Review article

Designed nucleases for targeted genome editing

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Summary
Targeted genome-editing technology using designed nucleases has been evolving rapidly, and its applications are widely expanding in research, medicine and biotechnology. Using this genome-modifying technology, researchers can precisely and efficiently insert, remove or change specific sequences in various cultured cells, micro-organisms, animals and plants. This genome editing is based on the generation of double-strand breaks (DSBs), repair of which modifies the genome through nonhomologous end-joining (NHEJ) or homology-directed repair (HDR). In addition, designed nickase-induced generation of single-strand breaks can also lead to precise genome editing through HDR, albeit at relatively lower efficiencies than that induced by nucleases. Three kinds of designed nucleases have been used for targeted DSB formation: zinc-finger nucleases, transcription activator-like effector nucleases, and RNA-guided engineered nucleases derived from the bacterial clustered regularly interspaced short palindromic repeat (CRISPR–Cas (CRISPR-associated) system. A growing number of researchers are using genome-editing technologies, which have become more accessible and affordable since the discovery and adaptation of CRISPR-Cas9. Here, the repair mechanism and outcomes of DSBs are reviewed and the three types of designed nucleases are discussed with the hope that such understanding will facilitate applications to genome editing.

Introduction
To understand how genotypes influence phenotypes, researchers have traditionally used targeted gene inactivation via homologous recombination (HR). However, this approach is time-consuming and challenging in plant cells mainly because the efficiency of such HR is extremely low (ranging from 1 in 103 to 105 of transformed cells) (Offringa et al., 1990; Paszkowski et al., 1988). Alternatively, targeted gene knockdown by RNA interference (RNAi) has become popular as a method for targeted inhibition of specific endogenous genes, because it is rapid, inexpensive and suited for high-throughput applications. However, knockdown of gene expression by RNAi is usually incomplete and only leads to temporary inhibition (Krueger et al., 2007). Furthermore, RNAi-based knockdown is often complicated with unpredictable off-target effects (Jackson et al., 2003).

A new genome-editing technology, based on designed nucleases that produce site-specific DNA double-strand breaks (DSBs), has emerged that enables precise and efficient targeted genetic modifications in various cells and organisms, including plants. In the absence of homologous templates, DSBs trigger error-prone nonhomologous end-joining (NHEJ), resulting in targeted mutagenesis (Bibikova et al., 2002; Rouet et al., 1994; Salomon and Puchta, 1998). In contrast, in the presence of an appropriate homologous template, DSBs can lead to precise homology-directed repair (HDR), which is at least two orders of magnitude more efficient than the conventional donor DNA-based gene inactivation method, which takes place in the absence of an appropriate DSB (Puchta et al., 1993; Rouet et al., 1994).

At the end of 2011, Nature Methods chose genome editing with designed nucleases, including zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), as the ‘Method of the Year’. Soon after, in January 2013, several groups independently reported the use of a novel class of nucleases derived from the bacterial clustered regularly interspaced short palindromic repeat (CRISPR–Cas9) system as a genome-editing tool (Cho et al., 2013; Cong et al., 2013; Hwang et al., 2013; Jiang et al., 2013; Jinek et al., 2013; Mali et al., 2013b). This latter class of nucleases, also referred to as RNA-guided engineered nucleases (RGENs), has been rapidly evolving since then.

Here, we review the three types of designed nucleases for inducing targeted DSBs. First, we will discuss the generation, repair and effects of DNA DSBs. Next, we will describe and compare the general features of ZFNs, TALENs and RGENs. Finally, we will speculate as to future directions and applications of designed nucleases for genome editing.

Double-strand breaks
The generation of DSBs is a key process in targeted genome editing. DSBs are a form of DNA damage that occurs when both DNA strands are cleaved. Genetically, DSBs result in discontinu-
ities of genetic information, leading to perturbation or inactivation of that information. Chemically, DSBs are discontinuities in the covalently linked carbon-phosphate backbones of both strands. Whereas some physiologic DSBs are generated in early-stage lymphocytes of the vertebrate immune system to produce antibody diversity, most DSBs are generated by pathologic causes that include ionizing radiation and oxidative free radicals (Lieber and Karanjawala, 2004; Lieber et al., 2003). DSBs in mammalian cells can be repaired by homologous recombination (HR) and NHEJ. Traditionally, one of the most popular methods for gene modification was based on using HR, a technique that has been widely employed in mouse embryonic stem cells to generate germ-line knockout or knockin mice. However, the efficiency of HR events is extremely low (ranging from 1 in $10^6$ to 1 in $10^7$) in higher eukaryotes. In 1994, the discovery that the introduction of a DSB increases the frequency of HR by at least 2–3 orders of magnitude (Puchta et al., 1993; Rouet et al., 1994) led to efficient HR-based genome editing using programmable nucleases that generate DSBs at specific loci. Furthermore, in the absence of a homologous template, NHEJ repair of DSBs can lead to targeted gene disruption due to the error-prone nature of this process (Bibikova et al., 2002; Rouet et al., 1994). Such HR- or NHEJ-mediated repair of DSBs generated with programmable nucleases allows exquisitely precise genome modifications, such as gene disruptions (knockouts), insertions (knockins) and corrections (substitutions), as well as chromosomal rearrangements (Figure 1).

**Repair of DNA double-strand breaks**

**Nonhomologous end-joining**

Nonhomologous end-joining is a natural pathway for repairing DSBs through the ligation of two broken DNA ends. NHEJ often terminates the repair with errors and can lead to the introduction of small insertions and deletions (collectively called indels) at the site of the DSB (Figure 1a). Small indels often induce frameshifts, causing gene knockout by a combination of two mechanisms: premature truncation of the encoded protein and non-sense-mediated decay of the mRNA transcript (the latter is not always particularly efficient). NHEJ can occur during any phase of the cell cycle. In higher eukaryotes, NHEJ, rather than HDR, is the dominant DSB repair system (Lieber, 2010; Puchta, 2005).

**Figure 1** Outcome of genome editing through designed nuclease-based generation of double-strand breaks (DSBs). (a) In the absence of donor templates, nuclease-induced DSBs can be repaired by error-prone nonhomologous end-joining, which consequently often results in small insertions or deletions (indels). With appropriate donor DNA or single-strand oligodeoxynucleotide (ssODN), DSBs can be repaired by homology-directed repair, which can lead to sequence insertion and nucleotide substitution. (b) When designed nucleases generate two different DSBs on a single chromosome, the flanking region can be deleted or inverted. (c) When designed nucleases generate DSBs on two different chromosomes, interchromosomal translocations can be induced.
Ligation of two DNA ends through NHEJ requires various repair enzymes. Both Ku-dependent and Ku-independent NHEJ sub-pathways exist. In classical, Ku-dependent NHEJ, the DNA end protection factors (which form the Ku70/80 heterodimer) bind to the ends of the DNA strand at the break site and recruit the repair enzyme ligase IV and its cofactor. During NHEJ, annealing of exact complementary single-stranded ends can result in accurate repair. However, most breaks occurring in the cell do not have complementary ends, and NHEJ frequently proceeds through the annealing of short (1–4 bp) microhomologous sequences. Often, DNA end processing leads to the formation of small (1–4 bp) insertions and/or deletions (indels) at the DSB site (Lieber, 2010). The alternative end-joining (Ku-independent) pathway can repair DSBs without Ku-dependent pathway factors. Microhomology-mediated end-joining (MMEJ) is a major Ku-independent NHEJ pathway. MMEJ uses 5- to 25-bp microhomology sequences during the alignment of broken ends before joining. MMEJ proceeds by annealing the microhomology regions, removing overhanging nucleotides and filling in the missing base pairs. Thus, MMEJ frequently produces a longer deletion at the DSB site than does Ku-dependent NHEJ (McVey and Lee, 2008).

Homology-directed repair

Homology-directed repair is a template-dependent pathway for DSB repair (Figure 1a). In contrast to error-prone NHEJ pathways, HDR is precise. The defining step of HDR is the pairing of a single-stranded DNA that is processed from a broken or damaged DNA site with its complement in a homologous region of undamaged double-stranded DNA (for example, the sister chromatid). This pairing is catalysed by the interaction of DNA strand exchange proteins such as RecA and Rad51 with a series of DNA substrates (Sarbajna and West, 2014). Unlike NHEJ, HDR is restricted to late S/G2 phases of the cell cycle.

Outcomes of DSB repair

Through the NHEJ mechanism

Small indels that are created at the target site through error-prone NHEJ can result in target gene knockout through the mechanisms discussed above. This process simply requires an appropriate designed nuclease; a homologous template is not needed. One of the standard methods for determining gene function is to observe the phenotype of knockout cells and organisms that lack functional copies of the gene of interest. NHEJ-mediated repair of DSBs that are generated by engineered nucleases has been widely used to produce various knockout cell lines (Kim and Kim, 2014; Segal and Meckler, 2013).

Two concurrent DSBs induced by two different designed nucleases, targeting regions far away from one another on a single chromosome, can give rise to chromosomal rearrangements or structural variations (Figure 1b,c). Deletions, inversions and translocations of large chromosomal segments (up to a few megabase pairs in length) have been achieved using three different types of designed nucleases (Carlson et al., 2012; Cong et al., 2013; Gupta et al., 2013; Kim et al., 2013a; Lee et al., 2010, 2012; Petolino et al., 2010; Qi et al., 2013b; Xiao et al., 2013). By inducing DSBs on two different chromosomes, inter-chromosomal translocations have also been made (Brunet et al., 2009; Cho et al., 2014) (Figure 1c). Recently, various cancer models containing chromosomal rearrangements have been generated using designed nucleases (Lagutina et al., 2015; Maddalo et al., 2014). This method has also been used to rescue a disease genotype caused by a chromosomal inversion (Lee et al., 2012; Park et al., 2014).

Through the HDR mechanism

In the vast majority of cases in plant and animal cells, transgene DNA integrates into nontargeted, random genomic locations. If the transgene integrates into undesired sites, it may inactivate essential genes or, in the case of mammalian cells, activate proto-oncogenes (Hacein-Bey-Abina et al., 2003). Also, randomly integrated transgenes can be epigenetically silenced depending on the site of integration. In contrast, targeted gene knockin using designed nucleases has several advantages. Targeted DSB generation with programmable nucleases allows the insertion of desired genes into predetermined locations such as ‘safe harbour’ sites with enhanced efficiency (Doyon et al., 2011; Hockemeyer et al., 2011; Li et al., 2011). ‘Safe harbour’ sites are locations in the genome where therapeutic transgenes can be integrated and expressed in a predictable manner without perturbing endogenous gene expression (Sadelain et al., 2012). To insert genes of interest into specific loci including genomic safe harbours, the nuclease is delivered into cells together with a targeting vector (donor DNA) that comprises the transgene and flanking arms that are homologous to the sequences near the target region (Figure 1a).

Point mutations can be corrected or single-nucleotide variations can be introduced in the target site of the genome through codelivery of designed nucleases and targeting vectors (Bibikova et al., 2001, 2003; Porteus and Baltimore, 2003) or single-stranded oligodeoxynucleotides (ssODNs) (Chen et al., 2011) (Figure 1a). In the case of donor DNA, the preparation is often cumbersome and time-consuming. However, ssODNs can be easily designed and synthesized (Chen et al., 2011). This ssODN-coupled point mutagenesis has been used in an easy, precise and efficient manner for the generation of disease models in animals (Cui et al., 2011; Wang et al., 2013; Wefers et al., 2013) and human cells (Soldner et al., 2011), for therapeutic purposes in an animal model of disease (Yin et al., 2014) and for introducing point mutations in the plant genome (Shan et al., 2013).

Three types of designed nucleases

Zinc-finger nucleases

Zinc-finger nucleases are composed of a zinc-finger protein (ZFP) domain, which is a designable, sequence-specific DNA-binding domain, and a nonspecific DNA cleavage domain derived from the type II restriction enzyme FokI (Kim et al., 1996) (Figure 2). The FokI nuclease domain must be dimerized to cleave DNA (Bitinaite et al., 1998); thus, two different ZFN monomers, each binding to a different strand, are required for an active nuclease. A ZFN is designed as a pair of monomers that recognizes two sequences, which flank the target site and are separated by a 5- to 7-bp spacer sequence (Figure 2a). One monomer binds to the forward strand and the other to the reverse strand.

The required dimerization of ZFN monomers expands the length of recognition sites, which substantially increases ZFN specificity. Each zinc-finger domain usually recognizes a 3-bp DNA sequence (Wolfle et al., 2000), and several domains arrayed in tandem can bind to proportionately longer nucleotide sequences (3–6 zinc-finger domains are used to generate a single ZFN subunit that binds to DNA sequences of 9–18 bp) (Figure 2a). Importantly, the specificity of a zinc-finger DNA-
binding domain can be altered by mutagenesis (Desjarlais and Berg, 1992; Rebar and Pabo, 1994). Such manipulation of ZFPs to alter their binding specificity is a key feature of constructing a designed nuclease. New ZFPs with desired specificities can be constructed by modularly assembling precharacterized zinc fingers (Bae et al., 2003; Bibikova et al., 2002, 2003; Kim et al., 2010;
Segal et al., 2003). Cell-based selection methods and modular assembly methods that consider context dependence between neighbouring zinc fingers have been developed to yield functional ZFNs (Bhakta et al., 2013; Gupta et al., 2012; Maeder et al., 2008; Sander et al., 2011). The use of obligatory heterodimeric FokI domain developed by modification of wild-type FokI domain significantly enhances specificity and reduces off-target effects (Miller et al., 2007; Szczepak et al., 2007). Nonetheless, it remains challenging to make efficient, specific ZFNs.

Zinc-finger nucleases have some disadvantages compared with newly developed programmable nucleases. First, compared with TALENs or RGENs, ZFNs have limited target availability. So far, there is no open-source collection of 64 (4 x 4 x 4) zinc fingers that covers all possible combinations of 3-bp subsites (Bae et al., 2003; Segal et al., 1999). Furthermore, not all engineered ZFNs create DSBs efficiently. Successful target sites are often in guanine-rich regions, consisting of 5'-GNN-3' (where N represents any nucleotide) repeat sequences. Thus, a single functional ZFN pair can be obtained per ~100-bp DNA sequence on average (Kim et al., 2009). This limitation is not too important for those intending to knock out a gene, because a frameshift introduced anywhere in the early coding sequence of the gene would suffice. However, generating a functional ZFN may be challenging if one particular target site is required, such as for creating a deletion, insertion or substitution at a particular site. Second, ZFNs often show low DNA-targeting activity (Ramirez et al., 2008) or are cytotoxic owing to off-target effects (Cornu et al., 2008). Third, it is difficult for nonspecialists to make ZFNs that target specific sites routinely. Although an academic consortium developed an open-source library of zinc-finger components and a screening protocol to identify ZFNs with high affinity and efficiency (Maeder et al., 2008, 2009), the library has not yet been widely accepted among researchers. However, ZFNs also have advantages compared with TALENs and RGENs. ZFN-encoding sequences (~1 kb x 2) are smaller than TALEN- (~3 kb x 2) and RGEN-encoding sequences (~4.2 kb for the protein + 0.1 kb RNA), facilitating delivery with viral vectors that have limited cargo size, such as the adeno-associated viral (AAV) vector. In addition, ZFPs are derived from mammalian proteins, whereas TALENs and RGENs have a bacterial origin. Thus, we speculate that ZFN immunogenicity is lower than that of TALENs or RGENs, although a careful comparison awaits further investigation.

**Transcription activator-like effector nucleases**

Like ZFNs, a TALEN consists of a designable, sequence-specific DNA-binding domain and a nonspecific DNA cleavage domain derived from FokI (Miller et al., 2011) (Figure 3). However, TALENs use a different type of DNA-binding domain known as transcription activator-like effectors (TALEs), which are derived from a species of plant pathogenic bacteria. Whereas each zinc-finger domain recognizes a 3-bp DNA sequence, there is a one-to-one correspondence between TALE domains and base pairs. TALEs are composed of tandem arrays of 33–35 amino acid repeats, each of which recognizes a single base pair in the major groove of DNA (Deng et al., 2012; Mak et al., 2012) (Figure 3b). The specificity of each repeat is conferred by the two amino acids at positions 12 and 13, known as repeat variable diresidues (RVDs) (Figure 3c). To recognize guanine, adenine, cytosine and thymine, RVD modules of Asn-Asn, Asn-Ile, His-Asp and Asn-Gly, respectively, are widely used. TALENs can be designed to target almost any given DNA sequence, which is a critical advantage over other types of nucleases.

Compared with ZFNs, TALENs are much easier to design and construct. TALENs are often built to bind 18- to 20-bp sequences. In fact, larger TALENs may result in lower specificity (Guilinger et al., 2011a). It is also tricky to construct longer TALE arrays because of the recombination that can occur due to the highly homologous TALE sequences (Holkers et al., 2013). Several methods have been developed for the assembly of custom-designed TALE arrays (Briggs et al., 2012; Cermak et al., 2011; Reyon et al., 2012; Schmid-Burgk et al., 2013). The target site binding affinity of an engineered TALE repeat array has been reported to be as high as 96% (Hockemeyer et al., 2011; Miller et al., 2011; Reyon et al., 2012). Additionally, genomewide libraries of TALENs that target protein-coding genes (Kim et al., 2013a) and microRNA-coding sequences (Kim et al., 2013b) have been constructed.

As mentioned above, the relatively large size of TALEN-encoding sequences can limit TALEN delivery and expression. This limitation is especially restrictive in mammalian cells, where viral vectors such as AAV are often used. Because of their low immunogenic potential and the low oncogenic risk from host-genome integration, AAV vectors are attractive as delivery vehicles for programmable nucleases. However, the cargo size of AAV is ~4.5 kb excluding the inverted terminal repeats, preventing delivery of a TALEN pair using this method. Furthermore, the highly repetitive nature of TALEN sequences may hinder their ability to be packaged and delivered by some viral vectors (Holkers et al., 2013). Although it is not a major issue for plant transformation, which is mostly performed using Agrobacterium T-DNA or plasmid DNA, the size of TALEN sequences would pose challenges for DNA assembly in multiplexing or multicolour targeting. The strategy of diversifying TALEN repeat coding sequences may be helpful for overcoming this problem (Yang et al., 2013).

**CRISPR-Cas9 (RNA-guided engineered nucleases)**

Zinc-finger nucleases and TALENs are relatively expensive due to the difficulty of synthesis. Genome editing became more accessible with the discovery and adaptation of the CRISPR-Cas9 system. This system, owing to its efficiency and ease of use, has now become the most popular genome-editing tool.

**CRISPR-Cas9 as an adaptive immune system in bacteria and archaea.** The RNA-guided DNA cleavage system naturally exists as an adaptive form of immunity against invading phages or plasmids in bacteria and archaea (Barrangou et al., 2007; Makarova et al., 2006). These organisms ‘remember’ the sequences of previously invading viral genomes and protect themselves by recognizing and cutting those sequences when they are encountered again. This type of acquired immunity proceeds via the capture of foreign DNA fragments (~20 bp) from invading phages or plasmids and the incorporation of these sequences (termed protospacers) into the bacterial or archaeal genome to form CRISPR. In type II CRISPR systems, these CRISPR regions (memory elements) are transcribed as pre-CRISPR RNA (pre-crRNA) and processed to form the target-specific CRISPR RNA (crRNA). Trans-activating crRNA (tracrRNA), a target-independent component, is also transcribed from the CRISPR region and is involved in the processing of pre-crRNA (Deltcheva et al., 2011). Both RNAs complexed with CRISPR-associated protein 9 (Cas9) form an active DNA endonuclease system, and destroy any DNA sequences that match the protospacer. In the case of the system from Streptococcus pyogenes, which is the
origin of the first engineered CRISPR-Cas9 system, the endonuclease can cleave a 23-bp target DNA sequence that is composed of a 20-bp guide sequence identical to the crRNA (protospacer) and a 5'-NGG-3' (or, to a lesser extent, 5'-NAG-3') sequence known as protospacer adjacent motif (PAM), which is recognized by Cas9 itself (Mojica et al., 2009). This PAM sequence can distinguish between 'self' (protospacers) and 'nonself' (invader) DNA sequences, priming the nonself sequences for a DSB at a site 3 bases before the PAM. Cas9 proteins derived from species other than *S. pyogenes* recognize different PAM sequences (Cong et al., 2013; Fonfara et al., 2014; Hou et al., 2013; Mojica et al., 2009; Shah et al., 2013).

CRISPR-Cas9 for genome engineering. Here, we use the term 'RNA-guided engineered nuclease (RGEN)' to represent a new type of genome-editing nuclease to avoid confusion with the natural type II CRISPR-associated adaptive immune system in bacteria.

In 2012, it was reported that guide RNA and purified Cas9 protein can cleave target DNA in vitro (Gasiunas et al., 2012; Jinek et al., 2012). In January 2013, several groups independently reported a new class of genome-editing nucleases (Cho et al., 2013; Cong et al., 2013; Hwang et al., 2013; Jiang et al., 2013; Jinek et al., 2013; Mali et al., 2013b), soon followed by their application in plants (Li et al., 2013a; Shan et al., 2013). The specificity of this system is determined by small guide RNAs rather

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**Figure 3** Structure of transcription activator-like effector nucleases (TALENs). Each TALEN domain and module is shown in the same colour in both (a), (b) and (c). (a) A schematic representation of a TALEN pair is shown. Each TALEN is composed of transcription activator-like effectors (TALEs) at the amino terminus and the FokI nuclease domain at the carboxyl terminus. Target sequences of TALEN pairs are typically 30–40 bp in length, excluding a 12- to 21-bp spacer. (b) A TALE protein in complex with target DNA. Each TALE repeat comprises 33–35 amino acids and recognizes a single base pair at the major groove through the hypervariable residues at positions 12 and 13, which are called a repeat variable diresidue. This model was prepared from crystal structures of TALE bound to DNA (Protein Database 3UGM) (Mak et al., 2012) and the FokI restriction endonuclease in the absence of DNA (Protein Database 2FOK) (Wah et al., 1998) based on a previous analysis. (c) Recognition of bases by corresponding repeat variable diresidues.
Disadvantages of RGENs. The coding sequence of _S. pyogenes_ Cas9 is ~4.2 kb. Even though the other designed nucleases act as dimers, this Cas9 sequence is longer than that encoding a TALEN monomer (~3 kb) or a ZFN monomer (~1 kb). Therefore, delivery of RGENs via viral systems is somewhat challenging. For proper transcription, a promoter and a polyadenylation sequence are required in addition to the Cas9 sequence. The sgRNA is approximately 100 bp, which must be delivered in parallel with the Cas9 sequence to produce an active RGEN. Inclusion of an RNA III polymerase promoter such as the U6 promoter for sgRNA. RGENs have been delivered to various types of plants using polyethylene glycol (PEG)-protoplast transfection, Agrobacterium-mediated transformation and microparticle bombardment, all of which have relatively large cargo capacities that are sufficient for RGEN accommodation (Bortesi and Fischer, 2015). The efficiency of RGEN-mediated genome editing clearly is affected by the delivery method in plant as well as animal cells. For example, PEG-mediated protoplast transfection resulted in a 10-fold higher mutation rate than that by Agrobacterium-mediated transformation in _N. benthamiana_ (Li et al., 2013a,b). Although it is less of an issue for plant biotechnology, efficient application of RGENs in mammalian cells sometimes requires the use of a specific DNA delivery system such as lentivirus that can accommodate the _S. pyogenes_ RGEN system; AAV, with its cargo size limited to less than 4.8 kb, cannot easily do so. Efforts have been made to reduce the size of RGEN-encoding sequences (using a short promoter and polyadenylation sequence) for use in AAV (Swiech et al., 2015). In addition, CRISPR-Cas systems from other species, some of which involve smaller Cas9 proteins, should be helpful in this regard. Recently, RGEN AAV, which contains the sequence encoding the smaller _Staphylococcus aureus_ Cas9 (more than 1 kb shorter than that encoding _S. pyogenes_ Cas9) and guide RNA in one shuttle vector, has been reported (Ran et al., 2015). These developments allow for the delivery of RGENs via AAV, which may be important for therapeutic applications that often require high delivery efficiency.

**Modification of designed nucleases**

**Designed nickases**

Nonhomologous end-joining-mediated repair of DSBs induced by designed nucleases inevitably causes the formation of uncontrolled and undesirable indels at the target site and, potentially, at off-target sites, even in the presence of a homologous donor template for HDR. In higher vertebrates and plants, DSBs are
primarily repaired by NHEJ. Altered (or ‘resistant’) sequences with indels at the cleavage site cannot be retargeted with the original designed nuclease. To correct these resistant sequences, they must first be identified; then, programmable nucleases that target each resistant sequence must be newly designed. Given that NHEJ-mediated indel formation is hard to predict, a variety of resistant sequences can be generated, making it difficult to design nucleases that target all of the resistant sequences. DSBs generated in the resistant sequences can then lead to the creation of a second set of resistant sequences, again through NHEJ-
mediated repair of the DSBs, preventing precise genome editing. To avoid the generation of unwanted mutations by designed nucleases, nickases that produce single-strand breaks (SSBs) rather than DSBs have been proposed as an alternative. Chemically, SSBs are discontinuities in the covalently linked carbon-phosphate backbone of one strand in the DNA double helix. A SSB can result in a discontinuity of the genetic information in the affected strand. However, SSBs perturb genetic information much less frequently than do DSBs because the other, intact strand can be used as a template to guide the correction of the damaged strand. Naturally, one of the most common sources of SSBs is oxidative attack by endogenous reactive oxygen species. A SSB can enhance HDR, although the efficiency is lower than that of the nuclease (Davis and Maizels, 2011; McConnell Smith et al., 2009). SSBs are repaired via the high-fidelity base excision repair (BER) pathway (Dianov and Hübscher, 2013) and do not activate the NHEJ pathway, preventing generation of unwanted indels. Thus, nickases can lead to precise genome editing.

The first designed nickases were modified ZFNs that consisted of one intact and one mutant FokI subunit with a mutation at the active catalytic site (Kim et al., 2012; Ramirez et al., 2012; Wang et al., 2012). ZF nickase heterodimers generate a SSB at the target site and do not cause undesirable DSBs at either the target site or off-target sites (Kim et al., 2012; Wang et al., 2012). ZF nickases induced precise genome editing via HDR with a lower efficiency than the corresponding ZF nucleases (Kim et al., 2012; Ramirez et al., 2012; Wang et al., 2012). Other designed nickases derived from TALENs have also been reported and tested in vitro (Gabsaillou et al., 2013).

Figure 5 Modifications of Cas9 as a genome engineering platform. (a) Cas9 nuclease. The Cas9 nuclease cleaves both strands of DNA via its RuvC and HNH nuclease domains, each of which makes a nick in a DNA strand, leading to the generation of blunt-end DSBs. (b) Cas9 nickases. Either catalytic domain can be inactivated to generate nickase mutants that each make single-strand DNA breaks. (c) RNA-guided FokI nuclease. Here, both of the Cas9 catalytic domains are inactivated to generate dead Cas9 (dCas9). Two FokI–dCas9 fusion proteins are recruited to adjacent target sites by two different guide RNAs to facilitate FokI dimerization, leading to a double-strand DNA cleavage between the two target sites by the activated FokI dimer.

Cas9 has two active catalytic domains, RuvC and HNH, which each cleave one strand and together generate a blunt-ended DSB (Figures 4d,e and 5a). Two types of Cas9 nickases have been constructed via the introduction of point mutations in RuvC (D10A) and HNH (H840A) (Figure 5b); both nickases have been shown to form SSBs (Gasiunas et al., 2012; Jinek et al., 2012; Supranaukas et al., 2011). RNA-guided engineered nickases (RGENickases) that contain the S. pyogenes Cas9 HNH–RuvC nickase mutant (D10A), which has better efficiency than the HNH/RuvC+(H840A) mutant, lead to high-fidelity HDR with negligible NHEJ-driven mutations (Cong et al., 2013; Fauser et al., 2014; Mali et al., 2013b).

To improve DSB specificity, paired nickases, like dimeric ZFNs and TALENs, can be used to increase the number of bases that are recognized. Because individual nicks in the genome are repaired with high fidelity through the BER pathway, off-target SSBs would be precisely repaired. Furthermore, because the probability that two nickases would make off-target SSBs that are close to each other in the genome is extremely low, the off-target mutation rate would be dramatically reduced. Paired nickases designed to make two SSBs, one on each of the two DNA strands, collectively generate a composite DSB, which will lead to indel formation through NHEJ. Properly spaced ‘paired nickases’ showed efficiency comparable to that of the corresponding nuclease with up to 500-fold reduced off-target activity in human and mouse cells (Cho et al., 2014; Kim et al., 2012; Mali et al., 2013a; Ran et al., 2013a). Comparable with the results from experiments using animal cells, a recent study on Arabidopsis found that the on-target mutagenic rate of paired nickases was the same as that of the Cas9 nuclease (Schiml et al., 2014).

RNA-guided FokI nucleases

An RNA-guided FokI nuclease, analogous to dimeric ZFNs or TALENs, is a fusion of a dimerization-dependent FokI nuclease domain as the cleavage domain and a catalytically inactive Cas9 (termed dead Cas9, dCas9) as the DNA-binding domain (Guilinger et al., 2014b; Tsai et al., 2014) (Figure 5c). As with paired nickases, highly specific gene targeting is feasible using RNA-guided FokI nucleases (RFNs) because of the increased number of bases that are recognized at a given site.

Transcriptional regulation using dead Cas9

Cas9 coupled with guide RNA has two key properties. One is the ability to bind to DNA at a targeted site, and the other is its catalytic function. A catalytically inactivated Cas9 (dCas9) has been repurposed to allow controlled transcriptional regulation of genes. Whereas transcriptional regulation using dCas9 has only transient effects, similar to RNAi, active Cas9 elicits permanent changes in the genome.

Although RNAi is a popular tool for knockdown of target gene expression, RNAi-based experiments are often complicated by inefficiency or unpredictable off-target effects. Transcriptional regulation using dCas9 represents a good alternative, which can elicit both up- and down-regulation of the expression of multiple genes simultaneously (Bikard et al., 2013; Gilbert et al., 2013; Qi et al., 2013a,b). Binding of a dCas9–guide RNA ribonucleo protein complex to an appropriate DNA element can repress transcription by blocking transcriptional elongation, RNA polymerase binding or transcription factor binding (Qi et al., 2013a, b). CRISPR-based interference using dCas9–guide RNA complexes themselves is less efficient in eukaryotes compared to prokaryotes.
Comparison of the three types of engineered nucleases

Efficacy

Not all newly designed nucleases are functional and equally efficient (Table 1), and it is difficult to predict the efficiency of newly designed nucleases. ZFNs usually exhibit relatively low efficiency for generating DSBs in cultured cells or organisms compared to TALENs or RGENs. Gene knockout efficiency with functional nucleases is difficult to predict and has been reported to range from 1% to ~60% in mammalian cells (Kim et al., 2013a; Reyon et al., 2012). RGENs also have shown a wide range of genome-editing activities (2.3–79%) in cultured cells (Cho et al., 2013; Cong et al., 2013; Ding et al., 2013; Jinek et al., 2013; Mali et al., 2013b). Both the target cell type and the delivery method seem to significantly affect the activity of all three classes of nucleases. Recently, a program was designed to enable approximate prediction of the activity of designed guide RNAs based on high-throughput efficiency data from 1841 guide RNAs (Doench et al., 2014). Although the standard, most accurate method for determining the activity of individual guide RNAs is cell-based analysis, this in silico analysis can be useful for selecting several highly active guide RNA candidates that could then be subjected to actual evaluation in cells, which requires much more labour, time and cost than in silico analysis. This in silico program-assisted approach can be useful for obtaining highly active guide RNAs for gene knockout, for which a large number of guide RNA can be designed. However, the users of this program should be aware of the possibility that sgRNA activity predicted by this program can be different from the actual sgRNA activity measured by cell-based analysis. Similar programs for plant genome editing are also available (Xie et al., 2014).

Safety

The specificity of ZFNs and TALENs can be determined by the number of zinc fingers and TALE modules the nucleases contain. More modules are generally thought to signify a higher specificity. However, too many modules can elevate the possibility of partial binding at many unwanted sites. Theoretically, nucleases should recognize DNA sequences of at least 16 bp to eliminate potential off-target effects in the human genome because the complexity of 16-bp sequences ($4^{16} = 4.3 \times 10^9$) is greater than the size of the human haploid genome (3.2 × 10⁹). However, modifying crop plant genomes that are larger than the human genome may require a longer target site sequence to minimize off-target effects. In reality, however, all three nucleases with target site sizes greater than 16 bp have shown some off-target effects (Fu et al., 2013; Gabriel et al., 2011; Hsu et al., 2013; Mussolino et al., 2011; Pattanayak et al., 2011). In the case of ZFNs, too many off-target cleavages are thought to cause cytotoxicity (Cornu et al., 2008).

Compared to dimeric ZFNs and TALENs, RGENs theoretically have lower specificity because of functioning as monomers. Several studies have examined off-target effects of RGENs (Cho et al., 2014; Cradick et al., 2013; Fu et al., 2013; Hsu et al., 2013; Mali et al., 2013a; Pattanayak et al., 2013). One study showed that RGENs can induce off-target mutations at sites that differ by up to five nucleotides from on-target sites, which implies that thousands of potential off-target cleavages can occur in the human genome for every RGEN (Fu et al., 2013), whereas some studies reported that off-target mutations were below the detection range when analysed by unbiased whole-genome analysis (Veres et al., 2014) or exome sequencing (Cho et al., 2014). Recent genomewide off-target analysis based on deep sequencing also revealed a broad spectrum of RGEN specificities (Frock et al., 2015; Kim et al., 2015; Tsai et al., 2015; Wang et al., 2015).

Several strategies have been suggested and found to minimize or prevent off-target effects (Koo et al., 2015). First, when designing a nuclease, choosing unique target sites that lack highly homologous sequences elsewhere in the genome is recommended. Many web-based programs have been developed for searching for potential TALEN or RGEN off-target sites (Bae et al., 2014; Heigwer et al., 2013, 2014; Hsu et al., 2013). Second, RGEN off-target effects can be modulated by controlling the level or duration of nuclease expression (Hsu et al., 2013). Third, the use of recombinant proteins and in vitro transcribed RNA, rather than plasmids encoding these components, can further reduce the frequency of off-target mutations due to the rapid degradation of the protein and RNA in cells (Gaj et al., 2012; Kim et al., 2014; Ramakrishna et al., 2014). Jin-Soo Kim’s group and we have recently reported that plasmid-free delivery of Cas9 protein and guide RNA can dramatically reduce off-target mutations without reducing efficiency (Kim et al., 2014; Ramakrishna et al., 2014). Additionally, sgRNAs truncated at the 5’ end (length less

<table>
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<th>Table 1 Comparison of three classes of designed nucleases</th>
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<td><strong>ZFN</strong></td>
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<td>Recognition site</td>
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ZFN, zinc-finger nuclease; TALEN, transcription activator-like effector nuclease; RGEN, RNA-guided engineered nuclease; CRISPR, clustered regularly interspaced short palindromic repeat; Cas9, CRISPR-associated protein 9; sgRNA, single-chain guide RNA.
than 20 bp) (Fu et al., 2014) or those with two extra guanine nucleotides at the 3’ end (Cho et al., 2014; Kim et al., 2015) are reported to reduce off-target mutations up to 5000-fold (truncated sgRNA) or 660-fold (sgRNA with two extra guanine nucleotides) without alteration of mutation efficiencies at target sites. Paired nickases (Ran et al., 2013a) or RNA-guided FokI nucleases (Guilinger et al., 2014b; Tsai et al., 2014) for knockout of genes can be good alternatives to nucleases with minimum off-target effects (Ran et al., 2013a; Tsai et al., 2014).

Conclusion

Generation of DSBs in a targeted manner using designed nucleases greatly facilitates genome editing. Recent breakthroughs in programmable nucleases have made genome editing an efficient and affordable process. Furthermore, technologies for ‘reading’ and ‘writing’ (that is, sequencing and synthesizing, respectively) genomes are currently being developed in parallel with genome editing.

Nonetheless, several aspects of programmable nuclease technology, including activity, off-target effects, ease of engineering and delivery, can be improved. Recently published analyses based on deep sequencing showed Cas9 nuclease-induced hard-to-predict off-target cleavages across the whole genome (Frock et al., 2015; Kim et al., 2015; Tsai et al., 2015; Wang et al., 2015), raising a safety issue. To increase the efficiency of precise genome editing, DSB repair pathways can be controlled either genetically or pharmacologically. Recently, several methods that improve HDR efficiency by reducing NHEJ have been developed (Chu et al., 2015; Maruyama et al., 2015; Yu et al., 2015). If more precise genome editing can be performed, breeders will be able to manipulate the genomes of plants and animals with reduced adverse effects. In addition, genome-scale libraries of designed nucleases or transcriptional regulators (Findlay et al., 2014; Gilbert et al., 2014; Koike-Yusa et al., 2014; Konermann et al., 2014; Shalem et al., 2014) can be used for deciphering new biological findings by enabling high-throughput loss- and gain-of-function studies. In the future, designed nucleases with improved efficiency and precision are expected to open a new era of biological research, medicine and biotechnology.

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Conflict of interest

The authors declare that they have no conflict of interest.

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