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МИРОВЫЕ ДОСТИЖЕНИЯ ГЕНОМНОГО РЕДАКТИРОВАНИЯ В ОБЛАСТИ СВИНОВОДСТВА

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Представлен обзор основных мировых достижений редактирования генома свиней с использованием системы CRISPR/Cas9, в частности модификации генов (MSTN, IGF2, ZBED6, UCP1, LGALS12, APOE, vWF), для повышения продуктивных характеристик и хозяйственно полезных свойств, а также генов устойчивости животных к заболеваниям (APN, CD163, SRCR5, RSAD2). Большой интерес представляет изучение опыта применения этого инновационного инструмента для получения свиней с заданными признаками. Развитие молекулярно-генетических исследований, открытие взаимосвязей ген – фенотип обеспечило платформу, необходимую для модификации конкретных генов, чтобы значительно сократить репродуктивные циклы и повысить эффективность разведения свиней. Появившаяся относительно недавно система CRISPR/Cas9 уже нашла применение во многих передовых областях исследований, однако в задачах развития свиноводства, в том числе за счет получения трансгенных пород свиней, применение этой технологии ограничено. Это связано с тем, что существуют этические вопросы и проблемы нормативно-правового урегулирования, связанные с генно-отредактированными продуктами и потенциальными нецелевыми эффектами CRISPR/Cas9, которые необходимо исследовать. Технология геномного редактирования активно развивается в мире. В России реализуется программа развития генетических технологий, рассчитанная на 2019–2027 гг. Основная цель программы состоит в комплексном решении задач ускоренного развития генетических технологий, в том числе технологий генетического редактирования. Получение результатов посредством геномного редактирования линий сельскохозяйственных животных с новыми улучшенными свойствами – один из целевых индикаторов программы. С использованием CRISPR/Cas9 могут быть улучшены такие продуктивные характеристики свиней, как устойчивость к болезням, терморегуляция, повышение выхода и качества мяса.

Ключевые слова: CRISPR/Cas9, редактирование генома, ген, мутация, свиньи

GLOBAL ADVANCES IN GENOMIC EDITING IN PIG BREEDING

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An overview of the main world advances in editing the pig genome using the CRISPR/Cas9 system, in particular the modification of the genes (MSTN, IGF2, ZBED6, UCP1, LGALS12, APOE, vWF) to improve productivity and economic properties as well as the disease resistance genes (APN, CD163, SRCR5, RSAD2) in pigs is presented. It is of great interest to study the experience of using this innovative tool to produce pigs with specified traits. The development of molecular genetic research and the discovery of gene-phenotype relationships has provided the platform needed to modify specific genes to significantly shorten the reproductive cycles and improve the efficiency of pig breeding. The relatively recent CRISP/Cas9 system has already found use in many advanced fields of research, but its application is limited in the challenges of pig breeding, including the production of transgenic pigs. It is due to the fact that there are ethical and regulatory issues associated with genetically-edited

products and the potential non-target effects of CRISPR/Cas9 that need to be investigated. Genomic editing technology is actively developing worldwide. Russia is implementing the 2019-2027 genetic technology development program. The main goal of the program is to comprehensively address the problems of accelerated development of genetic technologies, including genetic editing technologies. Obtaining results through genomic editing of farm animal lines with new, improved properties is one of the program's target indicators. CRISPR/Cas9 can be used to improve pig performance characteristics such as resistance to disease, thermoregulation, improved meat yield and quality.

Keywords: CRISPR/Cas9, genome editing, gene, mutation, pigs

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Using selective breeding, people have developed breeds of pigs with beneficial characteristics for agriculture, although traditional selection is a lengthy process. However, this process can now be accelerated through genetic modification, including random transgenesis, gene knockouts, and knock-ins [1, 2]. Enhancing productivity traits and the quality of the products contributes to the efficient development of pig farming. Yet, alongside this, there are significant challenges related to pigs' susceptibility to diseases. Commercial pig farming has suffered massive economic losses due to various diseases such as African swine fever, classical swine fever, porcine reproductive and respiratory syndrome, among others. Modern molecular-genetic technologies allow us to identify specific causes that significantly contribute to the health and productivity traits of farm animals. One of the approaches for further use of this data is local DNA-level changes - genome editing. The ability to generate specific changes in the genome enables researchers to pose fundamental questions about gene functions, transfer allele variants between breeds or species, or create new phenotypes. Current achievements in this area show promising results related to enhancing productivity traits [3]. Moreover, it is believed that genome editing is one of the effective solutions to counter infectious diseases and reduce the heavy reliance on pharmaceutical drugs for treating pigs [4, 5].

The purpose of this article is to review the main global achievements in pig genome editing using the CRISPR/Cas9 system.

CRISPR/Cas9 is a component of the adaptive immunity of bacteria against phages and other invasive nucleic acids. The RNA-guided CRISPR/Cas system consists of a CRISPR repeat-spacer array and the Cas nuclease, which cleaves foreign viral DNA. The Cas9 nuclease, together with a single guide RNA (sgRNA), induces targeted and efficient double-strand breaks (DSBs) in the DNA. CRISPR/Cas9 is a relatively inexpensive system and offers means for broad application in various fields due to its simplicity and efficiency [6]. Mammalian cells were first subjected to gene editing using CRISPR/Cas9 in 2013, and by 2014 the technology was applied to pigs. Since then, CRISPR/Cas9 has found applications in livestock research, including in pigs [7].

Modification of specific genes to enhance the productive characteristics and economically beneficial properties of pigs. The demand for high-quality, low-fat pork is growing worldwide. The CRISPR/Cas9 system serves as a valuable tool for improving the quality of pork through gene manipulations aimed at increasing muscle mass and reducing fat tissue in commercial pigs. One of the achievements in molecular breeding

is pigs with a knockout of the myostatin (MSTN) gene. The MSTN gene inhibits the development of skeletal muscles, making it a suitable target to increase the muscle mass of livestock. Numerous studies have documented an increase in muscle mass with a reduction in fat tissue in pigs with the MSTN gene knockout, resulting in high-quality lean pork. However, a problem encountered with European commercial breed pigs with the MSTN knockout was the low survival rate of piglets after birth. In contrast, Chinese pig breeds carrying homozygous MSTN gene mutations exhibited good survivability. The second obstacle to creating transgenic pigs with a deficiency of the MSTN gene was the biological risks associated with selectable marker genes, such as the green fluorescent protein (EGFP) gene. Chinese scientists hypothesized that modifying the non-coding regions of the MSTN gene could be beneficial in promoting muscle development without significantly affecting the expression of MSTN and its related biological functions [8].

The insulin-like growth factor 2 (IGF2) gene has been successfully used as a regulator of muscle development in Chinese pig breeds [9, 10]. This gene activates a cascade of signaling pathways that regulate cellular proliferation, differentiation, and apoptosis during both intrauterine and postnatal growth. The transcription and expression of the IGF2 gene are suppressed by the ZBED6 protein, which contains a BED domain with zinc fingers. ZBED6 is a transcription factor in mammals that can combine with the IGF2 gene, increasing its expression level, thereby reducing subcutaneous fat deposition [11]. It's shown that this transcription factor is closely associated with muscle growth and development and can inhibit the formation of muscle tubes during cell differentiation. Xiang G. et al. [9] were the first to demonstrate the improvement of the economic traits of livestock by genetically modifying non-coding regions in the genome. Mutations in the intron of the IGF2 gene significantly improved the meat productivity of the Bama pig breed [10].

Pigs are susceptible to various diseases in cold weather. Throughout evolution, these animals have almost lost the key element of non-shivering thermogenesis - the thermogenin protein (UCP1). Rodent studies have shown that mice have the UCP1 protein, and they use brown fat to maintain internal body temperature through non-shivering thermogenesis [12]. A group of Chinese scientists led by Q. Zheng demonstrated the possibility of creating a UCP1 gene knockout in pigs via CRISPR/Cas9-mediated insertion of mouse UCP1 into the pig's endogenous locus [13]. Mating of the male obtained in this way with wild-type females confirmed the Mendelian segregation rule of transgenes among the F_1 offspring and also showed no impact of CRISPR/Cas9 elements on fertility. The resulting pigs exhibited improved thermoregulation and a reduction in the deposition of white adipose tissue, a priority in some countries' pig breeding programs. It's also reported that a total of 2,553 cloned embryos were transferred to the oviducts of 13 surrogate recipients. Three pregnancies were established, which reached term. Twelve male piglets were born naturally from three litters [14].

Adipose tissue performs various physiological functions, including storing excess energy as fat, protecting internal organs from physical impact, retaining heat, and secreting adipokines. Due to their well-developed adipose tissue, pigs are considered an ideal model for studying adipogenesis. A group of scientists has presented new data on fat deposition and a fat tissue-specific promoter element in genetically modified pigs. The galectin 12 gene (LGALS12) showed the highest specificity in pig adipose tissue. However, literature on the pig LGALS12 gene is scarce. According to bioinformatic analysis, five truncated fragments of the LGALS12 promoter were cloned. A 4 bp fragment (L-4 bp) exhibited promoter activity specific to adipose tissue. In these studies, it was shown that L-4 bp could control the expression of the apolipoprotein E (APOE) gene to perform its function in adipocytes. This data confirms the idea that LGALS12 is a candidate gene for genetic improvement of obesity-related traits in pigs [15].

Chinese scientists have used the CRISPR/Cas9 technology to create pigs with a knockout of the von Willebrand factor (vWF) gene. In humans, mutations in this gene cause Willebrand's

disease, which is characterized by spontaneous bleeding. To endow pigs with an economically beneficial trait, which entails active bleeding of the animal after slaughter, the authors introduced genetic constructs using cytoplasmic injection of CAS9 mRNA and shRNA (small hairpin RNA) into zygotes. This was followed by transplantation to surrogate sows, leading to the desired deletion in the vWF gene area in 62% of the piglets born. From a perspective of creating a large animal model for studying human genetic diseases, this research is useful and promising. However, from a food safety perspective, a problem arises: out of 76 embryos injected into five surrogate sows, only three pregnancies were successful. Only 16 piglets were born (2 of which died after birth), and only 10 had the economically valuable gene deletion [15]. It seems that these animals would have required special care, which would increase the cost of their maintenance, casting doubt on the profitability of the obtained beneficial trait.

Modification of specific genes to enhance pig resistance to infectious diseases. Susceptibility to infectious diseases in animals is one of the most serious problems in livestock farming. Many viral infections are associated with secondary bacterial infections, significantly contributing to the use of antimicrobial drugs in agriculture. The use of CRISPR/Cas9 is crucial for producing pigs resistant to coronaviruses. Coronaviruses are single-stranded RNA viruses found worldwide. They include viruses such as transmissible gastroenteritis (TGEV), porcine epidemic diarrhea virus (PEDV), and porcine deltacoronavirus (PDCoV) [16]. These coronaviruses cause significant losses in pig farming, as they result in high mortality rates in piglets due to malabsorptive diarrhea and dehydration. Research using CRISPR/Cas9 has confirmed that the aminopeptidase protein N (APN), present on the surface of intestinal villi, is the primary receptor for establishing a transmissible gastroenteritis infection in pigs. Newborn piglets with an APN deficiency are resistant to TGEV infection. However, an APN deficiency does not provide protection against PEDV infection [17]. Thus, there's an urgent need to identify the recognizing receptor for PEDV.

One of the essential receptors for infecting with the porcine reproductive and respiratory syndrome virus (PRRSV) is the cluster of differentiation antigen 163 (CD163). The CD163 protein performs several critical biological functions, including the recycling of hemoglobin/haptoglobin. CD163 has nine extracellular domains on the surface of monocytes and macrophages, and the virus specifically interacts with the fifth domain. By deleting exon 7, which encodes the entire fifth domain, it was possible to obtain expressing a modified protein CD163 that retains these functions. Pigs with knockout of the CD163 gene proved to be resistant to the PRRS virus [18]. However, the PRRSV virus has two forms: PRRSV-1 (European) and PRRSV-2 (Asian). PRRSV-1 and PRRSV-2 CD163 use different CD163 sites, so further research is needed.

Researchers aim at producing pigs with multiple resistances to viral pathogens. Oh J. et al. [19] developed a multi-resistance strategy for pseudorabies and PRRS using CRISPR/Cas9-mediated deletion of the CD163 gene and the integration of a small hairpin shRNA in pig fibroblasts. The integrated shRNA targets genes of the pseudorabies virus and PRRSV. However, pigs with dual resistance to the pseudorabies virus and PRRSV still need testing, as further *in vivo* studies are required.

Guo C. et al. [20] performed a specific deletion of a fragment from 41 amino acids containing a lipopolysaccharide-binding protein (LBP) in the cysteine-rich area phagocytic receptor 5 (SRCR5) CD163 in two breeds of pigs (small spotted Liang-Guang and Large White pigs). Then, the Large White pigs with modified genes in the F_0 generation were used for virus infection. These pigs with an edited genome were resistant to the porcine reproductive respiratory syndrome virus 2 (PRRSV 2).

Classical swine fever (CSF) is a viral disease in pigs characterized by fever, damage to blood vessels and hematopoietic organs, and diphtheritic inflammation of the mucous membranes of the colon. It causes tremendous economic damage to farms, with a mortality rate of 80-100%. The CSF virus induces immunosuppression, predisposing domestic and wild boars to sec-

ondary opportunistic infections of the gastrointestinal and respiratory systems. The use of CRISPR/Cas9 combined with RNA interference resulted in transgenic pigs resistant to the CSF virus. Through a CRISPR/Cas9 knock-in strategy, a specific antiviral shRNA was inserted into the Rosa26 locus of the pig to degrade the RNA of the CSF virus. Infection results in these pigs showed a significant reduction in CSF replication, clinical symptoms, mortality, and the transmission of CSF resistance to the first-generation piglets [21]. The Radical S-adenosylmethionine domain-containing protein 2 (RSAD2) is a cellular protein that exhibits broad antiviral activity against DNA and RNA viruses. Due to the broad antiviral activity exhibited by the RSAD2 gene, it was considered a potential candidate for CRISPR/Cas9 knock-in for developing virus-resistant transgenic pigs. The specific introduction of the RSAD2 gene into the pig's ROSA26 locus allowed for the creation of transgenic pigs, which exhibited a reduction in ASF and pseudorabies virus (PRV) upon infection [21]. Consequently, using several critical genes may lead to the emergence of multi-resistant pigs. However, limited knowledge of the interaction of these genes with receptors in many viral diseases may partially hinder the widespread adoption of this strategy.

Consideration of off-target effects. The off-target effect, which represents unintended DNA changes in non-target gene sites, poses a major problem for CRISPR/Cas9-mediated formation of genetically modified organisms (GMOs). Adverse changes can affect not only the phenotype of the parent animal but also the phenotype of subsequent generations. Off-target effects can be assessed by genome sequencing.

CONCLUSION

CRISPR/Cas9 generates significant public interest within the global community, but there are opposing views that will likely vary considerably depending on societal perspectives, including wealth, age, and religion of people. This affects the development and speed of implementation of these innovations in the agriculture of individual countries. Currently, China is the leader in the development of genome editing in the field of pig breeding. In Russia, a genetic technology development program is being implemented, scheduled for 2019-2027. The primary goal of the program is a comprehensive solution to the challenges of accelerated development of genetic technologies, including genetic editing technologies. In the near future, we can expect the emergence of the promising Russian developments in genome editing of various farm animals, including pigs. It should be acknowledged that gene editing can also cause unexpected side effects. Therefore, researchers must plan their experiments carefully. However, overall, the CRISPR/Cas9 system is being refined, becoming more accessible for use, offering unlimited possibilities and prospects for broader application in the future.

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