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Review

Endonucleases: new tools to edit the mouse genome ☆

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ABSTRACT

Mouse transgenesis has been instrumental in determining the function of genes in the pathophysiology of human diseases and modification of genes by homologous recombination in mouse embryonic stem cells remains a widely used technology. However, this approach harbors a number of disadvantages, as it is time-consuming and quite laborious. Over the last decade a number of new genome editing technologies have been developed, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats/CRISPR-associated (CRISPR/Cas). These systems are characterized by a designed DNA binding protein or RNA sequence fused or co-expressed with a non-specific endonuclease, respectively. The engineered DNA binding protein or RNA sequence guides the nuclease to a specific target sequence in the genome to induce a double strand break. The subsequent activation of the DNA repair machinery then enables the introduction of gene modifications at the target site, such as gene disruption, correction or insertion. Nuclease-mediated genome editing has numerous advantages over conventional gene targeting, including increased efficiency in gene editing, reduced generation time of mutant mice, and the ability to mutagenize multiple genes simultaneously. Although nuclease-driven modifications in the genome are a powerful tool to generate mutant mice, there are concerns about off-target cleavage, especially when using the CRISPR/Cas system. Here, we describe the basic principles of these new strategies in mouse genome manipulation, their inherent advantages, and their potential disadvantages compared to current technologies used to study gene function in mouse models. This article is part of a Special Issue entitled: From Genome to Function.

1. Introduction

Genome-wide association studies (GWAS) have been instrumental in the identification of single nucleotide polymorphisms (SNP)s associated with complex human diseases. The number of genetic associations has been steadily increasing each year since the introduction of this approach in 2005. Genomic regions marked by specific SNPs have attracted the attention of many researchers to potentially identifying the causal variant and understanding the pathophysiology of the disease [1,2]. These genomic regions can contain either protein-coding (direct protein variants) or non-coding regions that might regulate the expression of genes. However, discovering the causal variant and revealing the underlying biological mechanism of the associated disease is still a complicated process. For a number of reasons, the mouse is the most valuable and readily accessible animal model as a biological source to study genes within the candidate loci. The genome of the mouse has been fully sequenced, and most of the genes (~99%) in human are also present in mice. Mice are highly comparable to humans with respect to organs, tissues and physiological systems, enabling the study of gene-environment interactions in the whole organism. Furthermore, mice are easy to breed with a relatively short generation time, are small, and can be housed together, thereby keeping the costs relatively low. The discovery of gene editing via homologous recombination in mouse embryonic stem (ES) cells has further spurred the use of mice over other animal models [3–5]. Here, we will give an overview of the various tools for gene modification that have been developed during the last decades. Additionally, we will focus on new developments in mouse technology and the advantages these have over existing technologies to translate genetic findings into functional biological assessments.

2. Gene editing by homologous recombination

Most human diseases are studied from a candidate gene approach that has been identified by linkage or association studies, or deep

Abbreviations: CRISPR/Cas, clustered regularly interspaced short palindromic repeats/CRISPR-associated; crRNA, CRISPR RNA; DSB, double-strand break; dsDNA, double-strand DNA; ES, embryonic stem; FLASH, fast ligation-based automatable solid-phase high-throughput; gRNA, guide RNA; GWAS, genome-wide association studies; HDR, homology-directed repair; iPS, induced pluripotent stem; NHEJ, non-homologous end joining; OPEN, oligomerized pool engineering; PAM, protospacer adjacent motif; RVD, repeat variable di-residue; SELEX, systematic evolution of ligands by exponential enrichment; SNP, single nucleotide polymorphism; SpCas9, *S. pyogenes* Cas9; TALEN, transcription activator-like effector nuclease; tracrRNA, trans-activating crRNA; ZFN, zinc finger nuclease; ZFP, zinc finger protein

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sequencing approaches [6,7]. Although human diseases are usually very complex, typically involving gene-gene and/or gene-environment interactions, the most straightforward and commonly used method to study the function of candidate genes is by modifying these genes in mice. The development of gene targeting technology in ES cells was a major breakthrough that led to the generation of numerous mutant mouse models. The technique makes use of homologous recombination to mutagenize the genome in ES cells, which creates a deletion, insertion, or point mutation [8]. However, ~30% of all knockouts are embryonic or early postnatal lethal, which led to the development of other mutagenesis strategies, like Dre/Rox, Flp/Frt and the most widely used Cre/LoxP system. These systems provide the possibility of generating a tissue/cell-specific gene knockout (discussed below) [9–11]. Cre/LoxP is a site-specific recombination system that was discovered in bacteriophage P1 [11,12]. Cre recombinase drives recombination between two DNA recognition sites of 34 bp, also known as LoxP sites [8]. Genomic regions that are flanked by loxP sites in the same orientation, also termed a "floxed allele", will be excised in cells expressing Cre recombinase [13].

In general, this mutagenesis approach is commonly used if the gene of interest is vital for normal embryogenesis or if there is a necessity to investigate the function of the gene in a tissue/cell-specific context. Mice carrying the floxed alleles will be crossed with a mouse strain containing a transgene encoding the Cre recombinase under the control of a tissue-specific promoter, which results in conditional/tissue-specific knockout mice [14–16]. Over the last two decades, numerous, tissue-specific, Cre-driver lines have been developed. However, some drawbacks in the Cre/LoxP system have also started to emerge, which was recently extensively reviewed [16,17]. One major concern is the tissue-specificity of the chosen promoter that drives the Cre transgene. Expression of various genes assumed to be restricted to a specific tissue or cell type are actually expressed in multiple tissues/cells [16–18]. An additional problem is that Cre recombinase transgenic mice can have too high or low Cre activity, leading to toxicity or inefficient deletion of the gene, respectively [16]. Furthermore, Cre recombinase itself can also cause unwanted side-effects, such as random recombination, reduced proliferation and increased apoptosis, supporting the need to include the Cre recombinase transgenic mice as an additional control in the study design [16].

Another elegant method to examine gene function in a more physiological fashion is by engineering mice with reduced expression of the gene of interest. This can be accomplished by creating a hypomorphic allele that results in the expression of only a fraction of the normal protein levels. Combining a hypomorphic allele with either a wild-type, knockout, or hypomorphic allele enables generation of a series of mice with a gradual reduction in protein levels [19]. For example, this strategy has successfully been used to study the mitotic checkpoint proteins BubR1 and Bub1. Complete ablation of these genes results in embryonic lethality, but mice with reduced protein levels are born healthy and show an overt phenotype later in life [20,21]. The strategies to generate a hypomorphic allele have recently been described in detail [19,22].

Although gene editing by homologous recombination in ES cells is still the most widely used strategy to generate mutant mice the efficiency of homologous recombination is very low. Therefore this genetic editing method has to be performed in ES cells first instead of in the mouse directly. In addition, the availability of ES cells from different species is limited. All this combined has led to the development of new techniques, such as ZFNs, TALENs and CRISPR/Cas that harbor significantly improved efficiencies in gene editing [23–25]. The basic principles and advantages of these technologies will be discussed in the following sections.

3. Zinc finger nucleases

Zinc finger nucleases (ZFNs) facilitate genetic modification through the introduction of a double strand break (DSB) in a DNA sequence of interest. Subsequent DNA break repair then enables the introduction of the desired modification, which is discussed in detail below [26,27]. The DSB is produced by a ZFN, which is a sequence-specific endonuclease that can be designed to cleave at a precise DNA sequence [27]. A ZFN consists of a varying number of zinc finger proteins (ZFPs) or Cys_2His_2 fingers which are usually fused to the nuclease domain of *Fok*I, a restriction enzyme that cleaves non-specific DNA sequences [27–31] (Fig. 1A). Each ZFP is able to recognize a distinct three-base-pair DNA sequence and a typical ZFN consists of 3–6 fused zinc finger proteins. Optimal *Fok*I cleavage by ZFNs requires two independent ZFNs to bind on opposite DNA strands in the appropriate orientation and at the correct distance from each other [27,32] (Fig. 1A).

The introduction of a DSB by ZFNs at a predefined DNA locus provokes activation of a conserved DNA repair pathway, namely non-homologous end joining (NHEJ) or homology-directed repair (HDR) [33–35] (Fig. 1B). In most cases the DSB is repaired by the NHEJ pathway, which efficiently ligates the two broken ends. However, the NHEJ pathway is error-prone and the repair can result in small deletions and/or insertions (indels), which can lead to gene disruption [27,33] (Fig. 1B). Gene inactivation was initially applied by expression of two ZFNs directed against the *yellow* gene in the larvae of *Drosophila melanogaster*, which resulted in germline mutations [36,37]. Subsequently, ZFN technology has successfully been applied to mutagenize genes in various organisms, including zebrafish, rats and mice with varying frequency [23, 38–42]. For example, microinjection of engineered ZFNs in embryos was used to generate *Mrd1a* and *Tnfrsf9* knockout mice, respectively [23,42]. In addition to single gene disruption, ZFN technology has also been used to target two or three genes simultaneously in mammalian cells [43,44]. Furthermore, larger deletions, translocations, duplications and inversions can be introduced with ZFN [44–48].

HDR enables the introduction of single nucleotide changes (gene correction) after DSB induction by ZNF upon simultaneous delivery of a donor DNA repair template, which contains homology arms flanking the site of alteration [37,49,50] (Fig. 1B). This opens the possibility to study the functional consequences of human disease-associated point mutations in the preferred cells and/or model organisms [23,51–54]. In addition, this approach can be used to engineer larger modifications, including insertions of loxP sites, fluorescent proteins, antibiotic resistance markers, or other tags [52,55–59]. There are limitations to gene correction and gene addition via HDR: the need for co-delivery of a designed DNA donor template together with a specific-ZFN, and the strong preference of a cell for NHEJ over HDR-mediated repair of the DSB. Possible solutions are either to use ZFN nickases or a vector carrying multiple copies of linear donor fragments, which both increase HDR-driven genome editing while reducing unwanted mutations caused by NHEJ [60–63].

Importantly, ZFN-mediated gene modification has great therapeutic potential. ZFN has the advantage over known knockdown or blocking strategies because it is efficient and persistent, which could avoid the need for life-long treatment. For example, independent studies have shown that disruption of the *CCR5* and *CXCR4* gene, which encode HIV co-receptors, protects against HIV-1 infection *in vitro* and *in vivo*. Based on the *CCR5* studies, ZFN-mediated therapies for HIV have been designed and are currently being used in Phase 2 clinical trials [27, 64–68]. ZFN-induced HDR can also be exploited to correct genetic disease-causing mutations, as demonstrated in human induced pluripotent stem (iPS) cells carrying mutations underlying Parkinson's disease, α1-antitrypsin deficiency, or sickle-cell anemia [69–71]. Furthermore, ZFN-driven gene correction has been demonstrated to be effective in a mouse model of hemophilia, raising the possibility of *in vivo* genome editing by ZFN as a strategy for the treatment of genetic diseases [72]. The risk for potential off-target DNA cleavage when using ZFN technology raises some concerns. Increased ZFN specificity and simultaneous reduction of off-target cleavage can be achieved by linking more ZFPs in a ZFN, optimizing the orientation of protein-DNA interaction and using a heterodimer ZFN pair [51,73]. Although some reports have

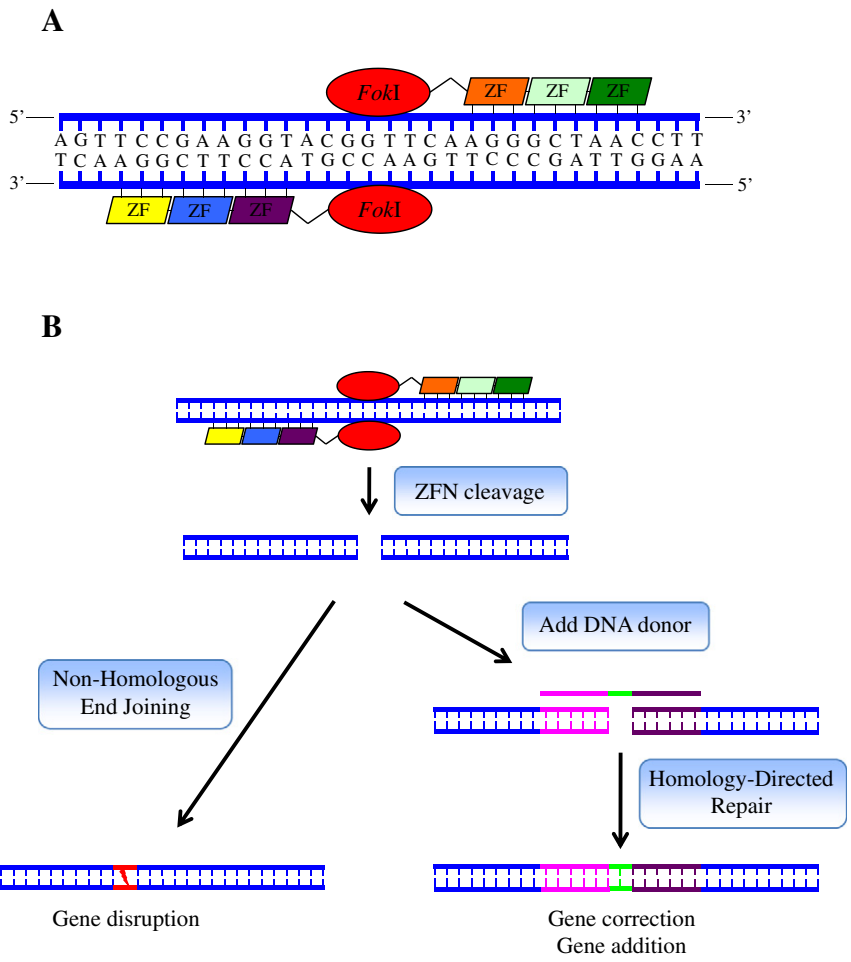

Fig. 1. Overview of ZFNs. A. ZFN consisting of three zinc finger proteins fused to the catalytic domain of *Fok*I restriction enzyme. Each zinc finger protein is able to bind to three nucleotides and can guide the ZFN to a specific target site in the genome. Two ZFNs targeting a specific sequence on opposite sides of genomic DNA are necessary to allow dimerization of two non-specific *Fok*I nucleases. B. Dimerization of two *Fok*I enzymes induces a DSB at the target site. Subsequent DNA-repair by the erroneous NHEJ pathway can introduce the desired genomic modification, like gene disruption. Alternatively, the addition of a DNA repair template can facilitate HDR-mediated genome editing and result in gene correction or addition. ZF = zinc finger protein.

demonstrated increased cytotoxicity issues when using heterodimers [50,74]. A systematic evolution of ligands by exponential enrichment (SELEX) protocol and unbiased genome-wide analysis can be utilized to determine the specificity for ZFP DNA binding and rank the risk of potential off-target sites [75,76]. Combination of the SELEX protocol with ultradeep sequencing confirmed the high ZFN specificity for the target site in *CCR5* and only identified rare off-target sites with low frequency [64].

There are currently a number of methods to fuse ZFPs with each other to generate new ZFNs, including modular assembly [77], Oligomerized Pool ENgineering (OPEN) system [78], a bacterial one-hybrid system [39], two finger modules [79,80] and context-dependent assembly [81]. However, the construction of ZFNs is challenging for non-specialist laboratories, especially with the bacterial one-hybrid and OPEN systems. Furthermore, most ZFNs fail to modify the gene of interest *in vivo*. This can be caused by either specificity issues through identical or very similar sequences in the genome or by the chromatin structure at the site of interest that prevents ZFN binding [27]. Current studies are focusing on improving the design and construction of ZFNs, increasing their specificity and thereby reducing off-target cleavage. In conclusion, ZFN technology appears to be a promising mutagenesis tool for generating mutant animals, including mice, and may have the potential to be used in therapeutic applications.

4. Transcription activator-like effector nucleases (TALENs)

Transcription activator-like effectors (TALEs) from plant pathogenic *Xanthomas* are virulence factors secreted via the type III system that can bind to host DNA and activate expression of effector-specific host genes [82,83]. TALEs contain a characteristic central domain of DNA-binding tandem repeats, a nuclear localization signal, and a C-terminal transcriptional activation domain [84–86]. A typical repeat is 33–35 amino acids in length and contains two hypervariable amino acid residues at positions 12 and 13, known as the "repeat variable di-residue" (RVD) (Fig. 2A). Two studies discovered that an RVD is able to recognize one specific DNA base pair and that sequential repeats match consecutive DNA sequences. They demonstrated that target DNA specificity is based on the simple code of the RVDs, which thus enables prediction of target DNA sequences [82,83]. Nucleotide specificity of repeats was shown for RVDs encoding NN, NI, HD and NG for recognition of guanine, adenine, cytosine and thymine, respectively [83,87]. The first successful generation of TALE nucleases (TALENs) was reported in two studies, in which they fused either native or modified TALEs to the catalytic domain of the *Fok*I restriction enzyme. These native and custom-made TALEN fusions were able to induce target-specific double-strand cleavage in yeast [88,89]. The essential TALEN architecture necessary for efficient genome editing in human cells was determined by linking TALE truncation variants to the catalytic domain of *Fok*I. This approach

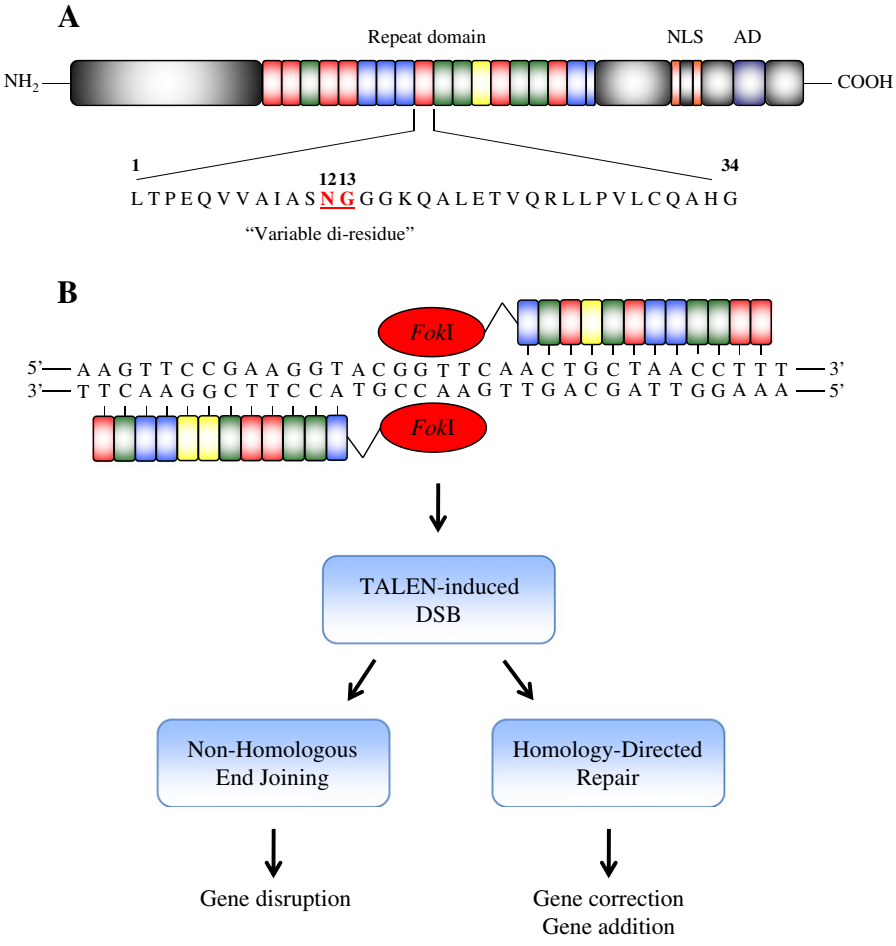

Fig. 2. A. Representation of a TALE characterized by an N-terminal domain, a central DNA-binding repeat domain and a C-terminal domain containing two nuclear localization signals (NLS) and the activation domain (AD). A typical repeat is 34 amino acids in length and contains two hypervariable amino acids at position 12 and 13 known as the RVD (highlighted in red). Each RVD is able to recognize one specific DNA base pair and serial repeats recognize specific DNA sequence and activate expression of specific effector host genes through the activation domain. B. Depiction of a TALEN. A TALEN containing 12 specific repeats that correspond to binding either thymine (red), cytosine (green), adenine (blue) or guanine (yellow) fused to the nuclease domain of *Fok*I. Simultaneous binding of a TALEN pair on opposite strands of DNA flanking the target site facilitates dimerization of *Fok*I and results in a TALEN-induced DSB. Ensuing DNA repair by the NHEJ or HDR pathway can be exploited to introduce the desired genetic modification.

revealed that the N-terminal 152 residues were dispensable and TALE variants containing 28 or 63 of the 278 original C-terminal amino acids were sufficient to drive efficient gene modifications of two endogenous genes. Furthermore, they demonstrated that the correct distance between the TALEN pair is essential for successful cleavage [87].

Similarly to ZFNs, TALENs enable genetic modification through induction of a double strand break (DSB) in a DNA target sequence. Ensuing DNA break repair by either the NHEJ or the HDR-mediated pathway can be exploited to introduce the desired modification (e.g. gene disruption, gene correction or gene insertion) [24] (Fig. 2B). TALENs have been utilized to efficiently introduce targeted genetic modifications in a number of model organisms, including *Drosophila melanogaster* [90], zebrafish [91–93], rat [94], pig and cow [95], rhesus and cynomolgus monkeys [96]. Most of these studies used TALENs to generate an NHEJ-mediated knockout animal, but two studies reported the use of two TALEN pairs to generate larger deletions and inversions in livestock fibroblasts and zebrafish [95,97]. Two designed TALEN pairs were also able to induce cancer-relevant translocations found in anaplastic large cell lymphoma [98]. Injection of TALEN mRNA specific for exon 2 of *Pibf1* gene and for exon 1 of *Sepw1* gene into the cytoplasm of mouse pronuclear-stage embryos resulted in founders carrying null mutations in the *Pibf1* and *Sepw1* gene. All mutations observed in F_0 mice were transmitted through the germline [99]. Increasing the amount of mRNA injected can produce a higher mutation rate and bi-allelic mutation frequency, but this can also result in fewer mutant mice due to the toxicity of high doses of mRNA [99]. Similar approaches using microinjection of TALEN mRNA targeting genes in mouse oocytes and zygotes have been successful in engineering knockout mice [100–105]. Exploitation of TALEN-mediated genome editing in mouse ES cells resulted in mice with targeted gene disruptions and insertions in two Y chromosome-linked genes, which was previously impossible with conventional gene-targeting technology [106]. HDR-driven insertion was introduced by simultaneous delivery of TALEN with a designed single-stranded DNA repair template in human pluripotent stem cells, somatic cells, zebrafish and rats [87,93,107–109]. For example, Hermansky-Pudlak syndrome and amyotrophic lateral sclerosis missense mutations in the *Rab38* and *Fus* genes, respectively, were introduced in mice [102,110]. Subsequently, TALEN-driven HDR was applied to correct the introduced mutation in the *Rab38* gene [110]. TALENs have also been successful in generating human stem cell-based disease models, and restoring expression of functional Dystrophin in cells from Duchenne muscular dystrophy patients [111,112]. New applications include TALEN-mediated generation of a conditional mouse model, human pluripotent stem cell line with conditional transgene expression, knockout of human microRNA genes and single base-editing of an intergenic region upstream of the *BUB1* gene [113–116]. Furthermore, TALE-DNA binding domains enables reversible modulation of mammalian endogenous gene expression and targeted epigenetic chromatin modifications [117–119].

Similar to ZFNs, a concern of genome editing by TALENs is the occurrence of off-target modifications. Extensive analysis of known TALEN/DNA cleavage profiles determined specificity scoring of each RVD/nucleotide association, which can be used as a guide in the design of TALENs [120]. TALENs can only tolerate limited position-dependent mismatches to keep detectable cleavage activity *in vivo*, demonstrating its high specificity [120]. In addition, newly synthesized TALEN variants have shown equal on-target cleavage activity and on average ten times lower off-target cleavage activity in human cells [121].

Currently, a number of methods have been developed for engineering of TALE repeats [24]: Standard cloning-based [91,92], "Golden Gate" cloning [122–124], iterative capped assembly [125], and the fast ligation-based automatable solid-phase high-throughput (FLASH) system [126], which has the advantage that construction is rapid, cheap and large number assemblies are feasible. More recent methods include ligation-independent cloning [127] and fairyTALE [128], which enable the high-throughput assembly of TALE repeats.

5. Clustered regularly interspaced short palindromic repeats/CRISPR-associated (CRISPR/Cas) system

In addition to TALENs and ZFNs, the CRISPR/Cas system has recently been introduced as an efficient and versatile tool for genome editing. CRISPR is an essential part of the immune system of bacteria and archaea directed against foreign nucleic acids [129–131]. Upon challenge with a viral or plasmid pathogen, bacteria and archaea integrate short fragments of foreign DNA (protospacers) into their own chromosomes at the proximal end of a repetitive element known as the CRISPR locus/array [129–132].

The CRISPR locus is characterized by a series of direct repeats of approximately 20–50 base pairs separated by unique spacers of similar length [130,131]. Transcription of the CRISPR loci into precursor CRISPR RNA (pre-crRNA) is followed by enzymatic cleavage, which results in short crRNAs that can bind with complementary sequences of foreign viruses and plasmids [132–135]. Each crRNA is packaged into a surveillance complex to protect the intracellular environment from invading viruses and plasmids. crRNA recognizes and mediates the destruction of foreign DNA sequences through complex formation with CRISPR-associated (Cas) protein that harbors nuclease activity [130–132,136]. Cas proteins are encoded by *Cas* genes and are localized in the vicinity of a CRISPR locus. The cleavage capability of Cas protein 9 (Cas9) was demonstrated on plasmid DNA containing a protospacer sequence and a protospacer adjacent motif (PAM) sequence, however, the addition of mature crRNA and a trans-activating crRNA (tracrRNA) are essential for proper cleavage. TracrRNA enhances crRNA binding to the complementary DNA strand and thereby activates crRNA-guided double-strand DNA (dsDNA) cleavage by Cas9 (Fig. 3A) (detailed description of the CRISPR/Cas system in bacteria and archaea has been reported in [130,132]). Cleavage is site-specific and occurs 3 base pairs upstream (arrows in Fig. 3A) of the PAM sequence. PAM is a very short stretch of conserved nucleotides in the immediate proximity of the protospacer, and is a determining factor in self versus non-self recognition. Engineering of a crRNA:tracrRNA chimera in the presence of Cas9 was sufficient to cleave a plasmid containing the GFP coding sequence *in vitro* [132]. Heterologous expression of a codon-optimized *S. pyogenes* Cas9 (SpCas9) nuclease, a designed tracrRNA and pre-crRNA have been shown to induce precise RNA-guided cleavage at genomic loci in human and mouse cells [25]. Similarly, targeted modification of loci with SpCas9 and a fusion transcript of crRNA-tracrRNA, also known as a guide RNA (gRNA), was successful in human and iPS cells [137,138] (Fig. 3B). A mutation in the RuvC I domain of SpCas9 converts it into a DNA nickase. DNA nickases introduces only a single-strand break or "nick" instead of a DSB, which facilitates homology directed repair with high fidelity [25,137].

CRISPR technology offers the ability to edit different loci simultaneously or to generate large deletions in mammalian genomes [25,137]. Co-injection into zygotes of *Tet1* and *Tet2* sgRNA with Cas9 mRNA resulted in mice carrying bi-allelic mutations in both genes with high efficiency and specificity [139]. The CRISPR/Cas system has been applied to modify single and/or multiple genes by either NHEJ- or HDR-mediated repair in numerous model organisms, including zebrafish [97,140–143], *Drosophila* [144–146], rats [147–149], mice [139,147,150–155] and cynomolgus monkeys [156]. Furthermore, co-injection into zygotes of Cas9 mRNA, various gRNAs and DNA vectors of different sizes encoding a tag or fluorescent reporter construct resulted in mice carrying small insertions or reporter genes. Likewise, using CRISPR/Cas together with a DNA repair template has been successful in creating a conditional floxed allele in mice [157].

Although analysis of potential off-target sites of five gRNAs in gene-modified mice and mouse ES cells identified off-target mutations with low frequency [157], the risk for off-target cleavage by CRISPR/Cas has become an important discussion point, especially since multiple mismatches are tolerated by the CRISPR/Cas system [158–160]. These off-target mutations, even in off-target sites harboring up to 5 mismatches, were often located within coding genes [158,161]. Yet, the accepted mismatch depends on the position of the gRNA-DNA interface, e.g. Cas9-mediated cleavage appears abolished when a single mismatch is present in the last 10–12 nucleotides near the 3' end of the gRNA-target site [25,132]. Off-target modifications have been observed in genes with strong homology. The CRISPR/Cas system targeting the *hemoglobin β* and *CCR5* genes revealed significant off-target cleavage in the related *hemoglobin δ* and *CCR2* genes in human cells, respectively [162]. Off-target modifications can be minimized by titration of the Cas9 and gRNA dosage and careful design of the gRNA [159,161]. Furthermore, both gRNA structure and composition can influence RNA-guide cleavage and diminish off-target mutagenesis [163]. Truncated gRNAs with shorter regions of complementarity can be used to reduce undesired off-target modifications and maintain similar on-target genome editing efficiencies [164]. Another method makes use of a Cas nickase instead of a nuclease. The advantage of this approach is that single-strand nicks in off-target sites are favorably repaired by the high-fidelity base excision repair pathway [165]. Cas nickases directed by a pair of gRNAs targeting opposite strands of a target locus can efficiently mediate DSBs while significantly reducing off-target activity in human cells [160,163,166,167]. In addition, utilization of double nicking has enabled highly efficient NHEJ-mediated DNA insertion, HDR and genomic microdeletions in human cells and mouse zygotes [167]. However, recent studies have demonstrated that single monomeric nickases can induce unwanted indel mutations as well [160,163,164]. Elucidation of the crystal structure of Cas9, either without or with binding to gRNAs and DNA, will enhance the functional understanding of Cas9 activity and possibly lead to increased specificity [168–170]. Fortunately, a free web-based application is available to facilitate CRISPR/Cas9-mediated mammalian genome engineering; it allows users to select and validate target sequences and identify potential off-target effects [159,171,172].

6. Concluding remarks

Nuclease technology for genome modification has brought a number of major advantages in comparison with conventional gene targeting by homologous recombination in mouse ES cells. The efficiency of nuclease-mediated genome editing is significantly higher, as demonstrated in a number of model organisms, and cell lines from different species. Nuclease technology can drastically reduce the time line to generate mutant mice. Direct injection of ZFNs, TALEN mRNA or gRNA with Cas9 into fertilized mouse oocytes can produce targeted mutations in founder animals with high efficacy. Most founder lines have subsequently been able to transmit the mutated alleles through the germline to their offspring. These approaches avoid the use of ES cells and the construction of large targeting constructs, which are laborious procedures. As demonstrated with the CRISPR/Cas system, multiple genes

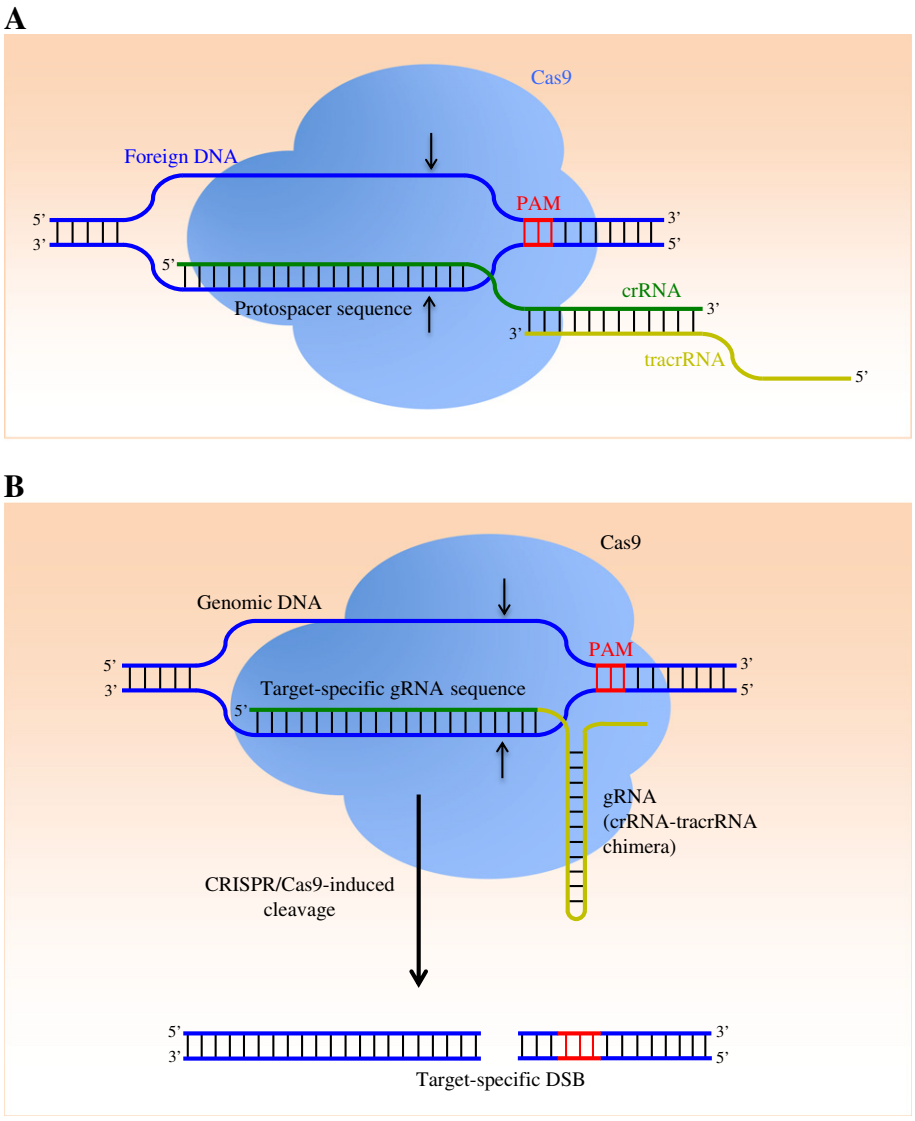

Fig. 3. Mechanism of the CRISPR/Cas system. A. Destruction of foreign DNA sequences. Upon challenge with viruses and plasmids, crRNAs recognize and bind to the protospacer sequence of foreign DNA with an adjacent PAM sequence. TracrRNA improves crRNA binding to the corresponding DNA sequence and thereby triggers crRNA directed double-strand cleavage through association with the Cas9 nuclease. Double-strand cleavage is site-specific and occurs 3 base pairs upstream of the PAM sequence, as indicated with the black arrows. B. Genome editing through the CRISPR/Cas system. A designed gRNA (chimera of crRNA and tracrRNA) recognizes the target sequence in the genomic DNA with adjacent PAM sequence, mediating the activation of Cas9 through complex formation and induction of a target-specific DSB. The subsequent DNA repair can then be exploited for editing the genome.

can be modified simultaneously, thereby avoiding the time-consuming crossing of single knockout animals [139]. In addition, nuclease technology has demonstrated the ability to produce mice carrying targeted disruptions and insertions into two genes located on the Y-chromosome [106], which was previously not possible. Conventional gene targeting by homologous recombination has mainly been successful in mouse ES cells, however nuclease technology can now be applied in a number of model organisms, including *Drosophila*, zebrafish, rat and pig [24,27]. Furthermore, nuclease technology can be applied in all genetic backgrounds, avoiding the need for extensive back crossing [23,41].

Since its discovery, TALENs has quickly overtaken ZFN technology due to its higher efficiency in target sequence cleavage, the development of easier and quicker construction methods for TALENs, and a greater flexibility to target specific sequences [124,126,173]. Similarly, the emergence of CRISPR/Cas system has demonstrated a remarkably high efficiency in making specific genetic modifications in mammalian cells and zygotes [25,137,139]. Excitingly, CRISPR can contain multiple gRNAs that enable simultaneous targeting of multiple genes in mice and other model organisms [139,143,147,149,153]. The design and production of gRNAs can be done quickly and easily through *in vitro* transcription of double-stranded oligonucleotides or by cloning of oligonucleotides in expression vectors, which is a clear advantage over the generation of ZFNs and TALENs [25,137]. However, the CRISPR/Cas system contains two major pitfalls, namely the requirement of a PAM sequence adjacent to the 3'-end of the target sequence and the high frequency of off-target cleavage [135,158–161]. The requirement of a PAM sequence limits the number of specific target sequences. CRISPR/Cas has utilized two Cas9 proteins derived from *S. pyogenes* or *S. thermophiles*, but recent exploitation of Cas9 orthologs from other species that recognize different PAM sequences will increase the flexibility in genome editing [135,174–177]. A typical TALEN target sequence usually contains around 30 nt that is unique within the mouse genome, while CRISPR/Cas allows multiple mismatches in the guide sequence and therefore increases the likelihood of off-target effects [178]. A possible solution is the use of Cas nickases guided by a pair of gRNAs targeting opposite strands for genome editing, as this significantly reduces off-target effects [160,163,167]. In addition, the use of DNA nickases can partially shift the balance from NHEJ towards HDR-mediated repair and thereby increase the efficiency of gene addition and gene correction [166].

The emergence of nuclease-mediated genome editing holds great promise for future use in targeted mouse genome editing technology. The ease of design, construction, high efficiency, potential applications, and short generation time of mutant mice make nuclease-mediated genome modifications a very interesting tool for studying the function of candidate genes, putative *trans*- and *cis*-regulatory elements, and transcriptional factor binding site relevancy in the etiology of human diseases.

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