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REVIEW

Therapeutic application of the CRISPR system: current issues and new prospects

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Abstract

Since its discovery, the Clustered Regularly Interspaced Short Palindromic Repeat (the CRISPR) system has been increasingly applied to therapeutic genome editing. Employment of several viral and non-viral vectors has enabled efficient delivery of the CRISPR system to target cells or tissues. In addition, the CRISPR system is able to modulate the target gene's expression in various ways, such as mutagenesis, gene integration, epigenome regulation, chromosomal rearrangement, base editing and mRNA editing. However, there are still limitations hindering an ideal application of the system: inefficient delivery, dysregulation of the delivered gene, the immune response against the CRISPR system, the off-target effects or the unintended on-target mutations. In addition, there are recent discoveries that have not been yet applied to CRISPR-mediated therapeutic genome editing. Here, we review the overall principles related to the therapeutic application of the CRISPR system, along with new strategies for the further application and prospects to overcome the limitations.

Introduction

Since it was first reported, the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) system has been considered one of the most powerful genome-editing tools owing to its several features. The CRISPR system is easy to design in that it only requires replacement of guide RNA (gRNA) to target different sequences. In addition, the CRISPR system shows efficient genome editing along with its ability of multiplex genome editing. The CRISPR system originates from RNA-guided DNA cleavage systems in bacteria or archaea, and provides adaptive immunity for them (Barrangou et al. 2007; Garneau et al. 2010). Part of the viral or plasmid DNA is acquired and processed for insertion into the bacterial genome to form the CRISPR region (Xiao et al. 2017). In type 2 CRISPR system, pre-CRISPR RNA (pre-crRNA) and *trans*-activating CRISPR RNA (tracrRNA) are transcribed from the region, and form a complex with CRISPR-associated nuclease 9 (Cas9), which refers to streptococcus pyogenes Cas9 (SpCas9) here (Brouns et al. 2008; Deltcheva et al. 2011). This complex then binds to the target site, which is recognized by guide RNA and Cas9. The target site comprises of a 5' upstream protospacer, 20-base pair sequences for SpCas9, recognized by complement guide RNA, and a 3′ downstream protospacer-adjacent motif (PAM) sequences, recognized by Cas9 (Sternberg et al. 2014; Anders et al. 2014). For more efficient genome editing, crRNA and tracrRNA can be linked to form single-guide RNA (sgRNA) which simplifies the structure (Jinek et al. 2012). The CRISPR-Cas9 complex then induces a double-strand break (DSB) at the target site. Since its discovery, many researchers have contributed to the progress in this field of the CRISPR system research. Some researchers have been involved in description of various CRISPR-associated nucleases, like Cas12a (Cpf1) (Kim et al. 2017). Others have applied bioinformatics to CRISPR research, generating relatively accurate systems for describing biological actions of the CRISPR system (Allen et al. 2018; Bae and Kim 2018; Wienert et al. 2018). Collateral non-specific activity of Cas12a or Cas13 showed its potential for the detection and diagnosis of the pathogenic nucleic acids (East-Seletsky et al. 2016). Finally, efforts have been made to apply the CRISPR system for therapeutic purposes.

In that many diseases are caused by the abnormal gene expression, there have been struggles for regulating the

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genes to help reverse disease phenotypes. The first generation of gene therapy involved RNA interference (RNAi) or viral transgene delivery. Though these approaches showed therapeutic effects on several disease models (Gonzalez-Rodriguez and Valverde 2015; Dahlmann and Stein 2015; Bisset et al. 2015; Nanou et al. 2013), problems occurred. For example, RNAi raised the problems like off-target effects, insufficient suppression of target gene expression (Mansoor and Melendez 2008; Castanotto and Rossi 2009). Viral transgene delivery also raised some problems, which will be discussed later in this paper. Programmable nucleases such as zinc-finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN) and CRISPR-associated nuclease have demonstrated their therapeutic values in several diseases models. Several clinical trials are going on for the programmable nucleases, each of which has its own pros and cons. For instance, treating HIV infection using ZFN is being studied (Alkhatib et al. 1996; Deng et al. 1996; Maier et al. 2013). TALEN has been employed to chimeric antigen receptor (CAR) T cell therapy, an emerging immunotherapy against hematologic malignancies (Jung and Lee 2018). Clinical trials of CRISPR-Cas9 applied to anti-cancer cell therapy or against HIV infection are imminent (Reardon 2016; Cyranoski 2016). ZFN is composed of *Fok* I nuclease, which shows its activity by dimerization and DNA-binding zinc-finger protein (ZFP) (Kim et al. 1996; Bitinaite et al. 1998). Certain ZFP is known to target 3-base pair DNA sequences. However, there are not enough ZFPs to target all 64 combinations of 3-base pair DNA sequences, which means ZFN has limited targeting capacity (Bae et al. 2003; Segal et al. 1999). TALEN is composed of TAL effector DNA-binding domain and DNA cleavage domain of *Fok* I nuclease (Miller et al. 2011). While TALEN presents broad spectrum of target range, there are some difficulties. First, constructing TALEN can be challenging and time consuming. In addition, DNA sequences encoding TALEN is highly repetitive. This makes viral delivery of TALEN hard because of the potential genetic recombination between TALEN and the virus (Lau et al. 2014). In addition, TALEN is more sensitive to the DNA methylation than the other programmable nucleases (Bultmann et al. 2012). Though the CRISPR system has some limitations like off-target effects or restriction of target sites by PAM sequences, due to its advantages like easy design, efficiency or ability for multiplex genome editing (Wang et al. 2013; Niu et al. 2014), it has been widely applied to a broad spectrum of diseases. By localizing Cas9 to mitochondria, mitochondrial genome editing is possible (Jo et al. 2015). Fusing the adenosine deaminase acting on RNA (ADAR) with catalytically inactive Cas13, a sort of Cas enzyme, enabled successful mRNA editing (Cox et al. 2017).

Though the CRISPR system is broadly applied, it definitely has some limitations including off-target effects. Currently, there are still obstacles to the optimal delivery of the system to target sites. Meanwhile, therapeutic application of some recent discoveries, like targeted chromosomal elimination (Zuo et al. 2017), has not been well studied to date. In this review, we will discuss about the overall principles of therapeutic application of the CRISPR system, along with the limitations of the CRISPR system and related prospects for the solution. First, we will address the means of CRISPR system delivery to the target sites with considerations for therapeutic application. Then we will review genome-editing mechanisms by the CRISPR system, including DSB repairing by homology-directed repair (HDR), non-homologous end joining (NHEJ), micro-homology-mediated end joining (MMEJ) or single-strand annealing (SSA), as well as epigenome regulation, chromosomal rearrangement, base editing and mRNA editing with strategies for their further therapeutic application. Finally, we will discuss about immune responses and off-target effects induced by the CRISPR system.

Delivery: first step to successful gene therapy

Achievement of successful genome editing using the CRISPR system must be preceded by successful delivery of the system. This is applied not only to the CRISPR system but also to many other biomolecules, in that no matter how efficient the machineries are, they are not beneficial if they are not delivered to the target site.

Several barriers against successful delivery exist; in particular, many obstacles to in vivo delivery are needed to be addressed. There are several ways to deliver proteins or nucleic acids to the target site, which are briefly summarized in Table 1, with conventional pros, cons and the existing or potential improvements of each delivery. The current delivery methods can usually be classified into one of two categories: viral or non-viral delivery.

Viral delivery

Sorts of viral vectors

Viral vectors can deliver genetic materials into target cells, depending on the vectors' tropism. Currently, in spite of relatively expensive production costs (Pezzoli et al. 2012), viral vectors are being widely used for both in vitro/ex vivo and in vivo delivery because of its delivery efficiency. Generally, four types of viruses are used as viral vectors to deliver the CRISPR system. Integration-deficient lentivirus (IDLV) vectors can endure inserts of a large size and exhibits low immunogenicity. Genetic materials delivered

Table 1 Methods of biomolecule delivery

	Pros	Cons	Improvements
Viral vectors	Efficient delivery Target cell/tissue specificity (tropisms)	Abnormal transgene expression Immune response Relatively expensive	**The abnormal transgene expression** Choose non-integrative viral vectors Safe harbor delivery Combining with non-viral delivery methods Inducible CRISPR system **The immune response** Immune screening of the patients Viral modification Immune suppression Plasmapheresis
Overall non-viral delivery	Transient expression of the delivered biomolecules Relatively low immune response	Low-target cell/tissue accumulation, specificity (except for the electroporation and micro-fluidic methods) Failure of endosomal escape	**The low accumulation, specificity** Site-specific US disruption of microbubble carrying the biomolecules Specific molecular interaction Collagenase (for the accumulation in the fibrotic tissue) **The failure of endosomal escape** Cationic or gas-generating particles
Electroporation	Efficient delivery	Cellular damage Usually in vitro/ex vivo	**The cellular damage** Preserving the zona pellucida (zygote delivery)
Micro-fluidic methods	Transfection of the 'hard-to-deliver' cells	Cellular damage Usually in vitro/ex vivo	**The cellular damage** TRIAMF
Hydrodynamic injection		Low efficiency of delivery Transient adverse effects	
Endocytosis-based methods	Non-integrative delivery	Inappropriate for the in vivo application Low-target cell/tissue specificity	**The incompatibility for the in vivo application** Employment for the delivery to kidney tissue **The low specificity** Receptor-mediated endocytosis
Lipid nanoparticles	Efficient delivery	Unstable in serum or solution (especially for cationic liposomes) Immune response (minor problem)	**The instability** Surface charge optimization Chemical modification **Recent improvements** Excellent efficiency by gRNA modification and high-dose administration Safe and tolerable, by designing biodegradable particles
Polymeric nanoparticles	Efficient delivery	Prolonged tissue deposition	**The tissue deposition** Biodegradable design **Improvement in target cell/tissue specificity** Magnetic nanoparticle
Cell-penetrating peptides	Assisting other delivery methods	CPP's instability CPP's mechanisms not fully understood	**The instability** Protein modification
Common limits of both viral and non-viral delivery		Renal clearance Extravasation problem	**The renal clearance** Vectors in complex with megalin or cubilin **The extravasation problem** US-mediated vessel cavitation Local hyperthermia Vasodilation drugs

US ultrasound, *TRIAMF* transmembrane internalization assisted by membrane filtration, *CPP* cell-penetrating peptide

by IDLV vector show transient expression. This can be an advantage in that continued expression of CRISPR-Cas9 can lead to unwanted off-target effects or genome instability. IDLV shows broad tropism, which means the CRISPR system can be delivered to many types of cells or tissues using IDLV vectors. CRISPR-Cas9 delivered by IDLV vectors showed effective genome editing both in vitro and in vivo (Ortinski et al. 2017; Chen et al. 2016; Waehler et al. 2007). Adeno-associated virus (AAV) vectors have low immunogenicity and small carrying capacity, less than 5 kilo-base pairs of nucleic acid (Chen et al. 2016; Wang et al. 2016). To carry large biomolecules like the base editor, the AAV vector requires the delivery molecule to be 'split'. This was validated in an adult mouse model of metabolic liver disease, whereby successful point mutation correction and restoration of normal phenotypes have been achieved by delivery of two AAV vectors carrying each part of the base editor (Villiger et al. 2018). Conventionally, AAV vectors were considered to have relatively longer expression duration of delivered transgenes than other viral vectors. However, recent study demonstrated that delivery of self-targeting the CRISPR-Cas9 system can achieve controlled expression of the system while effectively editing target sequences (Li et al. 2019). AAV vectors are applicable to both in vitro/ex vivo, and in vivo and have broad tropisms. Tropisms of AAV vectors vary according to the serotype. Certain AAV serotypes in different organisms may show different tropisms (Srivastava 2016; Lisowski et al. 2015). Furthermore, for each tissue, different AAV serotypes can show different delivery efficiency (Aschauer et al. 2013; Wiley et al. 2018; Hickey et al. 2017). Thus, for more successful delivery by an AAV vector, careful selection of the serotype is essential. Several clinical trials of AAV-based gene therapy are being done for diseases such as Duchenne muscular dystrophy, limb girdle muscular dystrophy type 2D, Becker muscular dystrophy and hemophilia (Mendell et al. 2010a, b, 2015; Al-Zaidy et al. 2015; Doshi and Arruda 2018). In 2017, gene delivery using AAV2 vector for RPE65-ssociated retinal dystrophy was approved by Food and Drug Administration (FDA), which is the first FDA approval of the gene therapy product targeting a disease caused by mutations in a single gene (Rodrigues et al. 2019). Adenoviral vectors can insert a large size of biomolecules and can be applied to in vitro, ex vivo and in vivo delivery. Though in vivo application of adenovirus vectors is generally known to be limited to the liver, there are some exceptions. For example, adenovirus serotype 49 showed effective gene transfer into human vascular cells (Dakin et al. 2015). Just like AAV vector delivery, expression duration of adenoviral vector-delivered genes tends to be long. In addition, immunogenicity of the vector is known to be relatively high (Bouard et al. 2000). There was an death case of adenoviral gene therapy because of the immune response. Thus, the immune response against gene therapy must be taken very seriously (Marshall 1999). Finally, baculoviral vectors can pack large size of biomolecules. Baculoviral vectors enable transient expression of delivered transgenes (Kost et al. 2005; Ames et al. 2007). Delivery of CRISPR-Cas9 system using baculoviral vectors exhibited successful genome editing in human cells (Hindriksen et al. 2017). In addition, using the baculoviral vectors, the CRISPR-dead Cas9 (dCas9), which is catalytically inactive Cas9, effectively suppressed the expression of certain microRNA (miRNA) in mouse model, by blocking the related regions of the genome (Luo et al. 2016).

Limits of the viral delivery, and the solutions

Tropisms of the vectors

Though viral vectors are widely employed for both in vitro/ex vivo and in vivo delivery, they have some serious flaws; choosing viral vectors can be restricted by tropism. Modifying viral structure could be a solution to this problem. For example, capsid modification of AAV2 led to successful cell entry of AAV2 into human keratinocytes, which are not the original target of AAV2 (Sallach et al. 2014). Coating AAV with reactive polymers was proven to be sufficient for ablating the original tropism of the virus (Carlisle et al. 2008). Using error-prone PCR, generating diverse forms of viral vectors with diverse tropisms is possible (Maheshri et al. 2006). While diverse types of viral vectors can deliver the CRISPR systems to various cell types, the CRISPR system ironically showed potential to generate diverse viral vectors. In *E. coli*, error-prone repair of the DNA nicks, using the CRISPR-nickase Cas9 in complex with the error-prone DNA polymerase, showed directed evolution of the target genome (Halperin et al. 2018). Repeated modification of genome sites where virus is integrated using CRISPR-nickase Cas9 and the error-prone DNA polymerase may lead to generation of modified viruses showing modified tropisms. Employing viral vectors other than the conventional ones such as IDLV, AAV or adenovirus, may be a solution to delivering proteins and nucleic acids to various tissues with extreme efficiency and tissue specificity. For example, herpes simplex virus has already been studied (Wolfe et al. 2009). Human papilloma virus (HPV) has not yet been applied to molecular delivery, but successfully modified HPV without carcinogenicity may serve gRNA as a great viral vector for gene therapy of gynecological diseases like cervical cancer. Indeed, analyzing genes or structures of so-called 'low risk' HPVs like HPV type 6 and 11 may help with generating a 'successfully modified HPV vector'.

Abnormal or prolonged transgene expression

Viral delivery may have another problem. Transgene delivered by viral vectors may be integrated into the genome, which may result in abnormal expression of the delivered gene or even tumorigenesis depending on periphery sequences or molecular environment, though with low probability. In fact, whether the problem really matters or not is controversial. In the case of AAV, one of the most popular viral vectors, the integration was observed at the probability of 0.1–1%. Some studies demonstrated that tumors can occur because of AAV-mediated integration in mouse models (Miller et al. 2006; Chandler et al. 2015). However, long-term studies in dogs or non-human primates showed that AAV delivery of the genetic materials is safe from the unwanted tumorigenesis (Niemeyer et al. 2009; Gil-Farina et al. 2016). For baculoviral vectors, no transgene integration was observed in human mesenchymal stem cells. In addition, baculoviral delivery did not induce any tumor formation in nude mouse model (Chen et al. 2011). Meanwhile, 'safety' is very important in the clinical field. Therefore, we can say that it is still worth to struggle to deliver the material to the site called 'safe harbor locus' on the genome (Hong et al. 2017). For example, in human genome, AAVS1, hROSA26 and CCR5 can serve as safe harbor locus (Pellenz et al. 2019). In mouse genome, there is ROSA26 (Wang et al. 2018). MYH9 gene locus also showed potential for being safe harbor locus in the mouse genome (Liu et al. 2018).

Viral delivery of genome-editing tools like CRISPR-Cas9, especially in the case of AAV or adenoviral vectors which have prolonged expression duration of the delivered gene, may result in continuous nuclease activity, causing unwanted off-target effects and subsequent genome instability. Combining viral and non-viral delivery methods could provide a solution. An AAV vector carrying sgRNA and lipid nanoparticle packing Cas9 mRNA was delivered together in a mouse model of human hereditary tyrosinemia. Due to relatively rapid degradation of Cas9 mRNA, off-target effects were below detection (Yin et al. 2016). Meanwhile, strategies to regulate the activity of Cas9 have been developed to address the problem. 'Split Cas9' can reduce activity of the CRISPR-Cas9 system, in which the N-terminal Cas9 fragments and C-terminal Cas9 fragments are delivered by different viral vectors and form dimers only by a certain kind of signaling. For example, dimerization can be induced by rapamycin-sensitive dimerization domains which are in complex with each fragment of Cas9 (Zetsche et al. 2015). Other 'inducible Cas9 systems' could be solution for the problem, in which the Cas9 gene is in complex with a special promoter and activated by certain molecules or physical stimulation. For example, small molecule-induced Cas9 (Davis et al. 2015) or photoactivatable CRISPR-Cas9 showed effective regulation of Cas9 activity (Nihongaki et al. 2015). Besides regulating Cas9, the CRISPR-Cas9 system with guide RNA (gRNA) induced by doxycycline also showed effective regulation of the system (de Solis et al. 2016). Meanwhile, in clinical medicine, ultrasound (US) provides fast, strong, and accurate treatment without major adverse effects to the target tissue (Wu et al. 2016; Kertzman et al. 2017). In human leukemia cells, some genes like BCL2-associated athanogene 3 (*BAG3*), DnaJ (Hsp40) homolog, subfamily B, member 1 (*DNAJB1*), heat shock 70 kDa protein 1B (*HSPA1B*), and heat shock 70 kDa protein 6 (*HSPA6*), showed increased activity by US stimulation (Tabuchi et al. 2007). Understanding the underlying molecular mechanisms related to how such genes are up-regulated by US, and delivery of the related molecules like certain promoters with gRNA or Cas9 may give us the chance to develop ultrasound-induced CRISPR-Cas9 systems for potential utilization in the clinical field. Other than biological intervention, chemicals like peracetate successfully inhibited transgene expression by AAV (Howard and Harvey 2017).

Immune response against the viral vectors

In vivo application of viral vectors can provoke immune responses to decrease its therapeutic efficiency. In addition, immune responses may accompany cytotoxicity, leading to local tissue damage or systemic adverse effects. Choosing vectors with low immunogenicity like IDLV or AAV vectors can alleviate this problem. However, though AAV vectors are known to be relatively less immunogenic (Chen et al. 2016; Wang et al. 2016), they could still provoke immune responses. For example, cellular immune responses against several serotypes of AAV vectors, and mediated by cytotoxic T cells, were observed in humans (Mingozzi et al. 2007). The humoral immune responses can also be a serious barrier against therapeutic application of AAV vectors in that the significant population has natural antibodies against AAVs (Calcedo et al. 2009). One study reported that anti-AAV 5 antibody was the least prevalent in rheumatic arthritis patients (Mingozzi et al. 2013). Thus, for gene therapy in rheumatic arthritis patients, employment of AAV serotype 5 may be considered.

Other solutions can be classified into viral modification or immune modulation. Mutagenesis of the viral epitope is one form of modification (Perabo et al. 2006). Generating a viral vector library containing viruses with various kinds of epitopes showed great potential for selection of appropriate vectors; such viral vector library can be generated by error-prone PCR (Maersch et al. 2010). In addition, as mentioned above, it may be generated by directed genome evolution using CRISPR-Cas9 and the error-prone nuclease (Halperin et al. 2018). Chemical modulation like PEGlyation (PEG: polyethylene glycol) is another option of viral modification

(Lee et al. 2005). Packing viral vectors with vesicle or liposome may also help; liposomal packing of bacteriophage increased its macrophage entrance by protecting the virus from neutralizing antibodies (Singla et al. 2016). Furthermore, the vesicle/liposome may be designed to express some immune inhibitory molecules like PD-L1 on the surface to help the vectors evade from inadequate immune responses. Immune suppression may show positive effects on successful viral delivery.

For the immune modulation, in the rheumatic arthritis patients, rituximab, an anti-CD20 monoclonal antibody, showed modest effect of anti-AAV antibody depletion (Mingozzi et al. 2013). In a mouse model with strong expression of regulatory T cells, immune responses against transgene delivery by AAV vector was prevented (Hoffman et al. 2011). However, immune suppression will require careful monitoring for adverse effects like infections. Such adverse effects could be overcome using specific antibodies against anti-viral vector-antibodies to some extent, specifically repressing humoral immune response against the vectors. Surprisingly, though immune suppression in AAV transgene delivery was usually proved to be effective, it sometimes resulted in the complete opposite manner, with absolute negative effect on gene transfer efficiency, which was due to the fact that the immunosuppressant was actually the antagonist for the regulatory T cells. Thus, careful selection of the immunosuppressant is required (Mingozzi et al. 2007). Indeed, understanding the cellular/molecular mechanisms of the immune response against viral vectors may provide us with new insights about the immune suppression. Meanwhile, viral modification can be used for subsequent modulation of the immune response. In a mouse model, pre-injection of an epitope of adenovirus serotype 5 containing a certain motif (thio-oxidoreductase motif) showed effective suppression of immune responses against injected adenoviral vectors (Miao et al. 2016). Additional trials in several viruses, using different epitopes with different kinds of motifs, may enable optimization of training the host immune system for efficient viral delivery. Finally, if all other options fail to suppress the immune response, radical methods like plasmapheresis can be considered. It was demonstrated that plasmapheresis before viral vector administration can efficiently eliminate pre-existing neutralizing antibodies, enabling efficient delivery (Monteilhet et al. 2011).

Non-viral delivery

Though non-viral delivery of proteins or nucleic acids is regarded to be relatively inefficient, it has recently made great progress. For example, delivery of CRISPR-Cas9 RNP (ribonucleoprotein) complex by electroporation showed efficient genome editing with minimal cytotoxicity on HPSCs among several delivery methods, including plasmid and lentiviral delivery (Lattanzi et al. 2019). Transient expression of delivery molecules is possible through non-viral methods, preventing unwanted off-target effects or genome instability. Non-viral delivery methods can be classified into two groups: physical methods and particle-mediated delivery.

Physical methods

Physical methods generally include electroporation, micro-fluidic methods, hydrodynamic injection and endocytosis.

Electroporation

In electroporation, electric pulses increase the permeability of cell membranes. This approach showed efficient in vitro/ex vivo genome editing by CRISPR-Cas9 in hematopoietic stem cells (HPSCs) (Mandal et al. 2014). Efficiency was improved by delivering the RNP complex or chemically stabilized gRNA (Kim et al. 2014; Hendel et al. 2015). In general, electroporation is known to cause cellular damage, restricting its therapeutic application. The profile of electroporation-induced cytotoxicity was shown to be dependent on the degree of voltage. For example, low voltage induced apoptosis of the target cells, while high voltage caused necrosis (Cvetković et al. 2017). On the other hand, recent study showed that by evading the weakening of zona pellucida of mouse zygotes, enhanced embroys' viability is possible while maintaining efficient genome editing (Tröder et al. 2018).

Micro-fluidic methods

Delivering CRISPR-Cas9 using microinjection, a kind of micro-fluidic methods exhibited successful genome editing in several kinds of cells such as mouse embryonic stem cells, mouse zygotes and human zygotes which were donated as surplus to infertility treatment. Researchers also succeeded in generating mutant mouse from the mutated mouse zygotes (Wang et al. 2013; Fogarty et al. 2017). Microinjection can directly deform cell membranes, and may be the last way to deliver biomolecules to hard-to-deliver cells (Han et al. 2015). However, because of its physical disruption of the cell membrane, subsequent cellular damages should be of concern (Horii et al. 2014). Recently, a new kind of micro-fluidic device has been developed; transmembrane internalization assisted by membrane filtration (TRIAMF), in which intracellular delivery of biomolecules is made possible by cell permeabilization that occurs when the cell goes through a filtration membrane, showed successful genome editing in HPSCs with reduced cytotoxicity (Yen et al. 2018). However, delivering biomolecules by this method to animal models or humans seems inappropriate since direct approach to

target cells within the body is challenging in reality. Consequently, micro-fluidic devices are suitable for in vitro/ex vivo delivery or embryonic delivery.

Hydrodynamic injection

In hydrodynamic injection, biomolecules are injected into an organism with a large volume of fluid, enabling the molecules to reach the target site. However, genome-editing efficiency by hydrodynamic injection is relatively low. In addition, injection of excessive fluid resulted in tissue damage in several cases, even if it is transient (Liu et al. 1999; Khorsandi et al. 2008; Kamimura et al. 2014; Ogawa et al. 2017). Hydrodynamic injection into the human body may lead to inevitable volume overload. Volume overload may subsequently result in exacerbation of several diseases, like heart failure (Hassan et al. 2016). Therefore, clinical application of hydrodynamic injection may be inappropriate, especially for patients with cardiovascular diseases, renal diseases or any others related to fluid circulation like liver cirrhosis. On the other hand, intra-portal hydrodynamic injection in small pigs did not show any sign of acute adverse effect like rise in liver enzyme (Stoller et al. 2015). Thus, further research considering several factors, like long-term follow-up after hydrodynamic injection and consideration of baseline diseases of the host, are required to determine possible adverse effects of hydrodynamic injection.

Endocytosis-based methods

Finally, endocytosis gave rise to a new way of biomolecule delivery (D'Astolfo et al. 2015; Fazil et al. 2016; Rouet et al. 2018). In induced transduction by osmocytosis and propanebetaine (iTOP), direct delivery of protein into a cell is possible by macropinocytosis, triggered by NaCl hypertonicity and a transduction compound called propanebetaine. Direct delivery of sgRNA and Cas9 protein using iTOP showed successful genome editing without an integrative manner (D'Astolfo et al. 2015). iTOP requires NaCl hypertonicity around target cells. However, in vivo manipulation of NaCl concentration within certain compartments of the host's body seems very challenging, which may hinder its in vivo application. However, there is an exception, whereby Henle's loop or medulla of the kidney exhibits an extremely salty environment, thus iTOP could potentially be applied to renal genome editing. In addition to iTOP, delivery of the engineered antisense oligonucleotides into human primary T cells through simple macropinocytosis showed efficient suppression of target gene expression (Fazil et al. 2016). Though it provides the possibility of being applied to therapeutic delivery into immune cells with the fact that pinocytosis is used for immune surveillance (Abbas et al. 2016), in vivo application of pinocytosis as a delivery method may exhibit low delivery efficiency because pinocytosis is comprised of 'non-specific' engulfment of biomolecules with fluid. Receptor-mediated endocytosis may provide a solution for this problem. Engineered SpCas9 harboring asialoglycoprotein receptor ligands (ASGPrL) showed increased accumulation in HEPG2 cells, a human hepatocellular carcinoma cell line, which express the corresponding receptors on their surface (Rouet et al. 2018).

Particle-mediated delivery

Particle-mediated delivery includes nanoparticles and cell-penetrating peptides (CPP). Nanoparticle-mediated delivery can be divided into two groups: liposome-mediated delivery and use of other nanoparticles like polymeric or magnetic nanoparticle.

Liposomal delivery

Liposomal delivery of Cas9 and sgRNA-coding plasmids to various cell lines has already been validated (Mali et al. 2013; Schwank et al. 2013). Compared to plasmids, liposomal delivery of RNP complexes of sgRNA and Cas9 protein showed more efficient genome editing (Zuris et al. 2014). Besides in vitro/ex vivo delivery, in vivo delivery of sgRNA, Cas9 and lipofectamine complex has succeeded in effective genome editing in mouse inner ear cells (Zuris et al. 2015). Liposomal drug delivery has already become prevalent in the market (Allen and Cullis 2013).

Despite its broad applications, liposomal delivery has been restricted by several factors. Designing the optimal size of a liposome is important because liposomal size can be related to delivery efficiency (Joshi et al. 2016). Liposomes can trigger the innate immune response, including reticuloendothelial system (Chrai et al. 2002) and complementary system (Szebeni and Moghimi 2009). Usually, liposomes can be divided into two groups, cationic and anionic liposomes, according to their surface charge. Though cationic liposomes generally exhibit more efficient intracellular delivery than anionic liposomes because of their electrostatic interaction with the negatively charged target cell's membrane, they are usually more instable in serum than the anionic ones (Neves et al. 2016). Optimization of the surface charge might contribute to the stabilization of the cationic liposomes. Low molecular weight heparin-modified cationic liposome of 5 mV surface charge showed increased stability in serum with better entrance into the target cell than anionic liposome, providing information about optimal surface charge of cationic liposomes for ideal delivery (Li et al. 2017). Generally, chemical modification can help to improve liposomes' stability, for the both cationic and anionic ones. PEGylated (PEG: polyethylene glycol) liposomes showed increased stability with reduced efficacy of gene delivery.

This is probably due to decreased electrostatic interaction between liposomes and cells. Re-tagging RGD peptides, which bind to integrin of the cell, to the PEGylated liposome led to the improved transfection efficiency (Majzoub et al. 2014). Since certain kinds of RGD peptides preferentially bind to certain kinds of integrin, re-tagging of the PEGylated liposomes with the peptides can enable efficient, target-specific delivery (Mas-Moruno et al. 2010). Other than PEGlyation, encapsulating liposome in ultrathin fibers showed positive effects on its stabilization (Li et al. 2014). Modifying liposomal surface using chemicals or proteins that inhibit phagocytosis of immune cells showed prolonged survival of liposomes (Rampersad et al. 2005; Johnstone et al. 2001). Further considerations for efficient and safe liposomal delivery can be made. Since a significant proportion of lipids are transported in the form of lipoproteins within serum, employing certain apolipoproteins can contribute to successful liposomal delivery. For instance, liposomes with apolipoprotein E on their surface enabled delivery of nerve growth factor across the blood–brain barrier without serious damage (Kuo and Lee 2016).

Recently, researchers reported quite ideal in vivo genome editing, both efficient and safe, in rat's liver using liposomal nanoparticles. In the study, they employed chemically modified sgRNA for the efficient activity of the CRISPR-Cas9 system. Next, they designed biodegradable lipid nanoparticles for the safe and tolerable system. Finally, high-dose administration of the system resulted in maximally 70% DNA editing rate, and more than 97% knockdown of the target protein (Finn et al. 2018).

Polymeric nanoparticle

Polymeric nanoparticles are efficient tools for intracellular delivery. Cas9 protein and sgRNA delivery by the nanoparticles named 'DNA nanoclews' reported successful genome editing, both in vitro and in vivo. DNA nanoclews were synthesized by rolling circle amplification in which short DNA or RNA primer is amplified into circle form by circular template (Ali et al. 2014). Developers of the DNA nanoclews coated the particle using a polymer called polyethylenimine to aid endosomal escape (Sun et al. 2015). Under magnetic field, delivering plasmids encoding sgRNA and Cas9 to porcine fibroblasts by magnetic nanoparticles showed efficient genome editing and low cytotoxicity (Hryhorowicz et al. 2019). Since the extracorporeal magnetic source may be able to control the nanoparticles within the body beyond physical barriers of several tissues, in vivo application of the magnetic nanoparticles has potential for efficient tissue-specific delivery or manipulation of the particle's distribution. This was proved by successfully expressing the CRISPR system only at the target organ, liver, after systemic injection, using magnetic field in mouse models (Zhu et al. 2019). However, designing biodegradable nanoparticles is important as failure of the particle's clearance could lead to several adverse effects like physical damage or unwanted immune response (Aragao-Santiago et al. 2016).

Cell-penetrating peptides

Conjugating CPP with the RNP complex of gRNA and Cas9, or TALEN, showed increased efficiency of genome editing in human cell lines and mammalian cells, respectively (Ramakrishna et al. 2014; Liu et al. 2014). Furthermore, CPP can promote intracellular delivery of several other biomolecules, including delivery nanoparticles. In a past study, liposomes in complex with CPP showed effective internalization of siRNA into melanoma cells (Asai et al. 2014). Artificially developed zinc-finger-based transcription factors harboring CPP showed effective delivery into the mouse brain, demonstrating potential for in vivo application of CPP (Ren et al. 2018). However, in vivo application of CPP might be challenging because of the protein's instability. Possible strategies to overcome this problem can be protein modification, conformational stabilization or shielding the protein (Fominaya et al. 2015; Prades et al. 2015; Lättig-Tünnemann et al. 2011). Meanwhile, the underlying mechanisms of CPP's action are still quite unclear. Some of the mechanisms like caveolae/clathrin-mediated endocytosis, macropinocytosis, direct translocation and concentration-dependent transport have been reviewed (Gestin et al. 2017). Since one of the previously studied mechanisms is macropinocytosis, then combining pinocytosis-based delivery like iTOP and CPP may result in positively synergic effects on the delivery efficiency.

The recently reported non-viral, particle-mediated methods

A new kind of particle-mediated delivery of Cas9-sgRNA RNP complex has been reported. Nanoblade, a murine leukemia virus-like particle, showed efficient in vitro genome editing in several cell lines. When nanoblade interacts with the target cell by endocytosis, mediated by ubiquitous membrane molecules, Cas9 is released from Cas9-Gag fusion in the target cell, resulting in the formation of the RNP complex. The genome-editing activity of the RNP complex was transient without immune response. Furthermore, nanoblades showed effective in vivo genome editing in a mouse tyrosinemia model by targeting the *Tyr* gene. Finally, generating nanoblades is extremely easy and economical when compared to AAV vectors. However, further studies in nanoblades like comparison of delivery efficiency between nanoblades and other vectors are required (Mangeot et al. 2019).

There are the other methods reported recently. A multifunctional vector, composed of several kinds of nanoparticles

like $CaCO_3$ or chitosans, exhibiting both ligand-mediated selectivity and peptide-mediated transmembrane function, showed efficient delivery of the CRISPR-Cas9 into tumor cells (He et al. 2019). Gold nanoparticle, in complex with donor DNA, CRISPR-Cas9 RNP and cationic polymers, succeeded in the delivery of the donor DNA and the CRISPR system into several types of cells. When locally injected into mice model of Duchenne muscular dystrophy, efficient correction of the pathogenic mutation has been observed (Lee et al. 2017). In addition, intracranial delivery of the CRISPR system RNP using gold nanoparticle alleviated disease phenotype in the mouse model of fragile X syndrome (Lee et al. 2018). Lastly, multistage delivery nanoparticles (MDNPs) for the CRISPR system have been developed, in which the CRISPR system is packed by 'multi-layers' of nanoparticles. Each of the layers is exposed according to the surrounding environment, enabling efficient delivery. Researchers systematically administrated the MDNPs into mice bearing tumor. At first, the outer layer of anionic nanoparticle is exposed, stabilizing the MDNP in the serum. After the MDNP reaches the tumor-microenvironment, the inner layer of cationic nanoparticles directly packing the CRISPR system is exposed, leading to the accumulation and internalization of the MDNP, into the tumor cells. Using the MDNP, successful in vivo delivery of the CRISPR system into tumor cells and therapeutic effects have been achieved, beyond several physiological barriers to reach the target site (Liu et al. 2019).

Common limits of the non-viral delivery methods, and the solutions

Non-viral delivery methods have some common limitations. For example, there is the problem of the poor accumulation of the vectors or the cargo biomolecules at the target site. Site-specific disruption of microbubbles carrying biomolecules using ultrasound (US) resulted in increased delivery efficiency without tissue damage (Zhu et al. 2016). Up-regulation of expression of several genes by US is discussed above (Tabuchi et al. 2007). Using US-responsive microbubbles to deliver gene fragments carrying US-reactive promoter may demonstrate ultimate target-specific genome editing. Specific molecular interaction between target cells and delivered materials may also promote the accumulation. For example, liposomes that have AA13, a ligand to the low-density lipoprotein receptor (LDLR), showed enhanced target specificity to acute myeloid leukemia cells, which express abundant LDLR on their surface (Liu et al. 2014). Collagenase can increase the accumulation rate of the vectors within fibrotic tissue (Eikenes et al. 2004). Even though the biomolecules get into the target cell, they can be trapped and degraded by endosomes, thus failure of endosomal escape can be a problem. Cationic vectors combined with particles like zinc or gas-generating nanoparticles showed effective escaping from endosomes (Pichon et al. 2002; Liu et al. 2015).

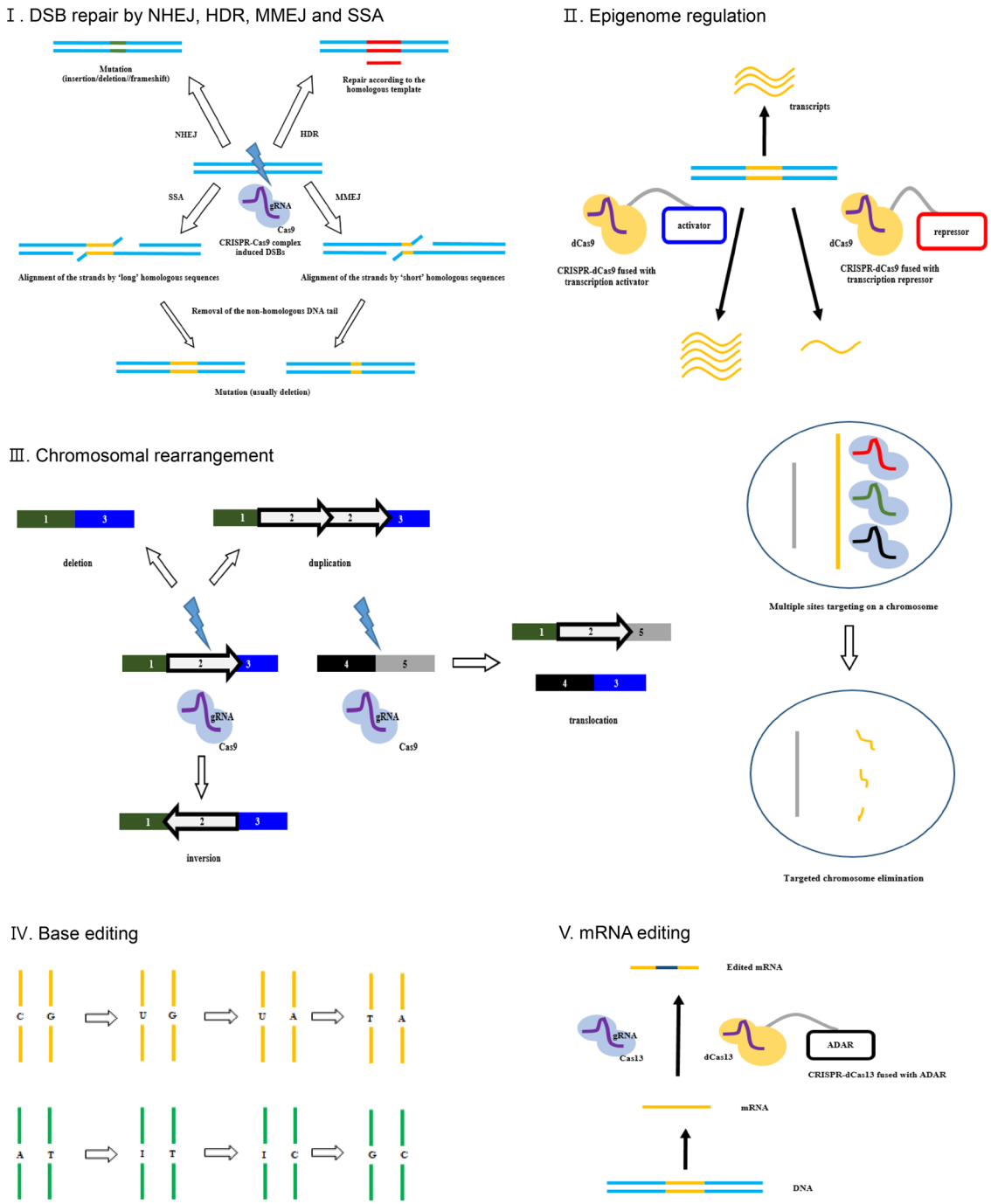
Common limits of the viral and non-viral delivery methods, and the solutions

Finally, both viral and non-viral delivery may encounter some common obstacles. Renal clearance of the vectors may reduce delivery efficiency. For intravascular injection of the vectors, selecting an appropriate injection site may help. For example, the renal artery must pass through the kidney, thus vectors directly injected into the renal artery could show low efficiency of delivery to the target site. In addition, understanding physiological and molecular mechanisms of renal secretion or reabsorption may provide insights into the solution for this problem. For example, megalin and cubilin are two receptors responsible for protein reabsorption in the proximal tubule of kidneys (Nielsen et al. 2016). Vectors or the cargo biomolecules expressing ligands for megalin or cubilin may show enhanced reabsorption in the kidney, which lead to increased delivery efficiency.

In addition to renal excretion, when the biomolecules are injected into the blood vessel, extravasation to get to the target tissue can be an obstacle to efficient delivery. Strategies for enhanced extravasation have been reported in the case of drug delivery to tumors; these include US-mediated cavitation enhanced extravasation whereby permeability of the blood vessel wall is increased by US (Yokoda et al. 2017), employment of appropriate drugs like angiotensin receptor blocker (Diop-Frimpong et al. 2011), as well as hyperthermia (Li et al. 2013). Though blood vessels adjacent to tumors show an increased degree of baseline permeability, such strategies may assist efficient delivery of the CRISPR system to various target tissues.

In vivo delivery: local injection vs. systemic delivery

Meanwhile, in vivo delivery of the CRISPR system can also be classified by some other criteria. For example, it can be locally injected around target tissues. Local injection has advantage of enabling delivering biomolecules to target site more specifically, especially beneficial to non-viral delivery methods in that they do not have concept of tropism. Intratumoral injection of the CRISPR-Cas9 showed effective inhibition of KRAS mutated tumor, which was subcutaneously injected into mouse (Kim et al. 2018). Intracranial or intrathecal, intramuscular, subretinal or intravitreal, intracardiac and intranasal or intratracheal injection can be considered when delivering the biomolecules to central nervous



system (CNS), muscle tissues, retina, heart and lung tissues, respectively (Swiech et al. 2015; Lin et al. 2018; Long et al. 2016; Yu et al. 2017; Hung et al. 2016; Johansen et al. 2017; Platt et al. 2014). In CNS delivery, localized injection is important because biomolecules may not reach the target site due to the presence of blood–brain barrier.

On the other hand, systemic delivery uses circulatory system to deliver biomolecules so that entire body is affected, possible by intravenous injection or oral delivery. Systemic delivery is relatively non-invasive. For AAV-mediated gene editing in mice's liver, systemic delivery is preferred in that AAVs finally end up in liver, distributed broadly within the target organ (Yang et al. 2016). Most of the systemic delivery of the CRISPR system had been done by viral vectors (Mout et al. 2017). However, in 2017, successful co-delivery of Cas9 mRNA and sgRNA in the mouse model was achieved by injecting the lipid nanoparticle, the ZAL (zwitterionic amino lipid) nanoparticle (ZNP), intravenously. The CRISPR-Cas9 effectively edited target genes in liver, lung and kidney

◂**Fig. 1** The genome-editing mechanisms of the CRISPR system. **I** CRISPR-Cas9 induces DSB at the target site, which can be repaired by several pathways, including NHEJ, HDR, MMEJ and SSA. Repair by NHEJ eventually leads to indel formation. The indel can cause indel mutation, usually leading to the production of a non-functional, abnormal protein. Thus, NHEJ is usually suitable for treatment of diseases caused by overexpression of the target gene. Meanwhile, NHEJ can induce a frameshift. Thus, restoration of the original reading frame is possible. Consequently, NHEJ may sometimes be applied to treating loss-of-function diseases caused by frameshifts. HDR enables precise genome editing according to the homologous repair template. Thus, HDR can be used for curing any diseases caused by altered genome sequences. However, HDR has some major limits like inefficiency, which will be discussed later. In MMEJ, broken strands are aligned according to the 'short' (5 ~ 25 base pairs) homologous sequences. The repair usually results in mutation like deletion. Thus, therapeutic application of MMEJ may be similar to that of NHEJ: treatment of diseases caused by gain-of-function mutation, and restoration of normal reading frame and normal gene expression. It appears that SSA resembles the MMEJ. One of their differences is the length of the homologous sequences, which are required for the alignment of DNA double strands. **II** When dCas9, which lacks the original nuclease activity, is in complex with transcription activator, repressor or histone modifier, the complex is able to modulate expression level of the target gene by epigenome regulation. Thus, CRISPR-dCas9 can be applied to treatment of several diseases with abnormal gene expressivity. **III** During the repair of the DSBs caused by the CRISPR system, chromosomal abnormalities such as insertion, duplication, inversion and translocation can occur. Thus, the CRISPR system can be applied to the treatment of the chromosomal disorders. It was reported that multiple targeting on a single chromosome using CRISPR-Cas9 with one or more guide RNAs can lead to the elimination of the targeted chromosome. Consequently, early treatment of hyperploidies like Down syndrome may be possible. **IV** Base editor enables substitution of C, G pair to T, A pair, or A, T pair to G, C pair. Thus, base editor could be applied to diseases caused by single-nucleotide substitutions. **V** Using CRISPR-Cas13, mRNA editing is possible. In addition, CRISPR-dCas13-ADAR enables the base editing on mRNA, which show the potential of the CRISPR system for the therapeutic application to diseases caused by transcriptional abnormalities. mRNA editing allows transient and reversible regulation of target gene's expression. *DSB* double-strand break, *NHEJ* non-homologous end joining, *indel* insertion and deletion, *HDR* homology-directed repair, *MMEJ* micro-homology-mediated end joining, *SSA* single-strand annealing, *dCas9* dead Cas9, *A* adenosine, *T* thymidine, *G* guanosine, *C* cytidine, *U* uridine, *I* inosine, *dCas13* dead Cas13, *ADAR* adenosine deaminase acting on RNA

which was confirmed by detecting the fluorescence (Miller et al. 2017).

Therapeutic CRISPR system: mechanisms of genome editing and therapeutic strategies

CRISPR systems are able to manipulate a target genome in various ways, and can be applied to various types of diseases. The CRISPR system's genome editing mechanisms are briefly summarized in Fig. 1. Guide RNA and Cas9 are joined to induce double-strand breaks (DSBs) on the target gene, repaired by several pathways which result in differently. Past reviews related to therapeutic applications of the CRISPR system were usually focused on the two pathways of DSB repairing: non-homologous end joining (NHEJ) and homology-directed repair (HDR) (Maeder and Gersbach 2016; Cox et al. 2015). However, DSBs can also be repaired by micro-homology-mediated end joining (MMEJ) or single-strand annealing (SSA) (Boulton and Jackson 1996; Lin et al. 1984). Furthermore, fusing guide RNA with dCas9 which has mutation at the nuclease domain makes various epigenome modulations possible (Gilbert et al. 2013). DSBs made by CRISPR-Cas9 can lead to chromosomal rearrangement (Choi and Meyerson 2014). Meanwhile, targeted base editing became possible first by conjugating gRNA, Cas9 variant and cytidine deaminase (Komor et al. 2016). Finally, the CRISPR system is able to edit mRNA, too (Abudayyeh et al. 2017).

DSB repairing by non-homologous end joining (NHEJ), homology-directed repair (HDR), micro-homology-mediated end joining (MMEJ) and single-strand annealing (SSA)

When CRISPR-Cas9 induces DSBs on the genome, they can be repaired by four mechanisms: non-homologous end joining (NHEJ), homology-directed repair (HDR), micro-homology-mediated end joining (MMEJ) and single-strand annealing (SSA). Repair of DSBs within a chromosome can lead to several consequences like insertion or deletion mutation, frame shift mutation, complete repair according to the homologous strand or gene integration, according to the repair pathway.

Non-homologous end joining (NHEJ)

In NHEJ, the broken ends of DNA strands are directly joined together without a homologous template. Though NHEJ is able to repair DSBs accurately, continuous DSBs and subsequent repairs by NHEJ will eventually form insertions and deletions (indels) on the genome, which is likely to result in frame shifts or generation of non-functioning, abnormal proteins. Thus, NHEJ is usually suitable for treating diseases caused by overexpression of certain genes. For example, NHEJ-mediated inactivation of the *HTT* gene in Huntington's disease or elimination of the *FGFR3* gene in achondroplasia showed therapeutic effect (Yang et al. 2017; Wojtal et al. 2016). *CCR5* gene-ablated human HPSCs exhibited increased resistance to HIV infection when transplanted into a mouse model (Xu et al. 2017). On the other hand, NHEJ can lead to recovery of normal gene expression in diseases caused by loss-of-function mutations. In cataract mice carrying a single-nucleotide deletion in the *Crygc* gene,

NHEJ-mediated insertion of an additional single-nucleotide insertion or deletion of five nucleotides showed recovery of normal histology in the eyes. The latter could be explained by recovery of the normal reading frame (Wu et al. 2013). In the case of Duchenne muscular dystrophy, restoration of reading frame by CRISPR-Cas9-induced DSBs and NHEJ showed partial recovery of normal dystrophin level within both patient-derived cells and the mouse models (Ousterout et al. 2015; Min et al. 2019; Nelson et al. 2016). Another case of the NHEJ-mediated recovery of the normal phenotype has been reported, using the CRISPR-SaCas9 (Cas9 from *Staphylococcus aureus*) system with two gRNAs. In the mouse model of congenital muscular dystrophy type 1A, the point mutation in intron2 of *Lama2* led to skipping of exon2 and truncation of the final protein product, resulting in muscle atrophy and hind-limb paralysis. SaCas9 and two different kinds of gRNAs were delivered to the mouse model to induce DSBs at both ends of the intronic region containing the pathogenic mutation. After the genome was repaired by NHEJ, the pathogenic region was successfully removed, demonstrating the therapeutic effect (Kemaladewi et al. 2017).

Homology-directed repair (HDR)

In HDR, DSBs are repaired by introducing homologous templates to the break site, which results in precise repairing according to the sequence of the templates. Thus, HDR-mediated repairing of DSBs is able to cure various genetic diseases that need accurate repair of mutation, regardless of whether they are caused by loss-of-function or gain-of-function mutations. One famous example is the repair of a point mutation on the *Fah* gene to treat hereditary tyrosinemia in mice (Yin et al. 2014). Correction of deletion or point mutations in the *CFTR* gene in organoids from cystic fibrosis patients has also been reported (Schwank et al. 2013). Finally, CRISPR-Cas9-induced DSBs and HDR showed effective correction of a point mutation in the *TGFBI* gene in human corneal keratocytes from granular corneal dystrophy patients without off-target effects (Taketani et al. 2017). Meanwhile, by recruiting the DNA strand homologous to both ends of the two different chromosomes, HDR can facilitate targeted chromosomal rearrangement (Spraggon et al. 2017). This will be discussed later in this review.

Since HDR is able to bring precise and predictable repair of DSBs, efforts have been made to increase efficiency of HDR. While NHEJ is active through the entire cell cycle, HDR only occurs in late S/G2 phases (Heyer et al. 2010). Thus, maximizing CRISPR-Cas9 expression in the late S/G2 phase can increase the relative rate of HDR when compared to NHEJ. Fusing Cas9 to a protein called Geminin made it possible by ubiquitination of Cas9 during the G1 phase (Gutschner et al. 2016). Optimization of delivery time of the Cas9-gRNA RNP complex also showed increased efficiency of HDR (Lin et al. 2014). Suppressing the expression of NHEJ-favoring molecules or promoting expression of HDR-favoring molecules showed increased rate of HDR (Chu et al. 2015; Canny et al. 2018; Shao et al. 2017; Li et al. 2017). Structural modification of guide RNA can improve efficiency of HDR. It is known that when the CRISPR-Cas9 complex cleaves the target genome, Cas9 asymmetrically releases the 3′ end of cleaved DNA strands first, before complete dissociation of the complex. Design of a single-guide RNA complementary to the 3′ end strand showed dramatic increase in the efficiency of HDR (Richardson et al. 2016). Finally, since the NHEJ directly joins cleaved strands, it hardly processes modified DNA ends. However, the pathways like HDR, or micro-homology-mediated end joining (MMEJ) is initiated by DNA end resection, thus they are able to process modified DNA ends (Aparicio et al. 2014). Co-delivery of the CRISPR-Cas9 system with a certain DNA end modifier may result in DSBs with modification at the end of the strands. This may inhibit the NHEJ pathway, thus more DSBs can be repaired by HDR.

Micro-homology-mediated end joining (MMEJ)

In MMEJ-mediated repair of DSB, 5 ~ 25 base pairs of micro-homologous sequences are used for alignment of the broken strands. The strands are then joined together, usually leading to subsequent formation of a deletion mutation, like in Fig. 1 (Truong et al. 2013; Mateos-Gomez et al. 2017; Ma et al. 2003). In that MMEJ is highly mutagenic (Glover et al. 2011; Sinha et al. 2017), it may be used to knock-down overexpressing genes, curing diseases cause by the gain-of-function mutation. In addition, the deletion mutation caused by the MMEJ may result in the frameshift. Thus, MMEJ may be able to cure the loss-of-function diseases by the frameshift mutation, like in the case of the NHEJ in the Duchenne's muscular dystrophy disease models (Ousterout et al. 2015; Min et al. 2019; Nelson et al. 2016). Meanwhile, a method for TALEN or CRISPR-Cas9-mediated gene integration using MMEJ, called precise integration into target chromosome (PITCH), has been reported to enable precise gene integration. Thus, MMEJ requires short homologous sequences (5 ~ 25 base pairs), and constructing the PITCH vectors is easier than constructing those of HDR, which usually contain long (500 ~ 1000 base pairs) homologous sequences. In addition, MMEJ is known to be active during M ~ early S phase, when HDR is inactive (Sakuma et al. 2016). Using the PITCH, successful gene cassette knock-in was reported in human cells and mouse zygotes (Aida et al. 2016). Finally, a software was developed for the design of the PITCH system (Nakamae et al. 2017).

Single-strand annealing (SSA)

SSA goes by several steps. First, the end resection is done at the DSB site, generating 3′ single-strand DNAs (ssDNA) Next step involves annealing of the strands by their homologous parts. Finally, 3′ non-homologous ssDNA tails are removed and the DSB is fully repaired (Lin et al. 1984; Rothenberg et al. 2008). SSA and MMEJ have some common features. First, their entire process described above resembles each other. Also, both of them occurs through the S and G2 phase of the cell cycle. However, SSA has much longer length of the annealing intermediate than MMEJ. SSA is mediated by RAD52, which is irrelevant to MMEJ (Ma et al. 2003; Morales et al. 2015; Bennardo et al. 2008; Yu and Gabriel 2003).

It is because the result of DSB repair by SSA is deletion mutation; therapeutic application of SSA may be similar to that of MMEJ in that MMEJ usually results in deletion mutation, too. In addition, MMEJ assisted targeted gene integration, and the PITCH system mentioned above, demonstrated its efficiency and accuracy. SSA may be employed to mediate target gene integration, too. Meanwhile, SSA between two different chromosomes can form translocation (Elliott et al. 2005; Manthey and Bailis 2010). Thus, conversely, SSA may be use to reverse the pathogenic translocation.

Epigenome regulation

Not only genomic sequences, but also epigenomes are related to various diseases (Lord and Cruchaga 2014; Nakatochi et al. 2017; Yang et al. 2017). Therefore, regulation of epigenome has great potential for therapeutic applications. Though dead Cas9 (dCas9), in which the nuclease domain is inactivated, cannot induce DSBs at the target site, dCas9 can be in complex with several molecules involved in epigenome regulation. CRISPR-dCas9 is a versatile tool of epigenomic regulation in a target-specific manner (Gilbert et al. 2013).

CRISPR-dCas9 with a transcription repressor or activator showed successful transcription regulation in various cell lines (Kearns et al. 2015; Thakore et al. 2015). Likewise, targeted histone modification and DNA methylation have been shown to be effective in mammalian embryonic stem cells or several cancer cell lines (Kwon et al. 2017; Liu et al. 2016). Epigenome editing by CRISPR-dCas9 is not just restricted to in vitro application. CRISPR-dCas9 with transcription activator succeeded in spontaneous conversion of astrocytes to functional neurons in a living mouse model (Zhou et al. 2018). Successful management of tumor progression has also been reported in mouse models (Braun et al. 2016; Xu et al. 2017). When delivered to the mouse hypothalamus, CRISPR-dCas9 with transcription activator showed therapeutic effect of reversing haploinsufficiency-induced obesity phenotypes by recruiting transcription activator to promote the expression of the *Sim1* and *Mc4r* genes (Matharu et al. 2019). Such reports suggest that CRISPR-dCas9-mediated epigenome modulation can be applied to the treatment of various diseases.

In vivo epigenome regulation using the CRISPR system has several advantages over the genome editing. First, epigenome regulation is suitable for dealing with various diseases caused by epigenome dysregulation. Second, the epigenome regulation does not affect the genome sequences, itself. Thus, it is safe from the unwanted mutation. Third, the regulation can be either permanent (Saunderson et al. 2017) or reversible (Braun et al. 2017). Finally, by fusing various effector molecules with dCas9, multiple options are possible for the regulation, as mentioned above. However, there are some drawbacks, too. For example, in the application of the multifunctional CRISPR system, SpCas9 with some large effector molecules like tripartite VP64-p65-Rta transactivation cannot be packed by AAV, one of the most popular viral vectors. This could be overcome by employing the split-Cas9 system, in which dual AAVs deliver the gene fragment of the each part of the dCas9, eventually ending up to make the intact and functional protein (Chew et al. 2016).

Chromosomal rearrangement

The CRISPR system and the chromosomal disorders

When DSBs are repaired, the broken genome fragments can be inserted into the original chromosome backwards. In addition, DSBs in two or more non-homologous chromosomes may lead to ligation between the fragments belonging to other chromosomes. Thus, CRISPR-Cas9 enables targeted chromosomal rearrangement. In vitro generation of inversion within chromosome 10 and certain translocations like *EML4-ALK*, *KIF5B-RET*, or *CD74-ROS1* have been generated using CRISPR-Cas9. Recovery of the normal structure from *BCR-ABL1* has also been validated (Choi and Meyerson 2014; Lekomtsev et al. 2016). Moreover, the targeted chromosomal rearrangement showed successful correction of chromosomal inversions in HPSCs from hemophilia A patients (Park et al. 2015). In vivo targeted chromosomal rearrangement by CRISPR-Cas9 was demonstrated too, succeeding in generating mice that carried oncogenic chromosomal rearrangements like *EML4-ALK* (Maddalo et al. 2014; Blasco et al. 2014). In the case of duplication, also a sort of chromosomal disorders such as inversion, translocation or deletion, successful generation (Li et al. 2015) or removal of the duplication was reported (Wojtal et al. 2016). These support the possibility of clinical application of CRISPR-Cas9 to the treatment of several chromosomal disorders,

including the oncogenic chromosomal rearrangements like *t*(9;22)(q34;q11), which is the Philadelphia chromosome.

However, efficiency of chromosomal rearrangement by CRISPR-Cas9 was too low, requiring selection and enrichment of appropriate clones containing edited genome or enhancement of efficiency. For the in vitro selection and enrichment, by co-delivering antibiotic-resistant genes with the CRISPR-Cas9 system, selection of clones resistant to antibiotics enabled enrichment of clones with edited chromosome (Vanoli et al. 2017). Fluorescence reporter system for quantitative description of chromosomal rearrangements has been reported (Li et al. 2015). The fluorescence reporter system can also be employed for the enrichment of the cells in which desired chromosomal rearrangement has occurred successfully (Ramakrishna et al. 2014). Finally, understanding the underlying mechanisms of chromosomal rearrangement and manipulation of the related molecules may be useful. It was reported that mutation of genes related to centromere 21 could result in trisomy 21 (Contreras-Galindo et al. 2017). Therefore, by editing the genes related to centromeres, we may be able to activate or inhibit certain chromosomal rearrangements.

Chromosomal elimination

Meanwhile, using one or more guide RNAs at once, CRISPR-Cas9 can eliminate target chromosome by multiple cleavages. Using this way, researchers have succeeded in generating mouse model with Turner syndrome by eliminating Y chromosome (Zuo et al. 2017). Thus, CRISPR-Cas9-mediated targeted chromosome elimination may be applied to the treatment of hyperploidies such as Down syndrome or Edwards syndrome.

Base editing

Base editor: from the first to fourth generation

Cytidine deaminases are able to convert cytidine to uridine in the target DNA strand, which can lead to several results including error-free repair, conversion of cytidine (C), guanosine (G) pair to thymidine (T), adenosine (A) pair, or DSB that may result in indels, which is very rare. The first generation of base editor (BE1) was generated by linking dCas9, XTEN 'linker', and rAPOBEC1, a type of cytidine deaminase. Since then, efforts have been made to increase the efficiency of base editing. Conjugating BE1 with uracil DNA glycosylase inhibitor (UGI) succeeded in inhibiting the base excision repair of the uracil generated by the base editor, which is the second-generation base editor. Meanwhile, nicking on the non-deaminated strand by CRISPR-Cas9 nickase showed preferential base excision repair on the nicked strand, promoting switching of the U:G pair generated from C:G, to a U:A pair, and eventually resulting in a T:A pair. Employment of CRISPR-Cas9 nickase into the base-editing system, which is the third-generation base editor (BE3), demonstrated more efficient targeted base editing with slightly increased rate (about 1%) of indels (Komor et al. 2016). Finally, pairing two UGIs with the conventional base editor, referred as the fourth-generation base editor (BE4), exhibited improved efficiency (Kim et al. 2017).

Utilization of base editors

Protein engineering to generate Cas9 variants with different protospacer-adjacent motif (PAM) recognitions contributed to utilization of therapeutic base editing (Kim et al. 2017). Base editing with cytidine deaminase which is in complex with inactive Cpf1 was also validated (Li et al. 2018). Since Cpf1 recognizes different PAM sequences from Cas9 (Kim et al. 2017), we can say that it contributed to further utilization of base editing for various target sites. Currently, conversion of the A:T pair to G:C pair is also possible by adenine base editor (Gaudelli et al. 2017). Finally, base editing in both mouse embryos to generate Tyr mutant mice with albino phenotype and an adult mouse model of Duchenne muscular dystrophy proved that base editing is effective in the broad spectrum of developmental stages (Ryu et al. 2018). Effective in vivo base editing in mouse inner ear cells demonstrated that base editing is also possible in post-mitotic cells (Yeh et al. 2018). Consequently, the base-editing system shows great potential for therapeutic application to many diseases caused by single-nucleotide mutations.

Successful base editing has been reported in mouse astrocytes, breast cancer cell lines (Komor et al. 2016), HEK293Ts (Kim et al. 2017), human iPSCs (Chadwick et al. 2017) and so on. Delivery of BE3 using AAV vectors in phenylketonuria adult mouse models showed correction of a pathogenic mutation in the *Pah* gene and restoration of the normal phenotype (Villiger et al. 2018). Finally, efficient base editing has been demonstrated in zebrafis (Zhang et al. 2017).

For the more efficient base editing!

Active base editor (BE) comprises of guide RNA, Cas9 variant, cytidine or adenosine deaminase and maybe UGIs (Komor et al. 2016; Kim et al. 2017; Gaudelli et al. 2017), which can exhibit a large delivery load for the vector (Villiger et al. 2018). Generation of simplified new BE, which has minimal number of domains from the original complex, may contribute to efficient delivery of BE, resulting in efficient base editing. Meanwhile, understanding the underlying molecular mechanism of base editing and the related molecules may also lead to efficient targeted base editing.

For example, DREAM complex, which is activated by p53 and p21 pathway, is known to inhibit APOBEC3B, a kind of cytidine deaminase (Periyasamy et al. 2017). Specific inhibition of DREAM complex may improve efficiency of the base editor, which employs APOBEC3B as its deaminase.

Ambush among the base editors: indels

Though indels formed by BEs are relatively rare (Komor et al. 2016), they may be a serious obstacle to clinical application of BEs since clinical application requires minimal risk of side effects. Employment of BE1 or BE2, rather than BE3, can reduce the possibility of indel formation (Komor et al. 2016). Several ways for reducing off-target effects of BEs are related to regulating the activity of the base editor, which means that they can also result in reduced frequencies of indel formation. Off-target effects will be discussed later in this review. Meanwhile, fusing BE with Gam protein, a bacteriophage protein that binds to DSBs and protects them from further degradation, showed reduced frequencies of the indels (Komor et al. 2017). Promoting HDR during base editing may also show reduced indel frequencies. This may be achieved by the optimization of the delivery time of BEs, up-regulation of HDR-related molecules, down-regulation of NHEJ-related molecules, or modifying the gRNA or the broken DNA's end during the base editing, same as in the case of CRISPR-Cas9 (Gutschner et al. 2016; Lin et al. 2014; Chu et al. 2015; Canny et al. 2018; Shao et al. 2017; Li et al. 2017; Richardson et al. 2016; Aparicio et al. 2014).

mRNA editing

Using the CRISPR system, targeted mRNA editing is possible. For the therapeutic application, mRNA editing has some advantages over DNA editing due to the temporary nature of mRNA molecule. First, even though the off-target effects occur, it can be under control (Gulei et al. 2019). Second, the reversible control of gene expression is possible. However, the nature of mRNA can also be the disadvantage in that to induce therapeutic change, extreme amount of the biomolecules may be required (Matsoukas 2018).

The CRISPR-Cas13 showed efficient RNA knockdown in mammalian cells. In addition, it was absolutely more accurate than the small hairpin RNAs (Abudayyeh et al. 2017). Fusing dCas13, catalytically inactive one, with ADAR (adenosine deaminase acting on RNA) showed efficient RNA editing. In addition, the CRISPR-dCas13-ADAR succeeded in correcting certain diseases mutations, found in X-linked nephrogenic diabetes insipidus and Fanconi anemia, efficiently in HEK293FT cells. Finally, protein engineering exhibited improved target specificity of the CRISPR-dCas13-ADAR. Interestingly, Cas13 does not require the PAM sequences, which means it can be flexibly applied to editing various sequences of RNAs (Cox et al. 2017).

Further studies including the in vivo application are required for the therapeutic application of the CRISPR-mediated mRNA editing.

Further considerations for the therapeutic application

Immune response by guide RNA and Cas9/Cas12a

Above, we have discussed about the strategies to evade the immune response induced by delivery vectors, especially the viral vectors. For example, selection of non-viral delivery methods, less immunogenic viral vectors, screening of the patients' immune profile before the injection, viral modification or immune suppression are possible. Indeed, nanomaterial-modified viral vectors reported its in vivo escape from the host's immunity (Zhong et al. 2008; O'Riordan et al. 1999; Wortmann et al. 2008). Liposomal packing of viral vectors also showed the potential for the in vivo application to evade the immune response (Mendez et al. 2014). Finally, co-delivery of additional empty capsid successfully absorbed pre-existing neutralizing antibodies against AAVs in mouse and non-human primate models (Mingozzi et al. 2013).

Meanwhile, besides the vectors, gRNA and Cas9 or Cas12a (Cpf1) are also able to induce the host immune response (Machitani et al. 2017). The immune response can lead to adverse effects which cause reduced efficiency of genome editing, tissue damage by inflammation, or immunological memory formation which can be a serious obstacle to subsequent genome editing; thus, thorough research in the field is required. In general manner, restricting the CRISPR system's activity (Xia et al. 2018) or humanizing the system (Wang et al. 2016; Riechmann et al. 1988) may reduce the host's immune response. However, the molecular mechanism of the immune response against gRNA and Cas9/Cas12a are not the same, as they are described in Fig. 2. In this section, we will discuss about the immune responses against the gRNA and the Cas enzymes, with the strategies to alleviate those.

Immune response against the guide RNA

gRNAs are often related to the innate immune response. Toll-like receptor 3 (TLR3) is able to response to the ssRNA (Tabeta et al. 2004; Wang et al. 2004). The preferred ligand of TLR3 is CpG sequences, and this was proven through reduced immune responses by CpG-depleted AAV vectors. Thus, it may be appropriate for the gRNA sequences to exclude CpG sequences (that is, appropriate choice of the

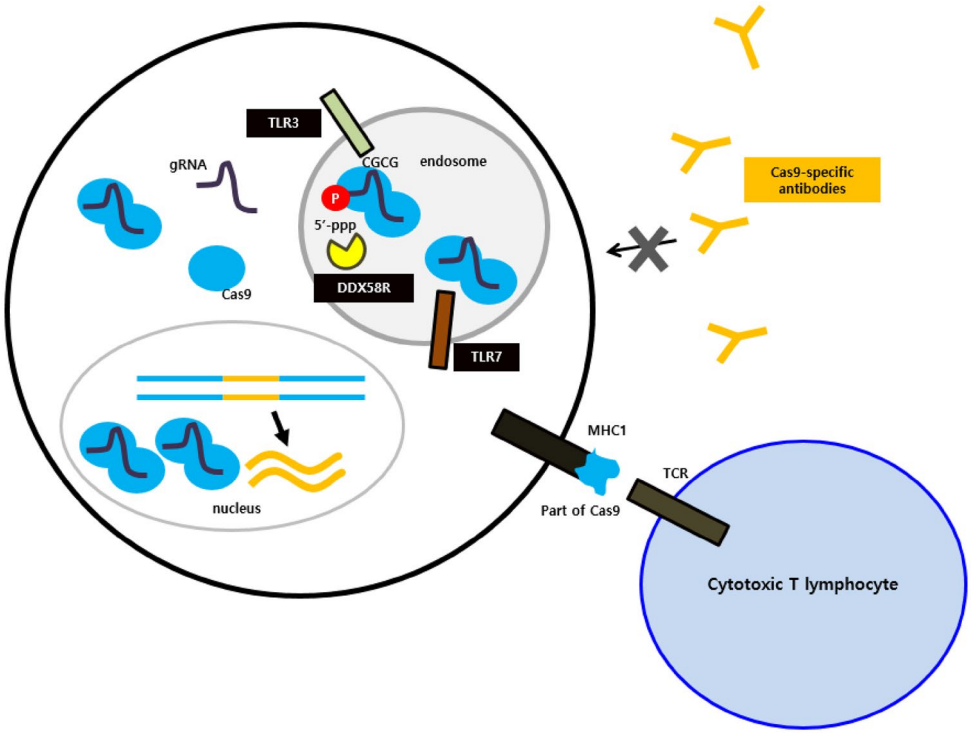


Fig. 2 Overall pathway of CRISPR-Cas9/Cas12a complex-induced immune response. Not only delivery vectors, but also gRNA or Cas9/Cas12a, can induce the host immune response. gRNA is usually related to the innate immune response. The hairpin structure of the gRNA, especially CpG sequences, is recognized by TLR3. Also, 5′ triphosphorylation of the gRNA is recognized by DDX58R, which is an RNA-sensing immune receptor. Finally, since TLR7 can immunologically recognize ssRNA, it may be involved in gRNA-induced immune responses. Cas9/Cas12a is more likely to be related to adaptive immune responses. Though a significant population of humans has Cas9-specific antibodies, which are extracellular proteins, in that Cas9/Cas12a is an intracellular protein, the mainly effective immune response to Cas9/Cas12a is cellular immunity, mediated by cytotoxic T lymphocytes. *TLR* toll-like receptor, *DDX58R* dead box polypeptide 58 receptor, *MHC1* major histocompatibility complex class 1, *ssRNA* single-stranded RNA

target sequences) to alleviate the immune response (Faust et al. 2013). TLR7 and TLR8 are involved in single-stranded RNA recognition which may be involved in the immune response against the gRNA of the CRISPR system (Diebold et al. 2004; Jurk et al. 2002). Chemical modification of gRNA is able to modulate the immune response. Transfection of 5′-triphosphorylated gRNA into human cells showed increased levels of interferon gamma and subsequent cytotoxicity, compared to that of 5′-hydroxylated gRNA. Thus, employing 5′-hydroxylated gRNA rather than 5′-triphosphorylated gRNA will enable efficient genome editing with a reduced immune response. Meanwhile, the immunological recognition of 5′-triphosphorylate RNA has been shown to be mediated by an RNA-sensing immune receptor called dead box polypeptide 58 (DDX58) receptor (Kim et al. 2018). Regulation of molecules such as TLR3/7/8, interferon gamma, or DDX58 receptor may contribute to reduced immune responses against gRNA, thus enabling efficient targeted genome editing without cytotoxicity or inflammation. In light of this, further screening for molecules involved in gRNA-induced immune responses is required. General immune suppression by drugs like steroid may also be beneficial (Broering et al. 2011); however, employment of immunosuppressants requires caution due to adverse effects like infection. In addition, selection of an appropriate immunosuppressant is essential for efficient genome editing because certain immunosuppressants can affect activity of the target gene. For instance, glucocorticoid is known to destabilize mRNAs of several integrins mRNAs in osteoblasts. Thus, combining targeted genome editing for the purpose of upregulating alphaVbeta3 integrin in osteoblasts with immune suppression by glucocorticoids can definitely lead to inefficient genome editing (Cheng et al. 2000). Inappropriate drug selection may also result in the unexpected negative effects on efficient genome editing, as mentioned above (Mingozzi et al. 2007). Finally, further in vivo studies about gRNA-induced immune responses are needed.

Immune response against the Cas9/Cas12a

Unlike gRNAs, Cas9 and Cas12a act as protein, which makes them usually related to adaptive immunity. A significant

proportion of the human population has antibodies and T cells against SpCas9 and SaCas9 (Charlesworth et al. 2019). However, since Cas9 and Cas12a are intracellular proteins and natural antibodies are extracellular proteins, the pathway of adaptive immunity which shows cytotoxic effects against Cas9/12a would be usually T cell-mediated cellular immunity, in which T cells recognize the part of Cas9 presented by the major histocompatibility complex (MHC) like in Fig. 2 (Crudele and Chamberlain 2018).

In vitro stimulation of human peripheral blood mononuclear cells by recombinant SpCas9 showed proliferation of T cells overall, but actually regulatory T cells had suppressed effector T cells (Wagner et al. 2019). Intramuscular delivery of AAV vectors carrying Cas9 and gRNA in mice only showed proliferation of immature T cells and minimal tissue damage. In contrast, Cas9 DNA delivery by electroporation provoked severe cellular immune responses and tissue damage, in which the damage is partially attributable to the cytotoxic effect of the electroporation. Consequently, employment of the appropriate mode of delivery may contribute to the evasion from the Cas9/Cas12a-induced immune responses (Chew et al. 2016). Localized expression of the CRISPR-Cas9 system may also prevent the unnecessary immune response (Hartigan-O'Connor et al. 2001). Meanwhile, localized expression of the CRISPR system can also be aided by several means, which were discussed earlier, in the delivery section. Delivery to less immunogenic organs like the brain or liver can alleviate the immune response (Swiech et al. 2015; Ran et al. 2015). Since the short duration of foreign biomolecule expression can prevent subsequent immune responses, delivery of the CRISPR-Cas9/Cas12a through non-viral methods rather than viral methods may be extraordinarily exceptional from the immune evasion. In addition, because adaptive immunity is eventually provoked by antigens, protein engineering to induce mutations on the potential immunogenic domain may alleviate the immune response (Chew et al. 2016; Yeung et al. 2004). Immune suppression like T cell inhibition (Chew et al. 2016) or plasmapheresis (Monteilhet et al. 2011) may be considered to suppress the immune response.

Predicting the immune response: toward the predictive medicine

For the therapeutic application of the CRISPR system, prediction of the potential unwanted immune responses is also important. In vitro pre-testing of the T cell immune response (Sette et al. 1994) or prediction of the B cell immune response based on 3D molecular structures of B cell receptors and their epitopes have been proposed for foreseeing Cas9/Cas12a-induced immune responses (Ponomarenko et al. 2008; Ponomarenko and Bourne 2007). Development of methods for predicting gRNA-induced immune responses may also contribute to successful therapeutic application. Based on numerous in vitro and in vivo studies, employment of machine learning will be of great help for the prediction of CRISPR system-induced immune responses.

Unintended mutations

Off-target effects: truly dangerous? Or negligible?

The CRISPR system can recognize sequences 'similar' to the target sequences, which can result in off-target effects (Hsu et al. 2013; Lin et al. 2014; Cencic et al. 2014). Off-target effects can lead to unwanted indel formation, epigenome modulation, chromosomal rearrangement or base substitution. Recently, genome-wide off-target effects of the cytosine, but not adenine, base editor in mouse embryo have been reported (Zuo et al. 2019). However, though numerous cases of the off-target effects have been detected, only a few have reported the actual cytotoxicity or tissue damage caused by off-target effects. Nevertheless, the efforts to reduce and detect off-target effects are important, since 'clinical application' of the CRISPR system definitely requires minimal adverse effect on the patients. Here, strategies to reduce the off-target effects are discussed.

Minimizing the off-target effects

Selection of the unique target sequences

To reduce the off-target effects, selection of unique and appropriate target sequences is essential. While selecting the target sequences, it has to be considered that in the CRISPR-Cas9 system, a certain number of base pair mismatches between gRNA and target DNA strand can be endured, especially when they are positioned far from the PAM sequence (Jinek et al. 2012; Cong et al. 2013). In addition, though SpCas9 usually recognizes the PAM sequence of 5′-NGG-3′, it can also bind to others like 5′-NAG-3′ (Zhang et al. 2014). Like in the CRISPR-Cas9 system, CRISPR-Cpf1 can endure base pair mismatches between gRNA and DNA strand to some extent, especially when positioned far from the seed sequence, which is adjacent to the PAM sequence. While Cpf1 preferentially recognizes 5′-TTTN-3′, except for 5′-TTTT-3′ as a primary PAM sequence, it does bind to 5′-CTTA-3′, too, as a secondary PAM sequence (Kim et al. 2017). Meanwhile, shorter gRNAs of 17 or 18 nucleotides showed preserved on-target activity and significantly reduced off-target effects in the CRISPR-SpCas9 system (Fu et al. 2014). Also, 2 guanines at the 5′ end of the gRNA showed reduced off-target effects of the CRISPR-SpCas9 (Cho et al. 2014). Currently, there are several programs assisting the most adequate gRNA design (Koo et al. 2015).

Engineering the Cas9 variants

SpCas9 with four amino acid substitutions showed reduced non-specific local interaction with the DNA strand and generated off-target effects below detection in several trials, while retaining high on-target activity, the SpCas9 high-fidelity variant #1 (SpCas9-HF) (Kleinstiver et al. 2016). There are some other examples of the high-fidelity Cas9 variants, such as enhanced specificity SpCas9 (eSpCas9) (Slaymaker et al. 2016), high-fidelity Cas9 (HiFiCas9) (Vakulskas et al. 2018), and hyper-accurate Cas9 variant (HypaCas9) (Chen et al. 2017), all aimed to reduce off-target effects while maintaining efficient on-target activity by reasonable protein engineering. In addition, a single amino acid substitution resulted in increased PAM specificity of Cas9 (Kleinstiver et al. 2015).

In the case of base editor, introducing mutations on non-specific, local DNA-interacting domains of BE showed decreased frequencies of off-target effects (Rees et al. 2017). BE with engineered APOBEC3A, a kind of cytidine deaminase that preferentially edits cytidines in specific motifs of the protein, also showed reduced off-target effects (Gehrke et al. 2018). Finally, BE of narrowed editing windows exhibited reduced off-target effects (Kim et al. 2017).

Employment of the dimeric nucleases

Employing the couple of CRISPR-Cas9 nickases was able to double the target specificity since a nick on the single strand can be easily repaired and not induce DSB (Ran et al. 2013). Similarly, instead of Cas9, employing dCas9 in complex with *Fok*I catalytic domain showed increased target specificity whereby *Fok*I shows DSB activity only when in the form of a dimer (Guilinger et al. 2014).

Inducible CRISPR system

Generating an inducible CRISPR system is another method for reducing the off-target effects since it can control the CRISPR system's activity, preventing the system's unnecessary activity which may lead to unwanted off-target effects. The inducible CRISPR system includes the chemical-induced or the photoactivatable CRISPR systems (Zetsche et al. 2015; Davis et al. 2015; Nihongaki et al. 2015; de Solis et al. 2016). For example, there is the split Cas9 system, where each fragment of the Cas9 dimerize, and becomes functional under the presence of certain chemicals, such as rapamycin, abscisic acid or gibberellin (Lo and Qi 2017; Bao et al. 2017; Gao et al. 2016). In the study dealing with the split Cas9 system dependent on rapamycin, researchers appended different localization signals to the each N-terminal and C-terminal fragment of the Cas9, preventing spontaneous dimerization of the fragments and successfully regulating the base line activity of the system in the absence of the chemical (Zetsche et al. 2015). Meanwhile, Cas9 in complex with the protein-destabilizing domain showed rapid degradation. However, expression of the stabilizing small molecules induced temporal genome editing of the CRISPR-Cas9, successfully regulating the system's activity (Senturk et al. 2017). Designing the inducible CRISPR system is also possible by modulating gRNA. Engineered gRNA was in complex with certain aptamer, responsive to certain chemicals such as tetracycline, theophylline or guanine, which prevented the gRNA from binding the target DNA sequences. Thus, the CRISPR system could work only in the presence of the chemicals (Liu et al. 2016; Tang et al. 2017). Recently, a new kind of inducible CRISPR-Cas9 was reported. In the protease sensing Cas9 (ProCas9) system, introduction of protease leads to activation of the Cas9 protein by dissociating the Cas9-circular permutants (CP) complex. Though further studies are required, the ProCas9 system seems to have pretty low-background activity, which means that without introduction of proteases, genome editing activity by Cas9 is extremely low, thereby contributing to prevention of unwanted off-target effects which can be pathogenic (Oakes et al. 2019). As mentioned before, US may be applied to the inducible CRISPR system (Tabuchi et al. 2007).

These strategies for inducible CRISPR-Cas9 could also be applied to generating the inducible base editor system. In fact, delivering each part of the base editor using AAV vectors, otherwise known as the split base-editing system, has already been conducted. However, this was attributed to the tight restriction of the maximal cargo size in AAV vectors (Villiger et al. 2018). Thus, further studies related to the split base-editing system are needed to demonstrate successful regulation of base editing and subsequent reduction of off-target effects.

Inhibition of the CRISPR system

Several molecules could be recruited for regulating the activity of the CRISPR system. Anti-CRISPR proteins are able to inhibit the CRISPR system in several ways. For example, AcrIIA4, a kind of anti-CRISPR protein, disrupts PAM sequence recognition of SpCas9, only when Cas9 forms a complex with its gRNA (Bondy-Denomy et al. 2015; Yang and Patel 2017). AcrIIA4 with CRISPR-Cas9 complex showed reduced off-target effects while conserving on-target genome editing (Shin et al. 2017). Since target recognition of BEs also involves gRNA and PAM recognition, anti-CRISPR may result in reduced off-target effects by BE, too. Adenosine/cytidine deaminase inhibitors like the DREAM complex, mentioned above, may serve as a tool for reducing the off-targets caused by BE (Periyasamy et al. 2017; Zhang et al. 2018).

Quick repair of the DSB

The third strategy for reducing the off-target effects is to repair them immediately. For CRISPR-Cas9, up-regulation of HDR can result in increased repair of the indels formed by the system, leading to higher probability of reversing the off-target effects. Strategies to up-regulate HDR are reviewed above (Gutschner et al. 2016; Lin et al. 2014; Chu et al. 2015; Canny et al. 2018; Shao et al. 2017; Li et al. 2017; Richardson et al. 2016; Aparicio et al. 2014). Co-delivery of CRISPR-Cas9 with a donor DNA strand for possible off-target sites may help with immediate repair of the off-target site cleavage. Determining the sequences of the donor strand may be assisted by use of programs for predicting potential off-target sites (Haeussler et al. 2016; Wang and Ui-Tei 2017; Lin and Wong 2018; Zhang et al. 2018).

On-target mutations

On-target mutations can also be the problem in therapeutic genome editing. In the mouse model of Duchenne muscular dystrophy, it has been shown that the CRISPR-SaCas9 can affect the target genome variously, resulting in unintended genomic or transcriptional alterations which should be considered for the therapeutic application (Nelson et al. 2019). Significant on-target mutations like large deletions, which may possess unexpected pathogenic potentials, have also been reported in several mouse and human cells (Kosicki et al. 2018). Finally, there was the actual adverse effect of the mutation caused by the on-target activity of the CRISPR-Cas9 in a mouse model (Simeonov et al. 2019).

Conclusion

The CRISPR system is being widely applied in the therapeutic field, showing strong potential to treat various diseases. However, as reviewed here, there are still several limitations for the therapeutic application of this system. For ultimate utilization in the clinical field, a CRISPR system with minimal concerns related to side effects is ideal. A lot of research is required for overcoming the limitations and considering a broad spectrum of strategies, including the ones reviewed here. If the limitations are successfully overcome, the CRISPR system will 1 day become a breakthrough approach for curing diseases from the fundamental, genomic level.

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