Advances In the Application of CRISPR/Cas9 Gene Editing Technology in Pig Breeding and Production

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Abstract. China is the richest pig breeding resource in the world. According to different conditions in different regions, pigs with different excellent traits were bred. Most pig breeds have the advantages of high fat content and strong environmental adaptability, but have the disadvantages of low lean meat rate and poor meat quality. Some pig breeds have poor resistance to disease and are prone to widespread infection. Only crossing with western pig breeds will introduce unfavorable genes, resulting in a sharp decline in the number of local pig breeds, but there are also shortcomings such as long crossing time. Gene editing (GE) technology has become a new way to regulate pig breeding. In recent years, with the exploration of the regular clustering structure and mechanism of the short palindrome repeat/CRISPR associated protein 9 (CRISPR/Cas9) system, GE technology based on CRISPR/Cas9 can improve lean meat rate and obtain disease resistance by knocking out specific genes and enhancing specific gene expression. This technology can also change the reproductive organs of domestic pigs to control their sex and improve the quality of meat. This article presents the application of CRISPR/Cas9 GE technology in pig breeding and production.

Keywords: CRISPR, Cas9, Pigs, Gene editing, Pig Breeding, BE.

1. Introduction

At present, pig breeding is an important livelihood industry in China, and also an important industry in animal husbandry, which is related to the development of the national economy. Because the body structure and physiological characteristics of pigs are similar to those of humans, they also have medical research value. At present, the goal of pig industry is to improve lean meat rate, reduce fat content, and improve pork quality and taste. At present, China's artificial pig breeds, such as Rongchang pig and Pudong white pig, have the advantages of high reproduction rate, good adaptability to living environment, easy feeding and high intramuscular fat content. However, there are disadvantages such as low leanness, long growth cycle, early maturity and easy fattening. Western lean pigs, such as Baksha or Duroc, possess the advantages of growing fast, high feed conversion efficiency and high lean meat percentage. All these can make up for the shortage of native pig breeds in China. Therefore, crossbreeding with western lean pig breeds can effectively improve the growth and lean traits of Chinese local pig breeds, but there are obvious disadvantages in crossbreeding. First of all, when pigs of different breeds cross, their offspring will introduce a large number of inferior genes and high-quality genes [1]. In order to select and cultivate excellent hybrids, a lot of manpower must be equipped and long-term observation and measurement must be carried out. This process of selection and reproduction must last for decades. The popular breeds in the world, such as Landrace, Large White and other famous breeds, have been successfully bred after nearly a century of breeding. The breeding process of new animal varieties is long, time is long, and economic cost is high. Secondly, traditional breeding methods cannot detect complicated features with difficulty of observing and measuring. For example, resistance traits cannot be observed without disease, and measurement and selection under continuous disease conditions may lead to major infection risk in breeding [1]. Thirdly, the law of hybridization shows that even after a long period of selective breeding, the inferior genes may not be completely eliminated from the hybrids. There are always bad genes and good genes linked together. GE technology allows precise modification of target traits. It is not affected by adverse genes in hybridization, and reduces the time spent in traditional hybridization due to generation spacing and selective breeding. It enables researchers to obtain the

required pig breeds in a shorter time. In the technology of GE, CRISPR/Cas9 system has developed rapidly for 13 years, providing new ideas for pig breeding. This paper first introduces CRISPR/Cas9-GE technology, and then summarizes the research status and progress of this technology in pig breeding and production. Finally, it gives an outlook on how this technology can be used in the future to improve the genetics of pig breeds more efficiently and comprehensively.

2. CRISPR

2.1. Development of CRISPR

CRISPR is a natural immune system derived from ancient bacteria, used to prevent the invasion of foreign nucleic acids or bacteriophages. In 1987, Ishino et al. discovered the CRISPR sequence when studying Escherichia coli, including the repetitive sequence [2]. However, they did not find the sequence of characters. In 2002, Jansen et al. named CRISPR to study its characteristics [3]. They also found three kinds of Crispr-related proteins, type I, type II and type III. In 2007, Barrangou R et al. confirmed in Streptococcus thermophilus that the defense system mediated by Crispr gene and Cas's protein can resist the invasion of foreign phages [4].

In 2012, Doudna and Charpentier successfully developed CRISPR/Cas9 GE technology, which won the Nobel Prize in 2020. In 2013, Zhang's team used this technology to modify mammalian genes for the first time [5]. This research has promoted the application of transgenic technology in animals and plants including pigs.

2.2. Structure of CRISPR

From the initial discovery of the sequence and its name CRISPR to its proven defensive role in paleontology and bacteria, its structure and function have been revealed [2-4]. This played a key role in its later transformation into a universal tool. CRISPR/CAS system is composed of a CRISPR sequence and a CAS protein. CRISPR sequence consists of three parts: a leading sequence, a number of highly conserved repeat sequences and a number of interval sequences. The leading sequence is located upstream of the system and acts as the CRISPR promoter. The repeating sequence forms a hairpin structure because it contains palindromes. The spacer sequence contains the DNA sequence of foreign invasion [6]. Cas's protein-related genes are located near the CRISPR sequence. When foreign DNA is invaded and cut, these genes are translated and transcribed into Cas's protein. At present, there are 14 kinds, the most common one is Cas9 protein.

Cas9 is the most commonly used GE and gene regulatory protein in the currently available CRISPR system. It consists of two structural domains, NUC and REC. NUC domains include HNH, RUVC, and PI domains. The HNH together with RUVC domains play a role in the foreign double-stranded DNA cleavage, and the PI domain is related to the recognition of the adjacent part of the original spacer (PAM) [7].

2.3. Mechanism of action of CRISPR

When foreign phages invade cells, the host cell's CRISPR/Cas9 system recognizes specific foreign sequences through PAM. The CRISPR motif is initially transcribed into precrRNA. Then mature crRNA can be processed, which combines with trans-activated crRNA (tracrRNA), thus forming a single guide RNA (sgRNA). It forms nucleic acid protein complex with Cas's protein and cleaves unknown nucleic acid by binding with PAM sequence. It forms a nucleic acid protein complex with Cas's protein, and cuts foreign nucleic acid by binding with PAM sequence [7]. In current research, crRNA and tracrRNA are usually converted into the required sgRNA. This sequence allows Cas9 protein to accurately identify the target DNA sequence and complete the cleavage, resulting in double strand breaks (DSB), and activate the host cell's DNA repair mechanism, homologous directed repair (HDR) and non-homologous terminal junction (NHEJ). When the donor DNA is present, it can accurately repair the break site according to the homologous segment. In the absence of donor DNA,

DSB will trigger NHEJ in the organism, which will insert or delete the gene fragment at the break [7,8].

2.4. Single gene editing technology

2.4.1 Cytosine base editor (CBE)

In 2016, Liu's team developed a CRISPR-based system for basic editor (BE), called CBE [8]. The system does not require both DNA strand to break. First, the cleavage domain of Cas9 protein is inactivated by a point mutation to dead Cas9 (dCas9). Then it connects with cytosine deaminase to form CBE system. The mechanism of action is the same as that of normal CRISPR system. The target gene is the target by sgRNA without cleavage. Cytosine deaminase changes the base C of the target site into U, while DNA replication changes U into T, thus changing C-G into A-T base pair. The change of C-T base was realized.

2.4.2 Adenie base editor (ABE)

In 2017, Liu's team developed the ABE system based on CBE [9]. In this system, when the fusion protein targets genomic DNA with sgRNA, adenine deaminase is able to combine with single-stranded DNA and deaminate a range of bases a to I, which is then read and copied as base G at the DNA level. Eventually, the direct substitution of a-T to G-C base pair can be realized. ABE system uses adenine deaminase to deaminase base A in DNA, but no natural deaminase can use DNA as a substrate. They evolved and engineered the protein of Escherichia coli tRNA adenosine deaminase (ecTadA), and finally obtained ABE7.10 after seven rounds of evolution and engineering. The selectivity of this method is more than 1000 times higher than the current Cas9 nuclease-mediated HDR product [9]. It completes the base conversion from A-T to C-G with higher efficiency and fewer unnecessary products.

2.4.3 Prime editor (PE)

The PE system allows four base conversions completed by CBE and ABE without using DSB and template DNA (C → T, g → A, a → G, t → C, C → A, C → G, g → C, g → T, a → C, a → T, t → A, t → G). The system uses primer editing guide RNA (pegRNA) to guide the fusion protein of Nickase Cas9 (nCas9) and reverse transcriptase to complete GE. PegRNA is composed of sgRNA, primer binding site (PBS) and reverse transcription (RT) template. Under the guidance of pegRNA, nCas9 binds to the target site containing PAM sequence to cut the single strand of DNA. The broken DNA single strand and PBS on pegRNA complement each other, and reverse transcriptase uses RT template for reverse transcription reaction. The 3 'flap of DNA double strand contains mutations, while the 5' flap has no mutations. It is easy to be recognized and removed by structure-specific endonuclease. The subsequent DNA connection and repair can achieve precise GE.

Single GE technology is based on CRISPR, but does not require DSB or template DNA. This is more effective and concise than the method based on Cas9 nuclease. It reduces the occurrence of deviation from the target effect and the generation of by-products. It provides a different way of thinking for raising pigs or other animals.

3. Status of pig breeding research

3.1. Improving growth traits of pigs

Mycostatin (MSTN) gene, also known as growth differentiation factor-8 (GDF-8), belongs to the TGF-β superfamily, which can negatively regulate mammalian muscle development, and inhibition of its expression can increase lean muscle mass and promote muscle growth. The special role of MSTN in skeletal muscle development makes it the target of gene editing to improve the lean muscle quality of domestic pigs and reduce obesity. Several research teams used CRISPR/Cas9 technology to improve meat production of pigs through Mycostatin gene knock-out. In 2020, Li et al. will introduce mutations (pdv20h and GP19del) in the MSTN signal peptide region of two piglets [10].

The study found that the mutation led to the down-regulation of MSTN gene expression as well as the increase of muscle fiber quantity, which promoted the muscle production of local pigs. This indicates that accurate GE of the signal peptide region of Mycostatin gene can promote muscle development in pigs. Zhu's team obtained Bama miniature pigs with MSTN gene, which increased the lean meat production of pigs. In his study, pigs edited by MSTN were 9.6% heavier than wild pigs at 6 months old [11]. Li et al. found that the weight of skeletal muscle of the MSTN knockout hybrid Meishan pig increased by 9% and the fat content decreased by 8.48% [12]. In order to produce an early stop codon and a nonsense mutation of MSTN in porcine fibroblasts, Wang et al. and Pan's experimental team created sgRNA for CT conversion at the sequence corresponding to the PAM site of MSTN. This early stop codon could also cause the MSTN gene to no longer be expressed, increasing the production of lean meat in pigs [13,14].

Animals' postnatal growth and development, as well as embryonic development, are significantly influenced by insulin-like growth factor 2 (IGF2). In Liangguang pig embryonic fibroblasts, Liu et al. disrupted the ZBED6 binding site motif in IGF2's intron 3, which significantly increased the production of IGF2 and improved the pig embryonic fibroblasts' capacity for myoblastic differentiation and cell proliferation [15]. In this study, IGF2-GE cells were used as donor cells to obtain well-lit piglets with 63bp ZBED6 binding site motif deletion. The muscle development of gene-edited pigs was significantly enhanced, which was 32% heavier than that of wild-type pigs in 338 days. Xiang et al edited intron 3 of IGF2 gene at 3072. They found that these mutations released the combination of ZBED6 and its motif, significantly improved the growth performance of Parmesan pigs, and increased the carcass weight by 33.35% compared with the wild type [16]. The regulation of IGF2 has been proved to be beneficial to the muscle development of pigs.

3.2. Improving disease resistance genes in pigs

China has about 50% of the world's live pig population. Highly infectious or fatal pig diseases may cause huge economic losses to animal husbandry. Disease prevention and control are crucial to the healthy development of pig industry. Applying CRISPR/Cas9 GE techniques for improving the disease resistance of domestic pigs can effectively increase economic benefits.

Porcine reproductive and respiratory syndrome (PRRS) is one of the most concerned diseases in the prevention and treatment of pig farms, also known as blue-ear disease. This disease is a high-risk infectious disease resulting from PRRS virus (PRRSV) infection. PRRSV infects the macrophages or monocytes of domestic pigs, resulting in miscarriage or stillbirth of pregnant sows in the late pregnancy, causing huge economic losses to pig production. In 2014, Whitworth et al. developed CD163 transgenic pigs using CRISPR/Cas9 technology [17]. Pigs having the CD163 gene deleted were entirely resistant to PRRSV infection in attack studies, indicating that CD163 is a crucial receptor. The CD163 protein is Scavenger Receptor Cysteine Rich (SRCR), as the team revealed in 2016. Exon 7 encodes the fifth of the nine SRCR structural domains, which when deleted resulted in type I PRRSV-resistant pigs by replacing it with a structural domain that is equivalent to human CD163. Subsequently, Guo's team deleted 41 amino acid fragments from the CD163 SRCR5 domain containing ligand binding pockets [18]. This led the gene-edited big white pigs to prove complete resistance to type II PRRSV in the virus attack test. Wang et al. designed multiple SgRNA in the sequence corresponding to CD163 PAM site, and carried out C → T transformation using single gene editing technology, resulting in nonsense mutation of CD163 in porcine fibroblasts [19]. This study provides new ideas for the prevention of viral infectious diseases in pigs.

3.3. Application to gender control

Piglet castration is an important part of pig production. It can reduce the energy consumption required for the development of male reproductive system. Because of the smell of male pig sex hormone, it can also eliminate the taste and quality of pork. However, castration of piglets is a complex operation, and it is also prone to wound infection, even later development. GE's technology enables all sows to produce female piglets, which will effectively avoid the problem of castration.

The sex determining region of Y (SRY) is an important genetic factor for male mammalian development. Male-to-female reversal syndrome has been linked to pathogenic mutation of the SRY gene in humans and other animal species. The disruption of testicular habitual expression, which determines testis, will alter the sex of pigs. In order to change the pig SRY's HMG domain into a female one, CRISPR/Cas9 technology was employed. In order to manage piglet sex without impacting pig development efficiency, Ry-KO piglets develop entire female reproductive organs with the same uterus and fallopian tube morphology as wild-type sows.

3.4. Simultaneous improvement of multiple genes

It uses a single GE technology. Song's team injected fertilized eggs directly with Cbes, introducing the beneficial expression allele of IGF-2 by adding stop codons to CD163 and MSTN genes. This disrupts the expression of the first two genes and upregulates IGF-2 expression. Real-time quantitative fluorescence PCR (qPCR) indicated that the expression level of IGF2 mRNA in heart, liver, lung, kidney, longissimus dorsi muscle tissue and semi-conscious tissue of piglets was significantly higher than that of wild-type piglets. CD163 and MSTN were not detected in piglets containing CD163 R312Stop and MSTN Q218Stop. This not only improves the growth performance of pigs, but also enhances their resistance to disease. Their current study has demonstrated the feasibility of using one-step CBE fertilized egg injections in pigs for basic editing of multiple sites that confer beneficial traits.

4. Conclusion

The CRISPR/Cas9 techniques have now played an important role in pig production and breeding, enhancing growth performance and muscle content by knocking out the MSTN gene and making a nonsense mutation in the MSTN gene by designing sgRNAs to complete single gene editing for C→T conversion, or by modifying the IGF2 intron. The system can make pigs immune to PRRSV virus by modifying the receptor binding domain of CD163 or deleting specific fragments in the binding domain, and can also complete the immunity to the transmission of porcine virus through nonsense mutation of a single GE in CD163 gene. The use of CRISPR/Cas9 technology enables male piglets to develop female reproductive organs, which helps improve the taste and quality of meat, and also avoids animal ethics and welfare problems. The single gene engineering technology has also proved the feasibility in the field of multi-gene editing, and improved the first two characters in this document. Either CEB or other methods can complete the modification of a single base while improving the efficiency.

This paper shows that the method of obtaining domestic pigs with high-quality genes through gene editing makes up for the shortcomings of cross breeding. Unlike cross breeding, which is affected by the inferior genes of other individuals, it also greatly shortens the breeding time.

Currently, the applied CRISPR/Cas9 GE techniques in pig breeding and production focuses on single loci with large genetic effects. There are few key gene loci affecting fertility, growth, meat yield, meat quality and disease resistance of domestic pigs. As the relationship between animal genomes and phenomena, including domestic pigs, is better understood in the future, multi-gene editing may become an important tool to improve single or multiple traits of domestic pigs. Therefore, in the future, it is necessary to improve gene editing tools or explore editing strategies to create a stable and reliable multi-gene editing technology to realize simultaneous editing of different gene loci that control pig production traits. The problems caused by off-target mutations in the non-C→T transitions generated during the gene editing process have also been the focus of subsequent researchers.

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