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Chapter

Introductory Chapter: Gene Editing Technologies and Applications

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1. Introduction

Gene editing is a type of genetic engineering in which DNA is inserted, deleted, modified, or replaced in the genome of a living organism. Unlike traditional methods that randomly insert genetic material into a host genome, current gene editing technologies target and change the specific genome locations. Zinc finger nucleases (ZFNs), transcription activator-like effectors nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPRs)/Cas9 nuclease system are the three common gene editing technologies. These technologies have been widely used in genome engineering to enable a broad range of mutation by inducing DNA breaks that stimulate error-prone repairs such as homologous recombination (HR) or nonhomologous end joining (NHEJ). They successfully make it possible to achieve site-specific editing, modification, and manipulation at specific genomic sites (**Table 1**) [1].

1.1 ZFNs

ZFNs are artificial restriction enzymes generated by fusing a zinc finger-specific DNA-binding domain to a nonspecific DNA cleavage domain. The specific binding domains of individual ZFNs typically contain between three and six individual zinc finger repeats and can each recognize between 12 and 18 base pairs. If the zinc finger domains are specific for their intended target site, then even a pair of three-finger ZFNs that recognize a total of 18 base pairs can target a single locus in a mammalian genome. The nonspecific cleavage domain from the restriction endonuclease *Fok I* is typically used as the cleavage domain in ZFNs. This cleavage domain must form a dimer in order to cleave DNA, and thus a pair of ZFNs are required to target nonpalindromic DNA sites [2] (**Figure 1**).

1.2 TALENs

Transcription activator-like effectors (TALEs) are proteins secreted by bacteria *Xanthomonas* via type III secretion system when they infect various plant species. TALEs are important virulence factors that act as transcriptional activators in the plant cell nucleus [3]. Each TALE contains a central repetitive region consisting of varying numbers of repeat units (about 17.5 repeats) of 34 amino acids [3, 4]. The DNA-binding domain contains a highly conserved 34 amino acid sequence with the exception of the 12th and 13th amino acids [3]. Only the 12th and 13th amino acids in TALEs are changeable and variable, the other amino acids are constant and

Technology	ZFN	TALEN	CRISPR/Cas9
Source	Extensively exists in the nature	Plant pathogenic bacteria *Xanthomonas*	An adaptive immune system in bacteria
Targeting specificity determinant	Zinc finger protein	TALE	SgRNA
Nuclease	*Fok* I	*Fok* I	Cas9
Restriction for a target sequence	Rich in cytosine	No special restriction	PAM
Target site	Two (left and right)	Two (left and right)	Only one
Length of target gene	12–18 bp	12–18 bp	18–23 bp
Mode of action	Two proteins act on two target sites	Two proteins act on two target sites	RNA and Cas9 act on one target site
Cleavage site	DSB with a sticky end	DSB with a sticky end	DSB with a blunt end
Efficiency	Medium	Medium	High
Ease of engineering	Low	Medium	High
Ease of characterization	Low	Medium	High
Cost	High	Medium	Low
Cytotoxicity	High	Low	Low
Off target	Yes	Yes	Yes

Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeat; DSB, double-strand break; Fok, Flavobacterium okeanokoites; PAM, protospacer adjacent motif; TALE, transcription activator-like effector; sgRNA, single guide RNA; and ZFN, zinc finger nuclease.

Table 1.
Comparison of different gene editing technologies.

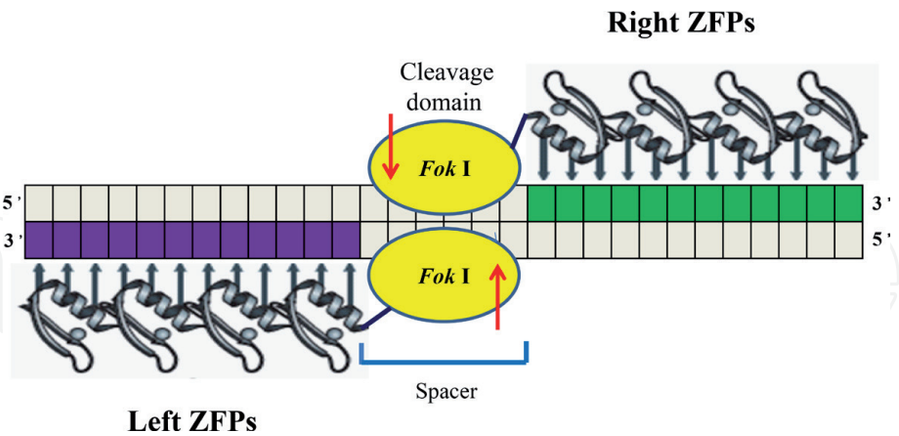


Figure 1.
ZFNs are chimeric nucleases consisting of specific DNA-binding modules linked to a nonspecific cleavage domain. Right and left ZFNs containing Fok I endonucleases link to an array of 3–6 zinc fingers that have been designed to specifically recognize target sequences (12 green and 12 purple boxes), respectively. The ZFN targets are separated by typically 5 or 6 bp. Two Fok I work as a homodimer to cleave the sense strand 1 bp and antisense strand 5 bp downstream of the binding site.

stable. These two locations—repeat variable di-residues (RVD) are highly variable and show a strong correlation with a specific nucleotide recognition by different frequency, for example, NI recognize A (55%), NG recognize T (50%), NN recognize G (7%), and HD recognize C(69%) [3]. Two amino acids have known to recognize one nucleotide after the breaking of code of DNA-binding specificity of

TALEs [3, 4]. The restriction endonuclease *Fok* I consists of an N-terminal specific DNA-binding domain and a C-terminal nonspecific DNA cleavage domain. The nonspecific DNA cleavage domain of *Fok* I cleaves the double-strand DNA (DSB) at a fixed distance of 9 and 13 nucleotides downstream of the recognition site [5]. TALENs are artificial restriction enzymes generated by fusing the specific TALE DNA-binding domain to a nonspecific *Fok* I DNA cleavage domain [6–8] (**Figure 2**).

1.3 CRISPRs/Cas9

The CRISPRs/Cas9 system, originally found in the bacteria, functions as an adaptive immune system against foreign virus or plasmid DNA. CRISPRs are DNA loci containing short repetitions of base sequences. Each repetition is followed by short segments of spacer DNA from previous exposures to the foreign DNA. CRISPR-associated protein (Cas9) is a DNA endonuclease whose structure is bilobed, composed of target recognition domain and nuclease lobes. The nuclease

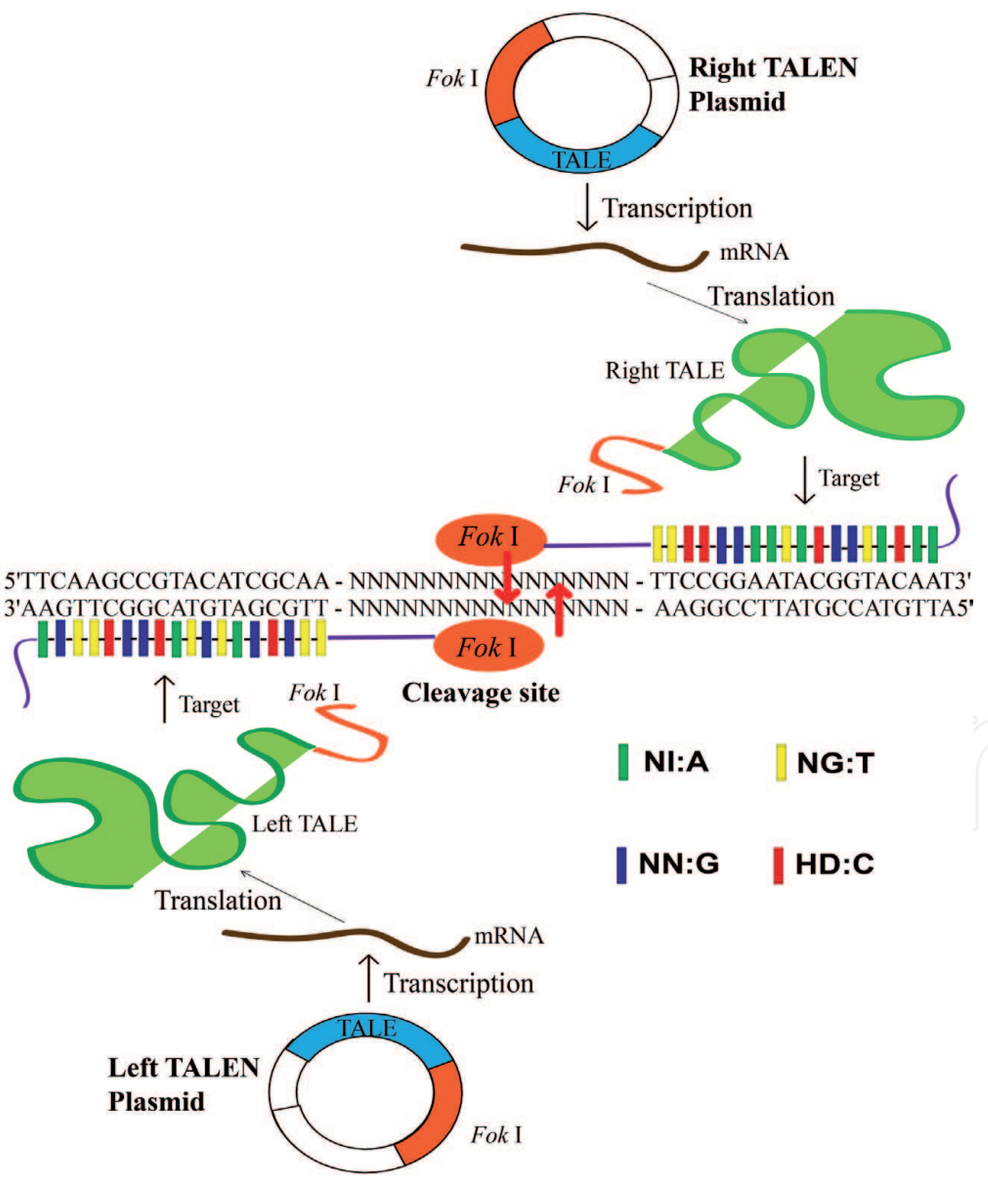


Figure 2.
The specific repeat variable Di-residues (RVDs) used to recognize each base are defined in the key (NI:A, NG:T, NN:G, and HD:C). Left and right TALEs recognize their target sequences and allow their associated Fok I endonucleases to work as a homodimer to cleave the sense strand 9 bp and antisense strand 13 bp downstream of the binding site. Binding of TALEs to the target sites allows Fok I to dimerize and create a double-strand break (DSB) with sticky ends within the spacer.

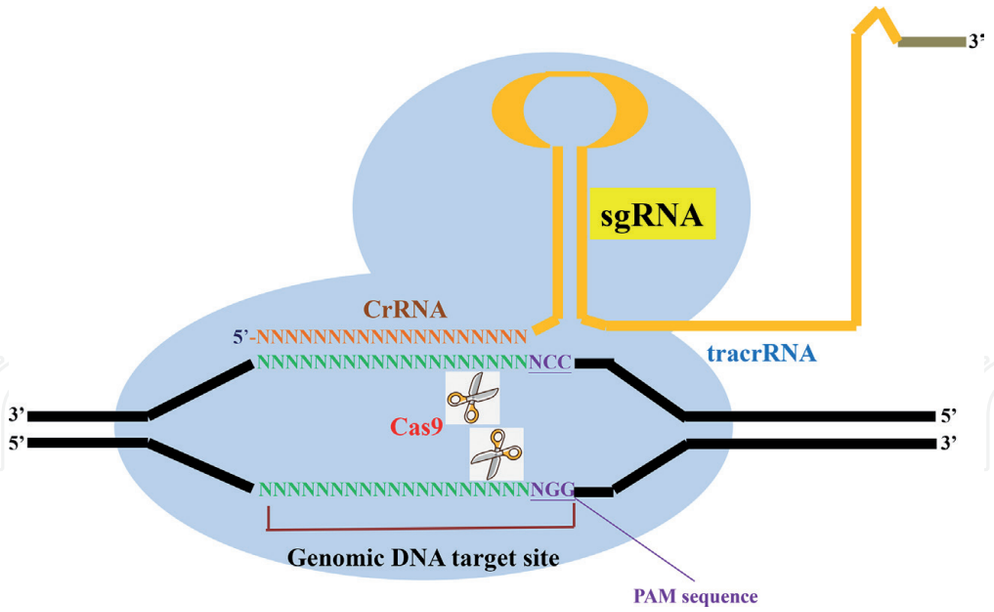


Figure 3.
A crRNA/tracrRNA hybrid acts as a single guide RNA (sgRNA) to recognize their target sequences and allow Cas9 endonucleases to cleave the sense strand 3 bp and antisense strand 3 bp upstream of the protospacer adjacent motif (PAM) sequence. Binding of sgRNAs to the target sites makes Cas9 create a double-strand break (DSB) with blunt ends on target sequences.

lobe contains nucleases RuvC, HNH, and a carboxyl-terminal domain for the protospacer adjacent motif (PAM) recognition [9]. The Type II CRISPRs system is currently limited to target sequences that are N12-20NGG, where NGG represents the PAM sequence [10]. Any potential target sequence must have a specific PAM sequence on its 3′ end. The CRISPR locus consists of Cas9 endonucleases, CRISPRs RNAs (crRNAs), trans-activating crRNAs (tracrRNAs), and precursor crRNAs (pre-crRNAs). tracrRNA is partially complementary to and pairs with a pre-crRNA to form an RNA duplex cleaved by RNase III. The crRNA/tracrRNA hybrid acts as a single guide RNA (sgRNA) for the Cas9, which cleaves the invading DNA. The DNA target sites can appear in multiple locations, all of which will be targeted by the Cas9 for cleavage. By delivering the Cas9 protein and appropriate sgRNAs into a cell, the organism's genome can be cut at most locations with the only limitation of PAM availability (**Figure 3**).

2. Application

2.1 Tools for basic research

2.1.1 Genetic cloning of living organisms

For conventional genetic cloning of animals, plants, and microbes, the target genes in the specific genome are cut using restriction enzymes. It usually takes lots of work and long time to clone and screen for the desired ones. Current gene editing technologies can be used to achieve the desired clones both quickly and accurately, without the limitation of restriction site availability [11].

2.1.2 Establishment of animal models

Genetic cloning, gene knock-in, and gene knockout are the most common methods to make induced or experimental animal models. The efficient approaches

include ZFNs, TALENs, and CRISPRs/Cas9. By these new gene editing technologies, specific animal models of many diseases for which there are no animal models available previously have been established with unprecedented efficiency and precision [12–14].

2.1.3 Development of testing tools and reagents

CRISPRs/Cas9 can be optimized by bacterial genotypes to be more adaptive to the variation of food pathogens, compared with traditional methods. The CRISPR locus of different bacterial species show high variance to be an ideal basis for genotyping [15]. The CRISPR/Cas12a(Cpf1) DETECTR (DNA endonuclease targeted CRISPR trans reporter) system can be used to diagnose gene mutations, cancers, and microbial infections and test microbial antibiotic resistance by analyzing specimens [16].

2.1.4 Discovery of drugs

The screening and identification of target sites are critical to drug discovery; thus, excellent and suitable platforms are needed. Gene editing technologies can work for the editing of functional genes and the screening of target sites. For example, CRISPRs/Cas9 was used to target the exons encoding functional protein domains. A screen of 192 chromatin regulatory domains in murine acute myeloid leukemia (AML) cells identified 6 known drug targets and 19 additional dependencies [17].

2.2 Nonhuman therapeutics

2.2.1 Agricultural products

The gene editing technology can be used to produce agricultural products in accordance with the need of humans. For example, crops were produced with high yield and resistant to diseases, insects, herbicides, and harsh environment [18, 19]; domesticated animals (pig, buffalo) were produced with double muscle phenotype [20]; and aquatic products (catfish) were produced with high level myostatin (MSTN) gene expression [21].

2.2.2 Food

We can make food more productive or have longer shelf-life by gene editing technologies. For example, CRISPRs/Cas9 was used to edit thermophilic bacteria *Streptococcus thermophilus* as a bacteriophage-insensitive mutant to improve the product (e.g., yogurt, cheese) yield by refraining from the infection of phages [22]. The white button mushroom *Agaricus bisporus* was engineered to resist browning using CRISPRs/Cas9. The effect was achieved by targeting to knock out the genes that encodes polyphenol oxidase—an enzyme that causes browning [23].

2.2.3 Industrial products

CRISPRs/Cas9 has been used to establish marine algae (e.g., diatoms) as useful in industrial applications as the carbon neutral synthesis of fuels, pharmaceuticals, health foods, biomolecules, materials related to nanotechnology, and bioremediations of contaminated water [24, 25]. CRISPRs/Cas9 was used to encode the pixel values of black and white images and a short movie into the living bacterial

genomes. By CRISPRs/Cas9, the technical limits of this information storage system can be optimized to be minimal. CRISPRs/Cas9 can capture and stably store many real data within the genomes of living cells [26].

2.2.4 Environmental protection

Marine microalgae are in charge of about 40% of primary production on earth and capture more CO_2 than rain forests. Diatoms are the most important unicellular eukaryotic microalgae and have dominant ecological significance. CRISPRs/Cas9 can be used to modify the diatom genome to achieve more effects in reducing the global warming [25].

2.2.5 Restoration of extinct animals

Woolly mammoths are different from current living elephants by adapting to the extreme cold environment. The mammoth TRPV3 gene, which encodes a temperature-sensitive transient receptor potential (thermoTRP) channel involved in thermal sensation and hair growth, could be achieved by modifying genes of Asian elephants [27]. The mammoth may be restored using CRISPRs/Cas9, if the modified embryo can be successfully transferred into the uterus of living elephants.

2.3 Human therapeutics

2.3.1 Medicine screening

The therapeutic strategies of Parkinson's disease (PD) are quite variable including drug and nondrug treatment. Therefore, it is necessary to find a suitable strategy to treat PD safely, efficiently, and quickly. A novel tool was established for monitoring endogenous alpha-synuclein (α-SYN) transcription by NanoLuc luciferase tag insertion at the 3′ end using CRISPRs/Cas9, and thus making it possible to screen for strategies rapidly that can be used for PD therapy efficiently [28].

2.3.2 Preparation for cell therapy or immunotherapy

The gene-editing technology can be applied to engineer induced pluripotent stem (iPS) cells and chimeric antigen receptor T (CART) cells. CRISPRs/Cas9 has been used to engineer iPS cells to evade immune rejection in full immunocompetent allogeneic recipients [29]. Because the CD19 CAR was successfully used in treatment, CRISPRs/Cas9 may further enhance the efficacy and safety of CART cells by engineering therapeutic T cells [30].

2.3.3 Potential application for disease treatment

2.3.3.1 Virus latent infection

Diseases caused by viruses are difficult to treat due to their high mutation rates and latent infections. It is almost impossible to eradicate latent viruses in the human host. However, TALENs and CRISPRs/Cas9 have been found to provide good strategies in targeting viruses and limiting their productive and latent infections *ex vivo* and/or *in vivo*, such as herpes simplex virus 1 (HSV-1), Epstein-Barr virus (EBV), human cytomegalovirus (HCMV), human immunodeficiency virus (HIV), and hepatitis B virus (HBV) [31–36].

2.3.3.2 Genetic disease

Genetic diseases can be cured by gene therapies such as sickle cell anemia, β-thalassemia, muscular dystrophy, α1-antitrypsin deficiency, Leber congenital amaurosis, and cystic fibrosis [37]. The gene editing technologies (e.g., CRISPRs/Cas9) potentially facilitate the progress of gene therapy, because many experiments have been successful *ex vivo* and *in vivo*, and some of them are being under clinical trials.

2.3.3.3 Neurodegenerative disease

By applying the Perturbing Regulatory Interactions by Synthetic Modulators (PRISM) to a yeast model of PD, sgRNAs were identified to modulate transcriptional networks and protect cells from α-Syn toxicity [38]. The APPswe (Swedish) mutation in the amyloid precursor protein (APP) gene causes Alzheimer's disease (AD). The mutant APP^{SW} allele can be selectively disrupted using CRISPRs/Cas9 both *ex vivo* and *in vivo* and thereby decrease pathogenic amyloid-β (Aβ) [39].

2.3.3.4 Cancer

CRISPRs/Cas9 were tried to inhibit hepatocellular carcinoma (HCC). miR-125b can suppress the expression of SIRT6 by directly targeting the seed-matching region of its 3′UTR. After the expression of *SIRT6* knocked out through CRISPRs/Cas9, HCC cells showed the decreased viability and invasiveness, which had the similar function upon the overexpression of the miR-125b [40]. CRISPRs/Cas9 was also tried to inhibit breast cancer. Cyclin-dependent kinases (CDKs) are established anti-cancer drug targets, and a new generation of CDK inhibitors provides clinical benefits to the patients. Breast cancer cells were genetically manipulated using a deactivated CRISPRs/Cas9 (dCRISPR) approach to strengthen the endogenous CDK18 promoter to express highly to exhibit an increased sensitivity [41].

3. Conclusion

The gene editing technologies, especially CRISPRs/Cas9, have been extensively used as tools in basic research for genome encoding, silencing, enhancing, and modification. Currently, they are further applied in manufacturing nonhuman therapeutic products and medicinal products. Particularly, the discovery of medicinal products using gene editing technologies will open a new era for human therapeutics and expect to bring a hope for patient recovery from being seriously sick. Many biotechnology companies and pharmaceutical plants have successfully produced products using gene editing technologies. For example, CRISPR has become an industry which is prosperously developing recently. Nonhuman therapeutic products are usually manufactured nonexclusively, while human therapeutic products are manufactured exclusively because they are highly technical, ethically concerned, and more profitable. Gene editing technologies are very promising in applications, though there are still many technical (e.g., off target effects, option of delivery tools, localization of function) and ethical challenges (e.g., evaluation of benefits and risks, compatibility of private interests and the public good, random manipulation of genes, commercialization of human therapy) unsolved. More and more products based on these technologies are approved for marketing. We also expect the challenges of safety concerns (e.g., genetically modified organism, tumorigenesis, etc.) and ethical issues will be overcome in the near future.

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