Principles of gene editing techniques and applications in animal husbandry

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Abstract

Gene editing techniques were developed chronologically, which include zinc finger nuclease, transcription activator-like effector nuclease and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas 9). In this review, the working principles of these techniques were first introduced, their advantages and disadvantages were then discussed, their application in animal husbandry were elaborated, and finally human concerns about gene editing were presented. Compared to the two former techniques, the third-generation gene editing technique CRISPR/Cas9 has higher targeting efficiency and accuracy, less off-target effect, lower cytotoxicity and lower costs for being easier for vector design and manipulation. Although some people may concern about social or ethical issues, the benefits of gene editing certainly outweigh its demerits. The three gene editing techniques have been successfully used to improve the production and quality of livestock products, animal fertility, resistance to diseases, and welfare in animal husbandry. With legislation and the development of gene editing technology per se, it is anticipatable that gene editing will have a broader utilization and make our lives happier.

Keywords Gene editing · ZFN · TALEN · CRISPR/Cas9

Introduction

Gene editing is a molecular technology developed to modify specific sites within the genome through gene deletions, insertions or conversions for the purpose of studying functionally unknown genes or conducting gene therapy. As modified genetic information is inherited to the offspring, gene editing is frequently employed to modify biological traits of organisms to establish new varieties or improve the economic return of agriculture and livestock production. Several gene editing techniques have been developed chronologically, which include zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas 9) (CRISPR/Cas9). All these techniques use a specific nuclease or

nuclease complex to recognize and cleave DNA at specific target sites to achieve DNA knock out or replacement through DNA repair machinery. In this review, the principles of gene editing were introduced, the advantages and disadvantages of different editing techniques were discussed, and their applications in the field of animal husbandry were reviewed and prospected.

Principles of gene editing techniques

ZFN technique

ZFN technique is a first-generation gene editing technique that utilizes a fused nuclease comprising a zinc finger protein (ZFP) and a Fok I restriction endonuclease (Kim et al. 1996). ZFP recognizes and binds to a specific DNA sequence while the dimerized Fok I, which possesses endonuclease activity, cuts the recognized sequence, thereby resulting in a DNA double-strand break (DSB) (Davis and Stokoe 2010). The DSB is subsequently repaired through non-homologous end-joining (NHEJ) or homologous recombination (HR) (Heyer et al. 2010). During the repair process in cells, mutations

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such as gene deletions or insertions are introduced, thereby achieving the goal of gene editing.

Zinc fingers are eukaryotic protein motifs that facilitate specific interactions between proteins and nucleic acids or different proteins. Multiple zinc fingers that recognize and bind to specific DNA sequence are tandem arranged and form ZFPs (Klug 2010). Every zinc finger specifically recognizes three consecutive nucleotides (triplets) on a single DNA chain. For example, a ZFP consisting of three zinc fingers are named from the N-terminal to the C-terminal as Finger 1 (F1), Finger 2 (F2), and Finger 3 (F3). These fingers bind to the target DNA along the 3'–5' direction. Each zinc finger consists of approximately 30 amino acid residues, of which the –1, +3, and +6 residues bind to the three bases of a triplet, thereby forming a 9 bp recognition site. The Fok I domain connects with the C-terminal of ZFP. When the distance between the two Fok I domains on the two sides of the double-stranded DNA is 5–7 bp, the two Fok I domains form an active dimer and cut DNA (Fig. 1) (Xiao et al. 2011; Pavletich and Pabo 1991).

TALEN technique

TALEN is a second-generation gene editing technique. Similar to ZFN, the core components of TALEN are a DNA binding protein and a Fok I restriction endonuclease. The difference is that TALEN utilizes transcription activator-like effector (TALE) instead of ZFP to fuse with the Fok I restriction endonuclease. TALE recognizes and binds to a specific DNA sequence, whereas Fok I, which possesses endonuclease activity after dimerization, cuts the recognized DNA to make a double-strand break. The double-strand break induces DNA repair through non-homologous

end-joining or homologous recombination, performing gene editing (Fig. 2).

The TALE protein is a secreted protein derived from the plant bacterial pathogen *Xanthomonas* (Bonas et al. 1989). As its structure is similar to transcription factors, TALE proteins regulate endogenous gene expression in host plants, thereby rendering sensitivity to infections (Bonas et al. 1989; Boch et al. 2009; Boch and Bonas 2010; Moscou and Bogdanove 2009). TALE proteins comprise three components: an N-terminal sequence for signal transduction, a tandem repeat sequence, and a C-terminal sequence that contains a nuclear localization signal and transcription activation domain (Boch et al. 2009). The tandem repeat sequence contains 12–33 repeat elements. Each repeat element is a sequence of 34 amino acid residues, of which the 12th and 13th amino acid residues are highly variable and referred to as repeat-variable di-residues (RVDs), but the rest residues are relatively conserved (Boch and Bonas 2010). RVDs determine the specificity of TALEs and are critical for targeting DNA. There are four common RVDs, which are NI (Asn–Ile), NG (Asn–Gly), HD (His–Asp), and NN (Asn–Asn). Each of the four RVDs has a fixed correspondence with A, T, C, or G. That is, NI identifies A, NG identifies T, HD identifies C, and NN identifies G (Boch et al. 2009; Boch and Bonas 2010; Moscou and Bogdanove 2009).

CRISPR/Cas9 system

CRISPR/Cas9 is a third-generation gene editing technique that involves artificially engineered immune system of bacteria and archaea, which mediates exogenous DNA degradation and therefore serves as a defense mechanism against viral infections (Boch and Bonas 2010; Bolotin

Fig. 1 The principle of ZFN technique (Xiao et al. 2011). A ZFN monomer (two monomers are illustrated in the opposite direction along the double strand DNA) is composed of a Fok I cleavage domain and a ZFP domain consisting of three zinc fingers. The ZFP domain binds to one strand of a double-stranded DNA. The N-terminal of ZFP corresponds to the 3'-terminal of the target DNA. The –1,

+3, +6 amino acid residue of each zinc finger recognizes three consecutive bases (a triplet) in the 3'→5' direction to form a 9-bp recognition site. The Fok I cleavage domain connects with the C-terminal of the ZFP; when the two Fok I domains in the opposite direction are spaced by 5–7 bp, they form a dimer and perform the cleavage function

Fig. 2 The principle of TALEN technology (Mao and Tao 2015). **a** RVDs of TALE protein target and bind to dsDNA, and Fok I cleaves dsDNA. **b** dsDNA breaks. **c** The broken dsDNA is repaired by NHEJ or HR

et al. 2005; Pourcel et al. 2005; Mojica et al. 2005). The clustered regularly interspaced short palindromic repeats (CRISPR) were first discovered in the genome of *E. coli* in 1987 (Ishino et al. 1987), but did not arouse attention until 2012 when it was reported that CRISPR and the CRISPR-associated protein Cas9 cut DNA duplex at specific sites in vitro (Jinek et al. 2012). This finding potentiated the application of CRISPR/Cas9 in gene editing. 1 year later, Cong et al. (2013) designed two CRISPR/Cas9 systems and demonstrated that Cas9 nucleases could be directed by short RNAs to induce precise cleavage at endogenous genomic loci in human and mouse cells. This is a breakthrough in gene editing research.

CRISPR comprises a leader sequence, several repeat sequences and several spacer sequences (Makarova et al. 2006; Jarman et al. 1993; Wei et al. 2015). The leader sequence is located upstream of the CRISPR gene cluster. It has no coding activity, but serves as a species-specific promoter. The repeat sequences are highly conserved palindromic sequences that can form hairpin structures. The repeat sequences are not tandem arranged but interrupted by spacer sequences. The spacer sequences are homologous to some sequences in the genome of phages or plasmids, which enables the cells to recognize and defend against the invasion of these corresponding phages or plasmids (Stern et al. 2010). Cas9 nuclease complexes comprised of a Cas9 protein, a specific CRISPR RNA (crRNA), and a transactivation CRISPR RNA (tracrRNA). The crRNA and tracrRNA form a dimer and bind to the Cas9 protein. The complex recognizes and cuts target DNA at specific sites to generate double-strand breaks that induce DNA repair in cells. In the absence of homologous DNA, repair occurs through NHEJ, which facilitates gene deletion (Ma et al. 2014). When homologous DNA is present, repair occurs through HR, which facilitates gene insertion (Fig. 3).

Advantages and disadvantages of different gene editing techniques

Compared to traditional HR, the ZFN technology has substantially high gene targeting efficiency (10–30%) (Table 1). ZFN technique does not require the use of embryonic stem cells and can be applied to various eukaryotic cells. ZFN is a first-generation gene editing technique and thus the most well established. However, the ZFN recognition domain is context-dependent. Its constituent amino acid repeats interact with each other, reducing the specificity and efficiency of gene targeting (Sander et al. 2011). In fact, it is difficult to design a suitable ZFN for any target gene. In another word, not all genes in the genome can be edited by ZFN. Besides, the ZFN technique has off-target effects, which in turn induces cytotoxicity (Sung et al. 2014).

Compared to the ZFN technique, the TALEN approach has even higher targeting efficiency (20–60%) (Table 1). This technique also has higher specificity with little off-target effect and low cytotoxicity. In addition, it is easier to design a pair of TALENs than ZFNs for a specific target DNA sequence. However, the assembly of TALE molecular modules is complicated and requires extensive sequencing, which in turn increases cost. The molecular weight of TALE proteins is higher than that of ZFP proteins, which makes it more difficult to operate at the molecular level, albeit its capability in targeting longer gene sequences (Wright et al. 2014).

The CRISPR/Cas9 system has the highest targeting efficiency among the three techniques (50–80%) (Table 1). The construction of the CRISPR/Cas9 system mainly involves the design of a RNA sequence that is complementary to the target DNA sequence. Compared to ZFN and TALEN, CRISPR/Cas9 is easier for target site design, vector construction and operation, and has lower cost. Both ZFN and TALEN

Fig. 3 The principle of the CRISPR/Cas9 system (Mao and Tao 2015). The transcribed pre-crRNA is processed by Cas 9 protein and RNase III to a mature crRNA. The mature crRNA, Cas 9 and the transcribed tracrRNA bind together to form a complex. The complex recognizes and opens the target DNA sequence which is complementary to the crRNA. The crRNA hybridizes with the complementary

DNA strand. Subsequently, the HNH activity site of the Cas9 protein cleaves the complementary DNA strand and the RuvC activity site cuts the other free DNA strand to form a DNA double-strand break. When protospacer adjacent motifs (PAMs) are present, HR or NHEJ mechanism is initiated to perform editing of the target gene

techniques target DNA by proteins, whereas the CRISPR/Cas9 system targets DNA through a base pairing mechanism using RNA. Therefore, DNA recognition by the CRISPR/Cas9 system is more accurate, with less off-target effect and lower cytotoxicity. However, this technology has been developed much later and is still in development. It may cause additional mutations in sites other than the target site (Ding et al. 2013; Fu et al. 2013). In addition, cutting of the target sequence depends not only on the matched crRNA sequence, but also on a few small PAMs. The Cas9 protein does not cut the target sequence if no PAM exists around the target sequence.

Applications of gene editing techniques in animal husbandry

To increase the production of livestock products

Myostatin (MSTN) negatively regulates muscle growth and development. Loss of MSTN function leads to the “double-muscle” trait. Using the ZFN technique, Qian et al. edited the MSTN gene in fibroblasts derived from Meishan pig fetuses. Through somatic cell nuclear

Table 1 Comparison of the three gene editing techniques

Gene editing technique	Generation	DNA recognition domain	Endonuclease	Advantages	Disadvantages
ZFN	First	ZFP	<i>Fok I</i>	Targeting efficiency usually 10–30% (Fujii et al. 2013, 2015; Liu et al. 2015; Lei et al. 2012); most well-established	Context-dependent; off-target effects; high cytotoxicity; complication in design
TALEN	Second	TALE	<i>Fok I</i>	Targeting efficiency usually 20–60%, high to 90% reported (Lei et al. 2012; Tesson et al. 2011; Jin et al. 2018; Nerys-Junior et al. 2018; Ding et al. 2013); not context dependent; little off-target effect; low cytotoxicity; easy to design; high specificity	Complicated TALE molecular module assembly; requires extensive sequencing; high cost; difficult to operate
CRISPR/Cas9	Third	crRNA	<i>Cas9</i>	Targeting efficiency usually 50–80% (Ding et al. 2013; Nerys-Junior et al. 2018; Sugano et al. 2018); easy to design and operate; low cost; little off-target effect; low cytotoxicity	Not well established; <i>Cas9</i> cannot cut target sequence if no PAM exists

transplantation, MSTN-mutated Meishan pigs were generated. Compared to the wild-type, MSTN mutated Meishan pigs have 11.62% higher lean meat production (Qian et al. 2015).

In addition, Yu et al. designed and transfected a pair of TALENs into goat fibroblasts, and obtained 272 monoclonal cells with MSTN gene mutations. Among these monoclonal cells, ten clones with different genotypes were used as donors for somatic cell nuclear transplantation, and three goat clones were obtained (K179/MSTN^{-/-}, K52-2/MSTN^{+/-}, and K52-1/MSTN^{+/+}). The goat with the mutated MSTN gene exhibited significantly higher meat yield. Compared to the wild-type (K52-1/MSTN^{+/+}), the double knockout goat clone (K179/MSTN^{-/-}) had 1.7-fold higher body weight while the single knockout goat clone (K179/MSTN^{+/-}) was 32% heavier than the wild-type (Yu et al. 2016). In addition, gene editing techniques have been employed in some other farm animal species to generate MSTN mutant animals with the aim to promote muscle development or to increase meat production (Wang et al. 2016b; Proudfoot et al. 2015; Luo et al. 2014; Li et al. 2016; Crispo et al. 2015).

Fibroblast growth factor 5 (FGF-5) is a regulatory factor that regulates hair growth. It converts hair follicles from the growing phase to the recessive phase. Mutation of FGF5 gene prolongs the growing phase and thus increases hair length. Using the CRISPR/Cas9 technique, Wang et al. microinjected MSTN- and FGF5-targeting vector Cas9 mRNA and sgRNAs into cashmere goat embryos and evaluated the top three economic traits in cashmere goat, including cashmere yield, cashmere fiber diameter and length.

Results showed that the GFG5 mutated cashmere goats had significantly longer cashmere fibers and higher cashmere yield (Wang et al. 2016a).

To improve animal fertility and disease resistance

Porcine reproductive and respiratory syndrome (PRRS), also known as blue-ear pig disease, is a challenging disease for worldwide pig production. It causes severe breathing problems in young pigs and breeding failures in pregnant females. In addition, it brings about abortions, early farrowing, increased number of stillborn piglets, and weak neonatal piglets. PRRS virus targets macrophages. CD163 is a molecule on the surface of macrophages and plays a key role in enabling the PRRS virus to establish an infection. Burkard et al. used CRISPR/Cas9 to delete a small sequence of the CD163 gene in the pigs' genome and generated 32 gene edited pigs. These pigs are resistant to infection when exposed to the PRRS virus. Only the sequence of CD163 that interacts with the PRRS virus was removed, so the CD163 proteins retains its other biological functions (Burkard et al. 2017).

Tuberculosis is a zoonotic disease and a leading health problem worldwide which accounts for about 1.5 million deaths annually (Gandhi et al. 2016). Bovine tuberculosis is a big threat to animal husbandry as no effective strategy is currently available to eliminate or control this disease. Expression of the mouse Sp110 nuclear body protein (Sp110) enhances macrophage apoptosis in response to *Mycobacterium tuberculosis* (Mtb) infection and upregulates host immunity to Mtb, providing a promising way to control

Mtb growth and transmission. Wu et al. successfully utilized TALEN technology to insert a mouse SP110 gene into the genome of Holstein–Friesian cattle. Transgenic cattle with SP110 gene knock in showed increased resistance to Mtb infection (Wu et al. 2015).

To improve the quality of livestock products

Cows' milk is rich in nutrients and serves as an ideal alternative milk source for infants other than breast milk. However, as the digestive system of infants is not well developed, the undigested β -lactoglobulin (BLG) in cows' milk can be absorbed and recognized by the infants' immune system as a pathogen, resulting in milk allergy. A BLG gene knockout cow was first generated by Yu et al. (2011) using the ZFN technique to reduce BLG antigenicity and immunogenicity. For the same purpose, Zhou et al. co-injected Cas9 mRNA and small guide RNAs (sgRNAs) into goat embryos and successfully generated BLG knock-out goats (2017), which had significantly lower expression of BLG in mammary glands when compared to that of the wild-type goats. These studies provide a method to improve the quality of milk as well as to reduce milk allergies.

Eating foods rich in n-3 polyunsaturated fatty acids (n-3PUFAs) can effectively reduce the incidence of cardiovascular and cerebrovascular diseases. The *fat-1* gene from *Caenorhabditis elegans* encodes a fatty acid desaturase which converts n-6 polyunsaturated fatty acids (n-6PUFAs) to n-3PUFAs when expressed within animal body. Thus, it is anticipated that *fat-1* animals could be used to produce n-3PUFAs. For this end, Li et al. (2018) tried to insert the *fat-1* gene from *C. elegans* into porcine Rosa 26 (pRosa26) locus via the CRISPR/Cas9 system and successfully generate *fat-1* knock-in pigs. Gas chromatography analysis confirmed the expression of this gene in pig tissues as that *fat-1* knock-in pigs exhibited a significantly increased level of n-3PUFAs, as well as an obviously decreased n-6PUFAs/n-3PUFAs ratio. These *fat-1* transgenic pigs provide promise for improving the nutritional value of porcine products and serving as an animal model to investigate therapeutic effects of n-3PUFAs on diseases. Even better, Zhang et al. (2018) inserted the *fat-1* gene into the goat MSTN locus using the CRISPR/Cas9 system to achieve simultaneous editing of the two genes. PCR and sequencing showed that the efficiency for simultaneous MSTN knockout and *fat-1* knock-in was as high as 25.56%, demonstrating that the CRISPR/Cas9 system is a potential gene engineering tool in safe animal breeding.

To improve animal welfare

For the safety of the milkers and other animals, cows' horns are commonly cut off shortly after birth. Cutting cows' horns

with an electric saw or burn off of cows' horns with a soldering iron is not only painful to the animal, but also difficult to manipulate. The CRISPR/Cas9 has ever been introduced to knockout genes responsible for angular growth of cattle. With the combined use of in vitro fertilization and embryo transfer techniques, two hornless Holstein dairy cattle have been bred to avoid the painful horn cutting to improve animal welfare (Carlson et al. 2016).

Applications of gene editing techniques in other fields

In addition to the various applications in animal husbandry, gene editing techniques have been more commonly employed in biomedical research and gene therapy for human diseases (Soldner et al. 2011; Qiu et al. 2013; Xu et al. 2017). As for plants, gene editing has been frequently performed to improve crop resistance to herbicides or insects, to improve plant growth property, agri-product yield or quality (Shukla et al. 2009; Townsend et al. 2009; Ricroch and Henard-Damave 2016; Zhang et al. 2017; Li et al. 2012).

Concerns and prospect

As the genomes of more species have been sequencing, identification of gene function has become the research focus and task for the post-genome era. Gene editing technology can be used not only for gene therapy in which a mutated gene is replaced by a wild-type gene, but also for animal model development, animal trait improvement, and gene function studies in which a wild-type gene is replaced by a mutated gene. The ZFN, TALEN, and CRISPR/Cas9 gene editing techniques have been successfully employed in these areas. The ZFN technique was rated by the American magazine "Wired" as one of the top ten scientific breakthroughs in 2008. The TALEN technique and the CRISPR/Cas9 system were rated by the journal "Science" as one of the top ten scientific breakthroughs in 2012 and 2013, respectively.

Compared to conventional gene targeting techniques, gene editing does not require the use of embryonic stem cells, is applicable to more species, more accurate and more efficient. However, these gene editing techniques are not flawless. The off-target effect, complication in vector construction and high cost are the common problems. Despite these issues, gene editing will continue to be a powerful gene engineering tool as the technique per se will be keeping improved with the advances of molecular biology. For example, the targeting accuracy of CRISPR/Cas9 has been improved recently with the introduction of mutation (N692A/M694A/Q695A/H698A) in the REC3 domain (Chen et al. 2017). It is anticipatable that gene editing technique itself will once again

have a revolutionary breakthrough to better benefit our lives and the world.

On the other side, concerns about gene editing have appeared (Ruan et al. 2017; Ledford 2017; Gaiind 2016). These include food safety, ethical issues and social equity. First, the general public is not so familiar with gene editing and worries that this technology may have a negative impact on gene-edited organisms. In addition, they concern that it is unsafe to eat the products of gene-edited organisms as the pros and cons of gene editing have not been fully assessed (Ruan et al. 2017; Ledford 2017). Second, some people argue that using gene-editing techniques to improve or create species violates natural laws of survival and ethics (Gaiind 2016). Finally, some people worry that this technology may become a privilege of the wealthy, with which only the rich people afford to replace their diseased organs or to knock-out their “harmful” genes, thus bringing about social inequity and even social unrest (Gaiind 2016). For all these concerns, laws and regulations should be made to ensure the safety and equity of gene editing. In fact, it is not a matter of the technology per se, but a matter of humans how people use it. With the development of science and technology, we believe that this technology will make the world and our lives better and happier.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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