genome may be easily modified by genome editing technologies, which are providing powerful research tools to dissect the basic mechanisms of infection and pathogenesis. Despite these advances, efficient intracellular delivery of exogenous biomolecules remains a major challenge for genome editing applications.

The CRISPR/Cas9 system has been optimized and adapted to fungi by using fungal constitutive or inducible promoters to drive Cas9 and sgRNA expression and codon-optimized Cas9 [99-104]. In addition, methods to achieve transient expression of Cas9 have been developed, in order to limit its cytotoxic effects [103]. The CRISPR-Cas9 complex has been delivered to fungi through transformation mediated by polyethylene glycol, agrobacterium, and blastospores [102]. CRISPR-Cas9 has been successfully applied in the genetic manipulation of several fungi, including model organisms, such as *Schizosaccharomyces pombe* [105] and *Saccharomyces cerevisiae* [106-108], and fungal pathogens, such as *Aspergillus fumigatus* [99,109], *Candida albicans* [110,111], other *Candida* species [112-114], and *Cryptococcus neoformans* [115,116], paving the way for applications in pathogenesis studies, characterization of mechanisms of drug resistance, and drug discovery.

6. Parasite infections

Studies that used genome editing technologies to target the genome of protozoa and nematodes aimed mainly at developing easy and efficient methods for gene knock out and genome engineering, with applications in

reverse genomics for the identification of virulence genes, new drug targets, and drug resistance mechanisms, while direct genome targeting has not been exploited so far for therapeutic purposes.

For example, ZFNs have been used to generate deletions, allelic replacement, and specific point mutations in endogenous genes of *Plasmodium falciparum*, including point mutations in the chloroquine resistance transporter gene [117]. CRISPR-Cas9 has been used to rapidly generate specific gene knockouts and single-nucleotide substitutions in *P. falciparum* [118-121], leading to the characterization of mutations associated with drug-resistance. Gene editing strategies to attenuate or kill malaria parasites for vaccine development and therapy have been proposed [122]. CRISPR-Cas9 allowed characterizing drug-resistance mutations and new potential therapeutic targets in *Toxoplasma gondii* [123-125]. In *Trypanosoma cruzi*, knock down of multiple genes was performed for functional genomics studies [126,127]. Proof of concept of CRISPR-Cas9-mediated genome editing was demonstrated for *Leishmania major* [128] and *Leishmania donovani* [129], with the introduction of loss-of-function insertion and deletion mutations.

The CRISPR-Cas9 technology has been also applied to the model organism *Caenorhabditis elegans* [130,131], a nematode that is widely used for the study of metazoan biology and for parasitology research. In multicellular parasite organisms, efficient *in vivo* delivery of genome editing constructs is even a more critical issue, in order to allow biallelic targeting of multiple cells in different tissues. Enrichment of genome editing events, which has been achieved in arthropods by using gene drivers [132] and mutagenic chain reaction [133], could be potentially applied to expand modified genetic traits in parasite populations.

7. Vector-borne infections

Vector-borne infectious diseases, which include infections caused by viruses, bacteria, and parasites, represent a relevant burden of disability and death, especially in developing countries. The most effective strategies to combat vector-borne infectious diseases are based on the control of arthropod vectors, which is hindered by increasing insecticide resistance. Novel approaches to control vector populations, especially mosquitoes, used genetic engineering technologies to generate genetically modified and sterile strains [134,135]. In particular, gene drive systems are exploited to rapidly spread these genetic modifications throughout vector populations, in order to promote their crash or to propagate alleles that make vectors less competent to pathogen transmission. Gene drives are selfish genetic elements that are transmitted to progeny more frequently than expected from Mendelian genetics. Example of gene drive systems found in nature are represented by transposable elements and homing endonuclease genes, and they have been already applied to generate transgenic mosquitoes [135,136]. The advent of CRISPR-Cas9 tools has allowed overcoming technical hurdles and to speed up the development of artificial gene drives, with relevant results already achieved [134-136], such as the introduction of anti-*P. falciparum* effector genes [137] and the inactivation of female fertility genes [132] in *Anopheles* mosquitoes to combat malaria. The risks related to potential off-target effects, the emergence of resistant strains of vectors, the ecological impact of suppression of mosquitoes and other vector

populations, and the spread in the environment of genetically modified organisms should be carefully evaluated before field implementation of these new technologies in vector control programs [135,138].

8. Expert commentary

8.1. Safety concerns

An important feature to keep in mind when designing a strategy with programmable nucleases is the risk of off-target effects, namely the possibility that other undesired loci of the human genome, homologous to the one to target, might be cleaved, giving rise to perturbations in the DNA that can lead to disease or tumor formation. Notwithstanding, in the human genome the recognition of a DNA sequences of 17 or more bp should minimize this event [139], actually, all the above mentioned nucleases can cause off-target mutations [20, 140-142]. The off-target activity of ZFNs, TALENs and CRISPR-Cas9 depends exclusively on the specificity of their DNA-binding domains, as their cleavage domain is not DNA-specific. ZFN-induced off-target effects are likely determined by the complex context dependence of the ability of ZF domains to bind DNA as well as the frequency and location of sites in the genome actually bound by ZFNs [141,143], thus being highly dependent on the ZFN being tested. As TALE domains are less tolerant of target sequence mismatches than ZFs, TALENs are likely to have higher specificity for target-binding site and cleavage [144]. Although several studies have shown that the CRISPR-Cas9 system can exert off-target activity *in vitro*, recent *in vivo* studies showed how this system is highly specific and rarely exert off-target effects [145,146]. In contrast, whole genome sequencing analysis indicated that sgRNAs might exert mutations *in vivo* in noncoding RNAs or other regulatory intragenic regions that are independent of the target loci and that could be harmful to key cellular processes [147]. The results raise important safety issue for clinical applications of CRISPR-Cas9, but need to be further investigated and confirmed. There are several strategies that can be applied in order to reduce the off-target effect of programmable nucleases. HEs usually show very high specificity for their DNA targets and exert less off-target cleavage, as demonstrated by low toxicity *in vitro*, even if expressed at high levels [23].

Significant reduction of off-target cleavage has been achieved by choosing unique target sites without highly homologous sequences within the human genome [148,149]. A further reduction was accomplished by approaches focused on the cleavage domain such as forcing *Fok*I subunits to interact as obligate heterodimers [150], by replacing it with a DNA-specific nuclease (such as HE) [151] or by using chimeric enzymes [149]. For example, HPV18 was successfully targeted by fusing the staphylococcal nuclease, cleaving DNA as a monomer, to a ZF protein thus not requiring dimerization [55]. Moreover, the avoidance of unwanted indels was achieved by converting *Fok*I or Cas9 into nickases exerting SSBs [148,153,154]. Such system proved to be successful in targeting HBV by designing two pairs of guides to introduce a DSB at target DNA with no apparent off-target mutations detected [155,156]. Furthermore, the use of truncated sgRNAs [157] or larger PAM sequences, demonstrated reduction of off-target cleavage with efficient on-target editing [158]. Finally,

the delivery of nucleases in form of mRNAs or proteins can lead to faster expression and lower toxicity compared to plasmids due to the rapid degradation into the host cells [159,160]. Despite the optimistic low rate of off-target events reported for all classes of nucleases, post editing verification such as next generation sequencing methods will be necessary in order to add confidence to results by increasing numbers and quality of reads of off- target sites.

Since all the four classes of nucleases originate in part from non-human organisms, they are potential immunogens that can redirect the immune-responses and trigger the clearance of the transfected cell. To date, few data are available on the *in vivo* toxicity or immunogenicity of programmable nucleases. Clinical trials with ZFNs showed no toxicity or immunogenicity, but the delivery of ZFNs to CD4+ T cells was done *ex vivo*, allowing for expansion of corrected cells before transplantation into patients [75]. In therapeutic mouse models of HBV and HPV infection, no *in vivo* toxicity was observed with TALENs [64,161].

Other important safety problems are represented by either the potential occurrence of multiple DNA DSBs in the same genome that might lead to genomic rearrangements or by the risk that cleavage of episomal genomes of viruses, such as HBV and HPV, with subsequent linearization, could promote their random integration into the target cells and hence the risk of tumor development. As the long-term effects of exposure to programmable nucleases *in vivo* remains unknown, safety procedures such as the use of tissue-specific, drug-inducible, or non-constitutive viral promoters, together with evaluation of the possible off-target effects and multiplexed targeting within the virus genome, should be taken into consideration to ensure the safety of the procedure.

8.2. Delivery challenges

When designing a therapeutic strategy with programmable nucleases, the delivery method is one of the most important hurdles to face. In fact, to obtain a therapeutic effect, nucleases must be delivered efficiently to infected cells and expressed at high levels. Several factors must be taken into consideration: the type of pathogen, the type of infected cell that need to be targeted, the accessibility of the anatomical site of infection, the dosing necessary to exert the desired effect, and the type of delivery system. Pathogens that are primarily found in a restricted anatomic site will be more suitable for the targeted delivery of programmable nucleases, whereas pathogens capable to infect blood cells or multi organs may be more challenging to treat *in vivo*, since they require systemic delivery. *Ex vivo* delivery is considered safer than *in vivo* delivery, but it has been applied so far only to treat disorders of the hematopoietic system. Several delivery approaches, based on virus-free system (such as electroporation, liposomes) and viral vectors (such as lentiviral vectors, adenoviral vectors, AAV vectors, phagemid vectors), have been developed and applied in clinical trials for delivery of therapeutic genes [162]. Methods based on non-viral delivery systems are less immunogenic and have a lower risk of chromosomal integration than viral vector-based approaches, but transfection efficiency is usually lower and not cell-type specific. For example, the liver is a good target for infection by AAV vectors, especially AAV8, which can be used to transduce HBV-infected hepatocyte *in vivo*. Viral vector have limitations in their capacity to accommodate foreign sequences, including those encoding programmable nucleases. Specifically, HEs are

the smallest class of programmable nucleases (about 250 amino acids) and this facilitates their insertion into any delivery vector. ZFNs, in turn, are smaller than TALENs and, unlike the latter, can be incorporated into small-capacity AAV vectors [46] or integrase deficient lentiviral vectors [163]. In addition, the highly repetitive nature of the coding sequences for ZFNs and TALENs represent a challenges in viral packaging, due to undesired recombination during the production process [164]. Since the enzyme Cas9 has a large size, smaller Cas molecules that can be efficiently packaged into small capacity viral vectors have been engineered, such as Cas from *S. aureus* [165-167].

Phages are ideal vectors for gene transfer in bacterial cells, since they have evolved to infect and introduce their genetic material into bacteria. However, their potential clinical use in humans is limited to the treatment of bacterial infections in external sites, such as external ear infections or burn wound-associated infections. In fact, after systemic injection in mice, most phages are rapidly sequestered by the liver and the spleen [168]; in addition, the narrow host range of phages limits their application to different pathogens, while the relatively large size hinders efficient diffusion into infected tissues; finally, induction of neutralizing antibodies prevents the possibility of repeated administrations of phages [169].

The use of non-viral delivery methods for bacterial cells is hampered by the cell wall. A recent study [170] developed a novel non-viral delivery method, called Cr-Nanocomplex, which was based on a nanocomplex of polymer-derivatized Cas9 protein and sgRNA targeting bacterial antibiotic resistance. In particular, in the nanocomplex, recombinant Cas9 was covalently modified with branched polyethylenimine, a cationic polymer, as the carrier for packaging sgRNA. At variance with approaches used for mammalian cells, based on the noncovalent encapsulation of Cas protein and sgRNA into nanoparticles and characterized by low loading and packaging efficiencies, the direct covalent modification of the protein allowed to minimize the amount of carrier material and hence its toxicity. *In vitro* experiments showed that a Cr-Nanocomplex targeting *mecA* could be successfully delivered into methicillin-resistant *S. aureus*, resulting in reduced growth of bacteria [170].

8.3. Emergence of resistant pathogens

Another concern about the use of programmable nucleases for the treatment of infectious diseases is the emergence of escape mutants that develop resistance to treatment. These mutants could be the result of the acquisition of genetic changes in target sites acquired during replication or may be induced by the nucleases themselves. This scenario is more likely to occur in error-prone RNA viruses than DNA viruses [171]. The emergence of endonuclease resistance can be prevented by multiple targeting of different regions of pathogen genome [175]. Replication-competent HIV-1 with mutation in nuclease target sites emerged after exposure to ZFNs targeting reverse transcriptase [172] or to CRISPR-Cas9 targeting HIV proviral DNA [173].

Anti-bacterial and anti-parasitic therapies based on programmable nucleases may also induce the emergence of escape mutants, characterized for example by a defective CRISPR-Cas, with loss of spacer sequences or deletion of the *cas9* gene [84,85,89]. Importantly, the occurrence of escape mutations in the targeted sequence

was not observed in experimental studies, indicating a low risk for the emergence of resistant bacterial strains [89].

9. Five-year view

Despite the great advances that have been made in the past few years with proof-of-concept studies demonstrating the feasibility of harnessing programmable nucleases for the treatment of antimicrobial diseases, many more are needed to concretely translate them into clinical and field applications. Obstacles that still should be overcome are mainly related to the safety issue. Thus, in a five-year view, advances are expected in the generation of nucleases lacking off-target effects and with higher cleavage efficiency, and in the development of sensitive diagnostic methods to detect the presence of off-target mutations. The applicability of genome editing-based antiviral therapy has been proven in clinical trials of ZFN-based inactivation of the *CCR5* gene to block HIV-1 infection [75], but further clinical studies are warranted in the next years to test safety and effectiveness of genome editing-based therapies. Another problem to be solved is the improvement in the efficiency and safety of delivery systems for programmable nucleases, especially if directing them for *in vivo* administration is the goal. Viral vectors have been widely used for genome editing in the research field, but it is expected that non-viral vectors will have more chances to be approved for clinical applications. Topical treatment of infections involving external sites, such as skin, mucosa, burn wounds and the eye, will probably have more chances of success than treatment of systemic infections. Development of strategies based on fine dosing and transient intracellular expression of the gene editing machinery is also preferable, since continuous expression of high-levels nucleases in the long run may increase the risk of off-target effects and the emergence of escape mutants. Definitely, before moving to the bedside, studies in suitable *in vivo* animal models will be necessary, but with the important limitation that animal models are not available for all infectious diseases.

Key issues

- Programmable nucleases, such as ZFNs, TALENs, CRISPR-Cas9 and HEs, are molecular scissors that can be targeted at predetermined loci in order to modify the genome sequence of an organism. The main mechanisms by which programmable nucleases can be employed for the treatment of infectious diseases is the introduction of mutations into the genome by the error-prone NHEJ mechanism.
- Programmable nucleases can be exploited as antiviral treatment by targeting essential viral genes or host factors that are required for viral infection and replication. Efficiency of these strategies has already been proven *in vitro* and *in vivo* models of HIV, HBV, HSV, and HPV infection. Phase I clinical trials have been initiated to tests safety and efficacy of targeted nucleases in patients with HIV infection and with HPV-related cervical lesions.

- As bacteria are particularly susceptible to genomic DNA damage and cell death compared to eukaryotic cells, the CRISPR-Cas9 systems has been redirected for lethal self-targeting, allowing selective elimination of targeted bacterial strains. In addition, CRISPR-Cas9 has been employed to target antibiotic-resistance genes and virulence genes. Furthermore, nuclease-deactivated Cas9 has been used to interfere with bacterial gene expression by blocking RNA polymerase or by hindering the binding of transcription activators.
- Feasibility of using programmable nucleases to modify the genome of parasites and fungi and to manipulate the genome of arthropod vectors has been demonstrated, opening perspective for therapeutic and vector-control applications.
- In the past few years, many progresses have been made in demonstrating the feasibility of programmable nucleases as therapeutic strategy for the treatment of infectious diseases. However, there are still many hurdles to overcome such as the safety issues, mostly dictated by the risk of off-target mutations, the raising of escape mutants, and the inefficiency of delivery methods, before translating results from preclinical studies into clinical and field applications.

Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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*** = of interest, ** = of considerable interest**

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Figure legends

Figure 1. Genome editing strategies for antiviral therapy. Targeting viral genome: programmable nucleases can be exploited for the direct targeting of virus genome leading to disruption of essential viral genes. **Targeting of integrated viral genome:** integrated viruses such as HIV can be targeted at both LTRs leading the excision of the provirus. Alternatively, programmable nucleases can act throughout NHEJ to disrupt essential viral sequences. **Targeting key host factors:** programmable nucleases can target viruses key host factors essential for virus entry/replication. **Activating viruses/restriction factors:** nucleases activation can be directed to induce virus reactivation in latently infected cells, triggering the immune system to recognize infected cells; the same system can be applied to activate host restriction factors that can block the virus at different biological levels.

Figure 2: CRISPR-based strategy as antimicrobials. Lethal self-targeting of bacterial genomes: by delivering, via engineered phages, self-targeting spacers, endogenous Cas3 exonuclease can be redirected, leading to chromosome targeting and degradation. **Targeting bacterial antibiotic resistance genes:** chromosome targeting and degradation can be achieved also by gRNA/Cas9 system to generate DNA DSBs, which can be edited using the endogenous DNA repair pathways. Alternatively the same approach can be adopted by using **nanosized CRISPR complexes** (Cr-Nanocomplex), polymer-derivatized Cas9 complexed with sgRNA targeting antibiotic resistance. **Blocking virulence gene transcription:** The block of transcription can be obtained by using a deactivated Cas9 (dCas9), able to bind but not cleave the target sequence.

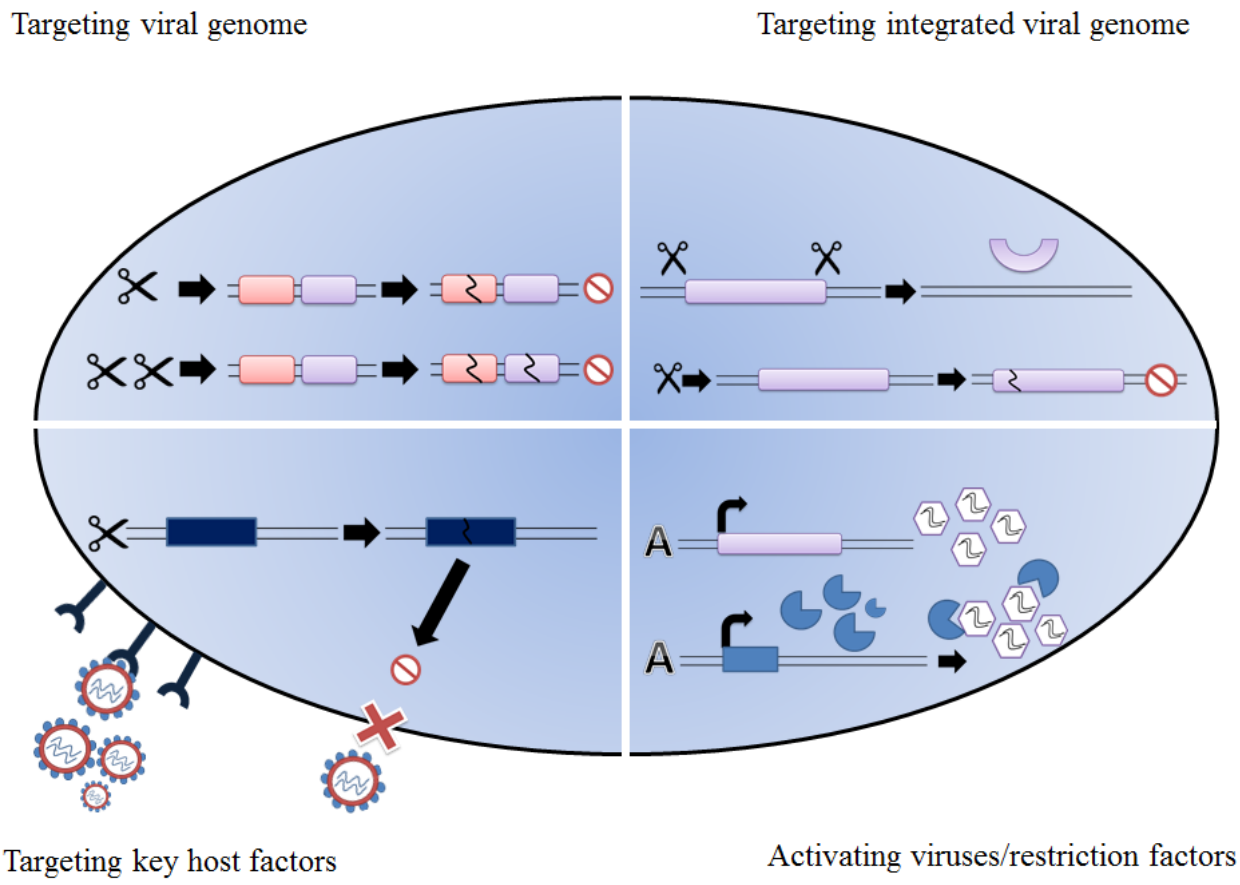
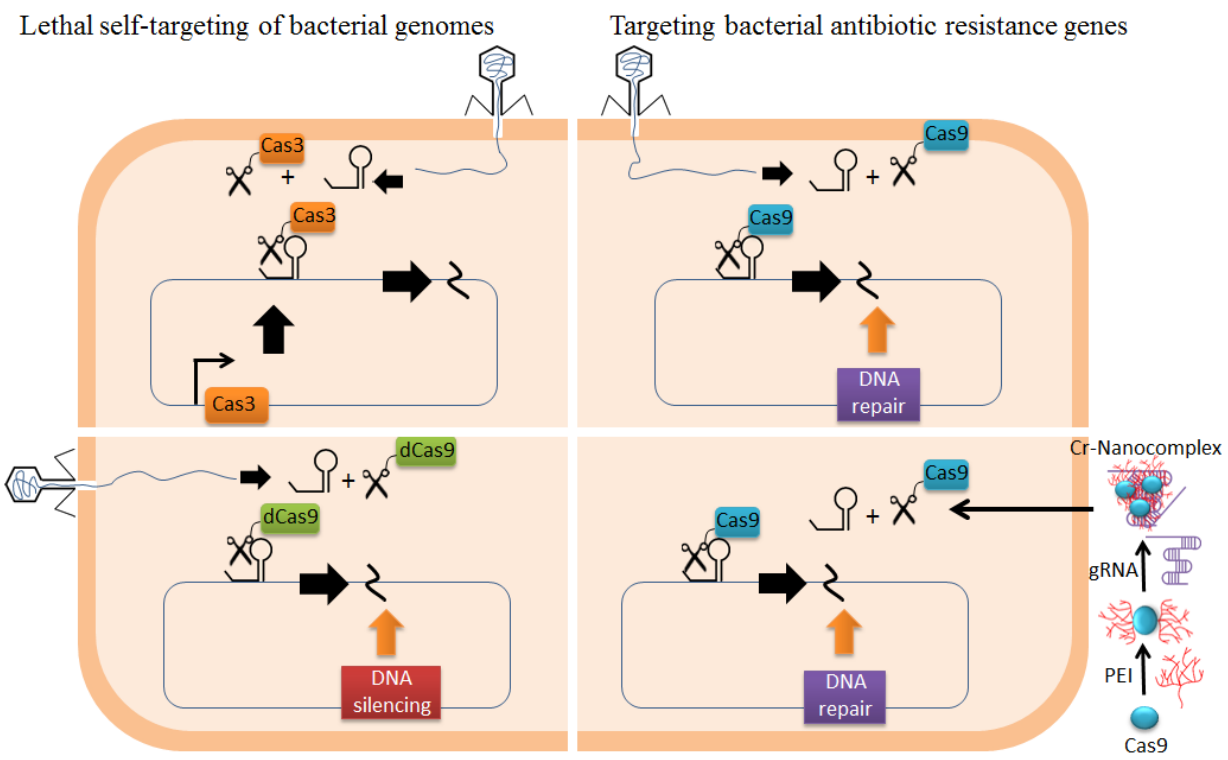

Targeting viral genome
Targeting integrated viral genome
A
A
Targeting key host factors
Activating viruses/restriction factors

Figure 1


Lethal self-targeting of bacterial genomes
Targeting bacterial antibiotic resistance genes
Cas3
Cas3
Cas3
Cas9
Cas9
DNA repair
dCas9
dCas9
DNA silencing
Cas9
Cas9
DNA repair
Cr-Nanocomplex
gRNA
PEI
Cas9
Blocking virulence gene transcription
Using nanosized CRISPR complexes

1

2 Figure 2