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Chapter

Application and Development of CRISPR/Cas9 Technology in Pig Research

Huafeng Lin, Qiudi Deng, Lili Li and Lei Shi

Abstract

Pigs provide valuable meat sources, disease models, and research materials for humans. However, traditional methods no longer meet the developing needs of pig production. More recently, advanced biotechnologies such as SCNT and genome editing are enabling researchers to manipulate genomic DNA molecules. Such methods have greatly promoted the advancement of pig research. Three gene editing platforms including ZFNs, TALENs, and CRISPR/Cas are becoming increasingly prevalent in life science research, with CRISPR/Cas9 now being the most widely used. CRISPR/Cas9, a part of the defense mechanism against viral infection, was discovered in prokaryotes and has now developed as a powerful and effective genome editing tool that can introduce and enhance modifications to the eukaryotic genomes in a range of animals including insects, amphibians, fish, and mammals in a predictable manner. Given its excellent characteristics that are superior to other tailored endonucleases systems, CRISPR/Cas9 is suitable for conducting pig-related studies. In this review, we briefly discuss the historical perspectives of CRISPR/Cas9 technology and highlight the applications and developments for using CRISPR/Cas9-based methods in pig research. We will also review the choices for delivering genome editing elements and the merits and drawbacks of utilizing the CRISPR/Cas9 technology for pig research, as well as the future prospects.

Keywords: applications, CRISPR/Cas9, delivery methods, gene editing, pig

1. Introduction

1.1 The status of pig production and current application of CRISPR/Cas9 technology

Worldwide, pig (*Sus scrofa domestica*) production accounted for 42% of total livestock production in 2018, and this percentage is expected to go up by the year 2050 [1, 2]. Pork, which makes up nearly 40% of all meat consumed by the world population, is clearly an important meat source for humans [3]. These production and consumption data reveal the significant implications of pigs for humans. Indeed, pigs bring many benefits for the convenience and survival of human beings. In light of the importance and necessity for pig production, researchers all around the world are using various methods to actively investigate this species.

Benefitting from the rapid development of genome-editing technologies during the last decade, many laboratories have applied this tool to animals, plants, and microorganisms in order to obtain both higher yield and better quality varieties. With the advent of the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 technique and the melioration of delivery methods, gene editing can be more successfully performed in livestock such as swine. In addition, evidence shows that, in addition to primates, pigs share many similar characteristics with humans such as organ size, genome length, blood glucose levels, and the complexity and composition of chromosomes [4, 5], as well as the early embryonic development trajectory [6]. Therefore, pigs are not only used as important domestic animals for food and pharmaceutical applications, but also served as ideal animal models for simulating various human diseases (e.g., diabetes, obesity, and cardiovascular disease). In this manuscript, we first introduce the historical perspectives of gene-editing technologies in pigs, review the latest advances in the utilization of CRISPR/Cas9 strategies for swine research, and then describe possible methods for delivering these genome-editing components, as well as the future perspective on pig studies by using this technology.

1.2 Historical background of gene editing in pigs

CRISPR, discovered in 1987, is a family of DNA sequences of short direct repeats interspaced with short sequences. Its mechanism of action has been confirmed to be related with acquired immunity of microbes [7–9]. By 2000, researchers had discovered that these specific sequences occurred in about 40% of bacteria and 90% of archaea [10, 11]. In 2002, this interesting architecture, initially named short regularly spaced repeats (SRSRs), was renamed as the clustered regularly interspaced short palindromic repeats (CRISPRs) [10, 12]. Between 2002 and 2009, a series of proteins associated with these palindromic sequences were identified as constituents of the complicated mechanism of microbial adaptive immunity [11]. In 2014, the X-ray crystal structure of *Streptococcus pyogenes* Cas9 (*Sp*Cas9) in complex with sgRNA was elucidated [13, 14]. Nowadays, *Sp*Cas9 endonuclease, which requires a protospacer adjacent motif (PAM) sequence (5'-NGG-3′), is routinely designed as a 'molecular scissor' guided by a single guide RNA (or dual-tracrRNA) due to simple structural characteristics, the advantages of easy operation, and high efficiency [11, 15]. Notably, the multiplex abilities of the Cas9-associated guided RNAs (gRNAs) and the diverse Cas9 orthologs (e.g., *Sp*Cas9, *Sa*Cas9, *St*Cas9) as well as the diversified Cas9 variants (**Figure 1**) have enabled CRISPR/Cas9 systems to be used in a wide range of research applications [16, 17].

As early as 1985, the first transgenic pig was created by direct DNA microinjection of the metallothionein-I/human growth hormone (MT/hGH) fusion gene into a fertilized egg [18]. Further technical enhancements occurred during the next 20 years, until, in 2011, Whitworth and his co-workers were the first to successfully apply ZFN technology to generate cloned eGFP knockout pigs [19]. Similarly, Carlson et al. (2012) pioneered the application of TALENs in editing the porcine genome, and they produced low-density lipoprotein receptor (LDLR) knockout pigs [20]. By 2013, the groundbreaking work of genome engineering in mammalian cells based on the CRISPR/Cas9 system had been achieved [21]. The first examples of genome-modified pigs engineered using the CRISPR/Cas9 technique were reported almost simultaneously by Hai et al. (2014) [22] and Whitworth et al. (2014) [23]. From then on, rapid and efficient CRISPR/Cas9-mediated genome editing in pigs has opened up many more possibilities for applications in biology and biomedicine.

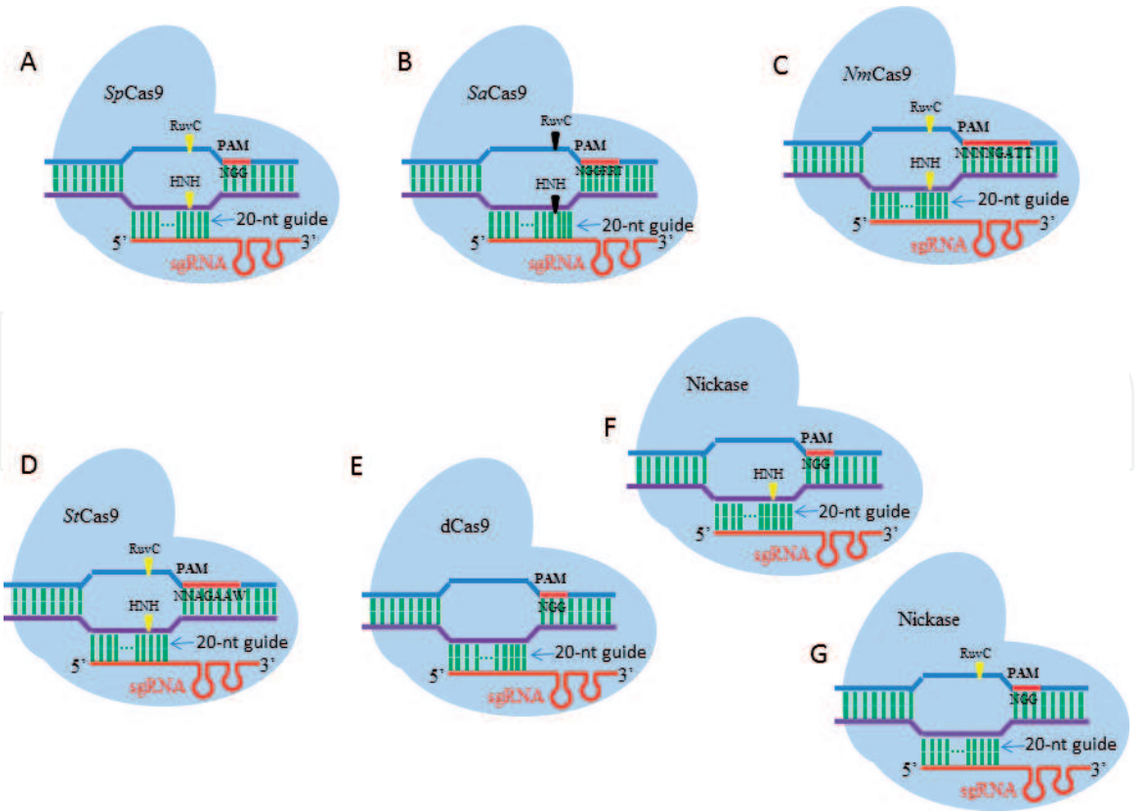

Figure 1.
Diagram illustrating different types of engineered CRISPR/Cas9 and its Cas9 variants. (A) The wild-type SpCas9 nuclease. (B) The wild-type SaCas9 nuclease. (C) The wild-type NmCas9 nuclease. (D) The wild-type StCas9 nuclease. (E) The dCas9 variant can bind DNA but cannot cut DNA strands. (F) The SpCas9 nickase that can only introduce a single strand break at the HNH nuclease domain. (G) The SpCas9 nickase that can only introduce a single strand break at the RuvC nuclease domain. (SpCas9, Streptococcus pyogenes Cas9; SaCas9, Staphylococcus aureus Cas9; NmCas9, Neisseria meningitides Cas9; StCas9, Streptococcus thermophilus Cas9; dCas9, catalytically inactive ("dead") Cas9; sgRNA, single-guide RNA; PAM, protospacer adjacent motif; W = A or T). Refer to [16].

2. Application and development

2.1 Applications in the antimicrobial and antiviral fields

Currently, the traditional methods for developing pig anti-viral vaccines are time-consuming and labor-intensive [24]. Cas9 endonucleases, as molecular DNA scissors guided by gRNA, are now used to target and cut exogenous DNA arising from virus or plasmids [25]. With the development of state-of-the-art biotechnologies, scientists now can utilize this revolutional tool to prevent domestic pigs from pathogenic bacterial and viral attack. In 2016, Liang and his colleagues developed a rapid vaccine development method based on the combination of CRISPR/Cas9 and the Cre/Lox system to fight against the re-emerging pseudorabies virus (PRV). The results demonstrated the protective efficacy of this candidate vaccine in swine and showed promise in controlling the outbreak of pseudorabies [26]. In another trial, Whitworth et al. (2015) employed the CRISPR/Cas9 system to directionally mutate the CD163 gene (cluster of differentiation 163 gene, a gate keeper gene associated with PRRSV) in order to create biallelic gene knockout pigs which had protective immunity against infection of porcine reproductive and respiratory syndrome virus (PRRSV) [27]. In 2018, Xie and his co-workers applied the combinational method of CRISPR/Cas9 and RNAi to generate anti-CSFV transgenic pigs and confirmed that these pigs could impede the multiplication of classical swine fever virus (CSFV). They further proved that the disease resistance traits presented in the

transgenic sows could be stably transmitted to their F1-generation offspring. This study suggested that the use of such transgenic pigs would offer potential benefits over commercial vaccination, could substantially reduce CSFV-related economic losses, and would also improve the well-being of livestock [28]. Compared to CSFV, African swine fever virus (ASFV) is a very acute, lethal viral pathogen for both domestic and wild pigs, but unfortunately, a vaccine candidate that effectively prevents ASFV infection remains elusive. HüBner et al. (2018) applied the CRISPR/Cas9 nuclease system to target the double-stranded DNA genome of ASFV. In vitro culture experiments showed that mediated targeting of the ASFV p30 gene using this system is a feasible strategy to fight against ASFV infection, and may also be applied to the natural animal host [29].

2.2 Applications to breeding and reproduction

Traditional breeding methods, which comprise selective breeding and cross-breeding, have clearly hit a bottleneck. Additionally, due to the long time, high cost, and high labor intensity of traditional breeding methods [30], researchers now hope to find other alternatives that are more convenient and efficient than previously. Genome-editing technology can help us to achieve a good result in a short time, and help better understand swine reproduction. Interestingly, many aspects of pig reproduction are suitable as translational models of reproduction in humans, including oocyte maturation, sperm-egg interaction mechanism, tubo-uterine contractility, early embryo development, pregnancy, fetal genome modification, and reproductive diseases [31]. Strategies that use the CRISPR/Cas9 technique to improve the reproduction in swine are becoming more prevalent. PRRSV, a virus associated with reproductive and respiratory disease, can cause severe unsuccessful reproductive outcomes in sows, decrease sperm quality in infected boars, and lower the birth rates of healthy piglets [32]. In 2016, Tao et al. generated efficient biallelic mutation in porcine parthenotes by cytoplasmic injection of Cas9/sgRNA mixtures. These data emphasize the function of parthenotes in revealing early embryonic development and assessing mutation efficiency [33]. In the same year, Whitworth et al. used CRISPR/Cas9 to generate CD163-knockout pigs to protect pig from PRRSV and reduce the incidence of reproductive disease, important for pig studies in both the fields of reproduction and anti-viruses [27]. In 2017, Park et al. utilized CRISPR/Cas9 technology to program the NANOS2 gene in domestic pigs to generate offspring with monoallelic and biallelic mutations. They found that NANOS2 knockout pigs presented the phenotype of male specific germ line ablation but other aspects of testicular development were normal. The exception was male pigs with one intact NANOS2 allele and female knockout pigs which both maintained good reproductive performance [34].

2.3 Applications in immunization and xenotransplantation

Swines, having many highly similar anatomical and physiological features to humans, are considered to be the excellent donors for patients in the case of a shortage of human organs for allogenic transplantation [35, 36]. However, several issues still need to be addressed such as hyperacute rejection which can develop in recipients within several minutes after organ xenotransplantations [36, 37]. The advancement of the CRISPR/Cas9 technique has greatly strengthened the ability to effectively manipulate porcine genome in order to evaluate and generate porcine organs that can assist in xenotransplantation.

An early study, undertaken by Sato and his research team in 2013, used a modified CRISPR/Cas9 system to knockout the porcine GGTA1 gene, whose protein

product is responsible for the biosynthesis of the a-Gal epitope, which leads to hyperacute rejection upon pig-to-human xenotransplantation. This trial not only demonstrated that CRISPR/Cas9 is a promising tool for producing knockout cloned piglets, but also paved the way for pig-to-human xenotransplantation [38]. Piglets with biallelic knockouts of GGTA1 gene were eventually created by Petersen and his colleagues [39] using the combined technologies of CRISPR/Cas9 and somatic cell nuclear transfer (SCNT).

Swine could also serve as an ideal animal model for investigating viral immunity and immune rejection in xenotransplantation if they are deficient in class I MHC. Research published by Reyes et al. in 2014 utilized the Cas9 endonuclease with chimeric gRNAs to generate class I MHC knockout piglets as promising experimental animals for immunological research [40]. In 2015, Yang and coworkers designed two Cas9 gRNA molecules to inactivate 62 copies of the pol gene required for porcine endogenous retrovirus (PERV) activity. This study performed on porcine kidney epithelial cell lines demonstrated that the modifications could greatly reduce in vitro spreading of PERVs to human cells, raising the hope of the eradication of such viruses from pigs for heterograft donors [41]. One year later, Yang's research team (2017) made further progress in employing CRISPR/Cas9 technology to inactivate all the PERVs in a porcine primary cell line and produced PERV-eliminated pigs using the SCNT technique. The experimental results addressed the safety problem in clinical xenotransplantation due to the success of impeding interspecific transmission of viruses [42].

2.4 Disease models and translational medical research

The CRISPR/Cas9 technology has both simplified and expedited biomedical modeling for some refractory human diseases. One way to combat human diseases is to create genetically modified animal models for investigating the mechanism of diseases enabling the development of safe and effective drugs. An effective animal disease model should appropriately simulate the *in vivo* environment under investigation and respond or react to stimuli in a similar manner to the human body [43–45]. Commonly used animal models in the laboratory include mice, rats, dogs, monkey, and swine. The pig models have been developed to faithfully mimic various human diseases including neurodegenerative diseases [46], cancers [45], and gastrointestinal (GI) tract diseases [47] as they share similar features to humans in terms of anatomy, physiology, and genetics [43]. Gene editing using CRISPR/Cas9 technology is proving an innovative and effective research tool, which is greatly revolutionizing our ability to manipulate the porcine genome to create appropriate disease models.

As early as 2013, Tan et al. used two custom endonucleases (TALEN and CRISPR/Cas9 system) to edit azoospermia-like (DAZL) and adenomatous polyposis coli (APC) loci in the pig genome. The results suggested that gene editing could be incorporated into selection programs to accelerate genetic improvement, with applications in animal breeding and human personalized medicine [48]. In 2014, Zhou et al. were the first to report that zygote injection of a customized CRISPR/Cas9 system could efficiently generate genome-modified pigs (biallelic knockout pigs) in one step, which provided an important animal model for the treatment of human type I and III *von* Willebrand disease [22]. At the end of 2015, Peng et al. adopted the CRISPR/Cas9 method to knockin human cDNA into the albumin gene locus in pig zygotes and successfully produced human albumin from porcine blood [49]. Additionally, Feng et al. (2015) reported the potential of using the combination of CRISPR/Cas9 and human pluripotent stem cells (PSCs) to harvest human organs from chimeric swine [50]. In 2016, Wang et al. performed a study in

which Cas9 mRNA and multiple single guide RNAs (sgRNAs), which respectively specifically target to parkin, DJ-1, and PINK1 gene loci, were coinjected into in vivo derived pronuclear embryos of Bama miniature pigs. There were only minor low off-target events. These results demonstrated the capability of using the CRISPR/Cas9 system to trigger genetic modification of multiple sites in pigs, yielding positive results with high medical value [51]. In the same year, Lee and his team utilized genome-specific CRISPR/Cas9 systems to target runt-related transcription factor 3 (RUNX3, a known tumor suppressor gene) to generate a pig model that can recapitulate the pathogenesis of RUNX3-associated stomach cancer in humans. The results demonstrated that the CRISPR/Cas9 system was effective in inducing mutations on a specific locus of the pig genome, resulting in the generation of piglets lacking RUNX3 protein in their internal organs. This system brings useful resources (RUNX3 knockout pigs) for human cancer research and the development of novel cancer therapies [52]. In 2017, Zhang et al. designed an experiment that applied the CRISPR/Cas9 system and SCNT technology to generate complement protein C3 targeted piglets, which could be a valuable large animal model for elucidating the roles of C3, a protein of the immune system that plays a central role in the complement system and contributes to innate immunity [53]. By 2018, following many years' efforts, scientists have now made significant progress in using CRISPR/Cas9-mediated knockin techniques to produce a Huntington's disease (HD) pig model, which assists in the investigation of the pathogenesis of neurodegenerative diseases and the development of appropriate therapeutics [54]. Recently (2018), Cho and co-workers successfully used the CRISPR/Cas9 and SCNT technologies to generate INS knockout pigs (insulin-deficient pigs) and demonstrated the efficacy of the CRISPR/Cas9 system in producing pig models for use in diabetes research and pharmaceutical testing [55].

2.5 Improvement of meat quality and food safety

Pig meat quality is controlled by multiple factors. To some extent, genetics are considered as the dominating factor influencing pork quality in the pig industry, although environmental conditions can also potentially influence the porcine genetics in the long term. In addition, fat and lean meat contents are both important for the palatability of the pork [56, 57] and diet considerations. Consequently, scientists now propose to improve pork traits to cater for the taste of the general public by using gene-editing technology. In 2016, Bi et al. constructed isozygous, functional myostatin (MSTN) knockout cloned pigs without selectable marker gene (SMG) by combined use of CRISPR/Cas9 and Cre/LoxP. The results showed that compared to the control group, the skeleton muscles were more pronounced and the back fat thickness decreased slightly in such gene-edited pigs [58]. In 2017, Zheng et al. established a CRISPR/Cas9-mediated homologous recombination-independent approach to efficiently insert mouse adiponectin-UCP1 into the porcine endogenous uncoupling protein 1 (UCP1) locus. The resultant UCP1 knockin pigs showed an enhanced ability to control their body temperature during acute cold exposure, lower fat deposition, and increased carcass lean meat [59]. In 2018, Xiang et al. used CRISPR/Cas9 technology to effectively edit insulin-like growth factor 2 (IGF2) intron 3–3072 site as the method of choice for the improvement of meat production in Bama pigs. The result showed that it was the first time to demonstrate that editing a noncoding region can ameliorate economic traits in livestock [60].

CRISPR/Cas9 gene-editing technology has multiple benefits. In gene detection fields, Zhou et al. developed a CRISPR/Cas9-triggered nicking endonuclease-mediated strand displacement amplification method (namely CRISDA) for amplifying and detecting double-stranded DNA [61]. CRISDA promises to be a

Authors/year/refs	Cells/organisms	Genomic loci	CRISPR/ Cas9 delivery platforms	Gene-editing modes	CRISPR/Cas9 formats	Comments/results
Hai et al., 2014, [22]	Zygote	*v*WF	Cytoplasmic injection	Knockout	Cas9 mRNA and sgRNA	Constructed pig disease modes using CRISPR/Cas9
Sato et al., 2014, [38]	PEFs	GGTA1	Plasmids/ transfection	Knockout/ CNT	CRISPR/Cas9 plasmids DNA and sgRNA	Efficiently mutated portion of GGTA1
Whitworth et al., 2014, [23]	PFF cells	eGFP/CD163/CD1D	Plasmids/ transfection/ microinjection	Knockout	Cas9 plasmids DNA and sgRNA	Generated GE pigs for mutating two genes
Chen et al., 2015, [68]	PFFs	J_H	Plasmids/ transfection/ electroporation	Knockout/ SCNT	Cas9-sgRNA plasmids	Generated a B cell-deficient phenotype in pig
Li et al., 2015, [69]	Liver-derived cells	GGTA1/CMAH/ iGB3S	Plasmids/ transfection	Knockout/ SCNT	Cas9 plasmids and multiplexed sgRNA	Modified multiple genetic in a single pregnancy
Peng et al., 2015, [49]	Zygotes	Alb	Microinjection	Knockin	Cas9 mRNA and sgRNA	Knockined Alb gene and produced albumin in the blood of piglets
Ruan et al., 2015, [63]	PFFs	pH11	Plasmids/ electroporation	Knockin	Cas9/sgRNA targeting plasmids	Inserted foreign gene into the pH11 locus
Wang et al., 2015, [70]	Oocytes/PPFs	MITF	Microinjection	Knockout/ knockin	Cas9 mRNA and sgRNA	Expanded the practical possibilities in pigs
Zhou et al., 2015, [64]	PFFs	TYR/PARK2 /PINK1	Plasmids/ transfection	Knockout/ SCNT	Cas9 plasmids and sgRNA	Gene-targeted pigs can be effectively achieved
Kang et al., 2016, [52]	PFFs	RUNX3	Plasmids/ transfection/ electroporation	Knockout	Cas9-sgRNA plasmids	Generated pig disease mode for cancer research
Petersen et al., 2016, [39]	Oocytes	GGTA1	Intracytoplasmic microinjection	Knockout	(Cas9 and sgRNA) expression DNA	GGTA1 knockout pigs could bring xenotransplantation closer to clinical application

Authors/year/refs	Cells/organisms	Genomic loci	CRISPR/ Cas9 delivery platforms	Gene-editing modes	CRISPR/Cas9 formats	Comments/results
Wang et al., 2016, [51]	Zygotes	parkin/DJ-1/PINK1	Co-injection	Knockout	Cas9 mRNA and multiplexing sgRNAs	Modified multiple genes in pigs
Yang et al., 2016, [65]	PFFs	pINS	Plasmids/ electroporation	SCNT	Cas9 plasmids/sgRNA	Generated the genetically modified pigs exclusively expressing human insulin
Yu et al., 2016, [73]	Zygotes	DMD	Plasmids/ microinjection	Knockout	Cas9 mRNA and sgRNA	Targeted of DMD gene in miniature pig
Chuang et al., 2017, [71]	Fertilized eggs	GGTA1	Plasmids/ microinjection	Knockout	CRISPR/Cas9 plasmids DNA	Firstly used porcine U6 promoter to express gRNA to generate GGTA1 mutant pigs with PBMCs
Gao et al., 2017, [74]	PFFs	GGTA1/CMAH	Plasmids/ handmade cloning (HMC)	Knockout	Cas9-coding DNA and sgRNA	Modified multiple genes in pigs
Huang et al., 2017, [75]	PEFs	ApoE/LDLR	Plasmids/ electroporation	Knockout/ SCNT	(Cas9 and sgRNA) expression DNA	Generated genetically modified pigs targeting the ApoE and LDLR genes simultaneously
Li et al., 2017, [76]	Oocytes/PFFs	TPH2	Plasmids/ electroporation	Knockout/ SCNT	(Cas9 and sgRNA) expression DNA	Tph2 targeted piglets were successfully generated
Park et al., 2017, [34]	Oocytes	NANOS2	Plasmids	Knockout	Cas9:GFP mRNA and sgRNA	Edited the NANOS2 gene to generate germline ablated male pigs
Whitworth et al., 2017, [72]	Zygote	TMRPSS2	Plasmids/ microinjection	Mutation	sgRNA and Cas9 mRNA	Successfully modified the target gene
Wu et al., 2017, [77]	Oocytes	PDX1	Microinjection	Knockin	Cas9 mRNA and dual sgRNAs	Xeno-generated of human tissues and organs in pigs
Zheng et al., 2017, [59]	FFAs	UCP1	Plasmids	Knockin	Cas9-sgRNA plasmids	Improves pig welfare and reduces economic losses
Borca et al., 2018, [78]	Primary swine macrophage	8-DR	Plasmids	Targeted deletion	Cas9 plasmids/sgRNA	Used CRISPR-Cas9 system to produce recombinant ASFVs

Authors/year/refs	Cells/organisms	Genomic loci	CRISPR/ Cas9 delivery platforms	Gene-editing modes	CRISPR/Cas9 formats	Comments/results
Cho et al., 2018, [55]	Porcine primary fibroblasts	INS	Plasmids/ electroporation	Knockout	Cas9:GFP mRNA and sgRNA	Demonstrated effectiveness of CRISPR/ Cas9 in generating new pig models
Hübner et al., 2018, [29]	ASFV-permissive WSL cells	CP204L	Plasmids	Targeted deletion	(Cas9 and sgRNA) expression DNA	CRISPR/Cas9 mediated targeting of the ASFV p30 gene is a valid strategy to convey resistance against ASF infection
Santos et al., 2018, [79]	Pig aortic endothelial cells	pTHBD	Plasmids	Knockout/ recombination	(Cas9 and sgRNA) expression DNA	Create pigs with human genes in orthotopic position (hTHBD was inserted into the pTHBD locus)
Sato et al., 2018, [80]	zygote	GGTA1	Microinjection	Knockout	Cas9 mRNA and sgRNA; plasmid encoding humanized Cas9 and sgRNA	Developing a technique that reduces mosaicism is a key factor for production of knockout pigs
Xie et al., 2018, [28]	Porcine kidney cell/PFFs	Porcine ROSA26	Plasmids/ electroporation	Knockin/ SCNT	(Cas9 and sgRNA) expression DNA	Successfully produced anti-CSFV pigs
Yan et al., 2018, [54]	PFFs	HTT	Plasmids/ electroporation	Knockout/ SCNT	(Cas9 and sgRNA) expression DNA	First time to produce HD pig models for investigating the pathogenesis of neurodegenerative diseases
Yang et al., 2018, [81]	PFFs	CD163	Plasmids/ electroporation	Knockout/ SCNT	(Cas9 and sgRNA) expression plasmids	Demonstrated that CD163 knockout confers full resistance to HP-PRRSV infection to pigs

Acronyms and abbreviations: apolipoprotein E (ApoE); albumin (Alb); cysteine-rich domain 163 (CD163); CMP-Neu5Ac Hydroxylase (CMAH); duchenne muscle dystrophy (DMD); huntingtin (HTT); insulin (INS); microphthalmia-associated transcription factor (MITF); pancreatic duodenal homeobox-1 (PDX-1); porcine aortic endothelial cells (pAECs); porcine fetal fibroblasts (PFFs); pig embryonic fibroblast cells (PEFs); PTEN-induced kinase 1 (PINK1); Huntington's disease (HD); runt-related transcription factor 3 (RUNX3); thrombomodulin (THBD); tryptophan hydroxylase 2 (TPH2); transmembrane protease, serine 2 (TMPRSS2); tyrosinase (TYR); von Willebrand factor (vWF); wild boar lung (WSL).

Table 1. *Examples for the applications of CRISPR/Cas9 technology in pigs.*

powerful isothermal tool for ultrasensitive and specific detection of nucleic acids in pig pathogeny detection and food safety. Consequently, by making good use of this precision editing engineered technology in agriculture, a reliable avenue for elite swine production could be guaranteed, potential biological risks can be minimized, and a higher food safety can be protected.

2.6 Applications in transgenesis and beyond

Pig transgenesis is an important facet for functional investigation of biological pathways, as well as for biotechnology in animal husbandry. As a promising tool, CRISPR/Cas9 now has the ability to accelerate the process of pig transgenesis. Several studies have successfully constructed a CRISPR/Cas9 system for targeting the pig GGTA1 gene [38, 39, 62]. Ruan et al. (2015) inserted a gene fragment larger than 9 kb at the newly named pH 11 genomic locus using CRISPR/Cas9 technology and then confirmed that it was highly expressed in cells, embryos, and animals [63]. Similarly, Zhou et al. (2015) worked on CRISPR/Cas9-mediated gene targeting in porcine fetal fibroblasts (PFFs), in which TYR, PARK2, and PINK1 loci were effectively edited [64]. In 2016, Yang and colleagues edited the porcine INS (pINS) gene in fibroblasts by using TALENs or CRISPR/Cas9 [65], and in 2017, Zheng et al. inserted a mouse adiponectin-UCP1 gene efficiently into the porcine endogenous UCP1 locus by the utilization of a CRISPR/Cas9-mediated homologous recombination-independent approach [59]. In the same year, Wang et al. applied the combined system of Cre recombinase and Cas9/sgRNAs to simultaneously inactivate five tumor suppressor genes (TP53, PTEN, APC, BRCA1, and BRCA2) and activate one oncogene (KRAS) to develop a rapid lung tumor model in pigs [66]. By 2018, Whitworth et al. had developed a method that utilized the CRISPR/Cas9 technology to remove a loxP flanked neomycin cassette by direct zygote injection of RNA encoding Cre recombinase. This new technique can be used to efficiently remove selectable markers in genetically engineered animals without the need for long-term cell culture and subsequent somatic cell nuclear transfer (SCNT) [67]. Almost certainly, it has a very promising future for transgenic pigs with the advantages of enhancing body growth and minimizing environmental pollution that would be created by the CRISRP/Cas9 method. **Table 1** shows applications of CRISRP/Cas9 technology in transgenic pigs.

3. Delivery methods of CRISPR/Cas9

3.1 The appropriate choices for delivery: viral systems or nonviral platforms?

In order to introduce precise and efficient genome modification, the proper delivery modalities of CRISPR/Cas9 genome-editing materials are a crucial factor in the generation of genetically engineered pigs. A variety of strategies have been used for delivering the CRISPR/Cas9 system which can be mainly divided into viral and nonviral delivery methods (**Figure 2**) [82].

Viral systems are the traditional tools that have been widely used for delivering genome editing materials (DNA or mRNA). To-date, three viral vectors including lentivirus [83], adenovirus, and adeno-associated virus (AAV) have been used for delivery of CRISPR/Cas9 components in various biological studies [84, 85]. However, there are several limitations associated with viral vectors including immunogenicity, packaging capacity, broad tropism, and difficulty in production.

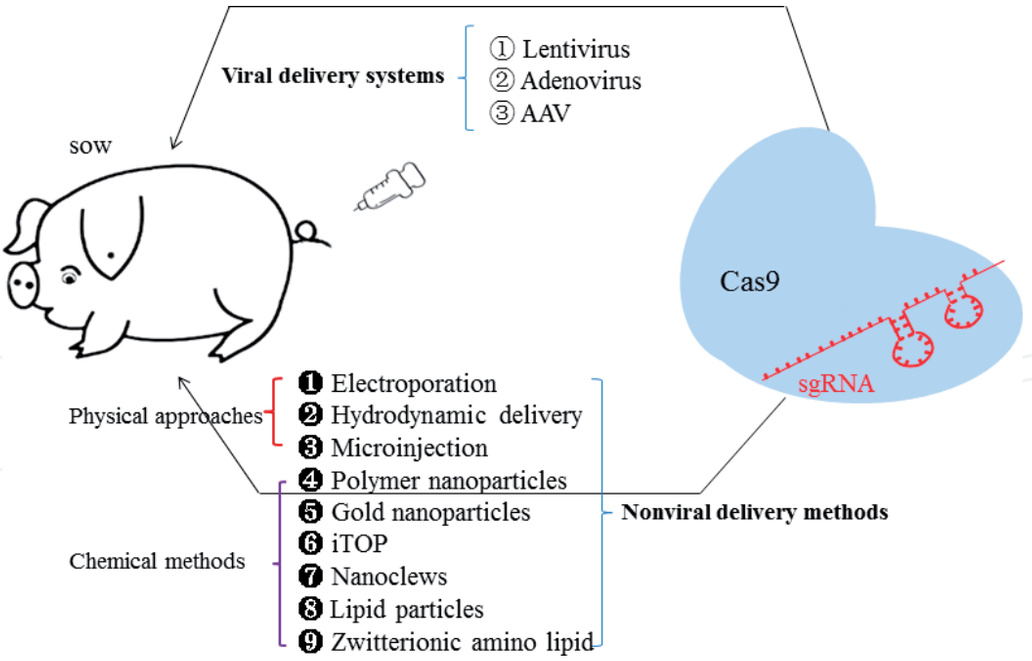

Figure 2.
Delivery techniques for the CRISPR/Cas9 system. (iTOP: induced transduction by osmocytosis and propanebetaine; AAV: adeno-associated virus).

Nonviral platforms for transferring the CRISPR/Cas9 components can be achieved by physical and chemical approaches. In contrast to viral vectors, nonviral vectors have lower immunogenicity, are not constrained by packaging sizes, are facile to synthesize, and are capable of carrying multiple sgRNAs simultaneously [86, 87]. In nonviral methods, genome editing reagents are delivered either as mRNA or as a combination of Cas9 endonuclease and sgRNA. To date, nonviral methods available include microinjection, electroporation [88], hydrodynamic injection, lipid particles, nanoclews, zwitterionic amino lipid (ZAL) nanoparticles, and iTOP as well as the combinations of viral and nonviral methods [82]. Herein, we compared the various methods for delivering the CRISRP/Cas9 system (**Table 2**).

Delivery methods of gene modification in the field of pig research have even used sperms as vectors for foreign genes (*e.g.* sperm-mediated gene transfer (SMGT), and intracytoplasmic sperm injection (ICSI)-mediated gene transfer), and delivery strategies such as retroviruses and lentiviruses are still current [100]. Somatic cell nuclear transfer (SCNT), a technique that consists of taking an enucleated oocyte and then implanting a donor nucleus from a somatic cell, is a remarkable breakthrough in the history of swine genetic engineering [101, 102]. SCNT combined with the rapid development of gene editing technologies such as TALENs and CRISPR/Cas9 has excellent prospects.

3.2 Challenges for delivering the CRISPR/Cas9 systems

The CRISPR/Cas9 system has been applied to genome modification in a variety of microorganisms, plants, and animals (including pigs), but the efficient transfer of such system is still a challenge that affects the precise genome-editing activity [103]. If the CRISPR/Cas9 systems are to effectively function in the targeted cells or organisms, choosing a suitable delivery system is of critical importance. According to existing research, the CRISPR/Cas9 system can be broadly divided into three kinds of packaging formats: Cas9 protein and sgRNA, Cas9 mRNA and

Delivery modes	Advantages	Limitations	Text refs
Lentivirus	Broad cell tropism; large capacity; long-term gene expression	Prone to insertional mutagenesis; transgene silencing; potential in carcinogenesis	[84], [89], [87], [90]
Adenovirus	High efficiency and versatility	Difficult to manufacture in scale; immunogenicity	[84], [91]
Adeno-associated virus	Minimal immunogenicity; non-pathogenic	Limited packaging size; potential to cause significant genomic damage	[14], [92], [93]
Electroporation	High transfection efficiency; suitable for all types of CRISPR-Cas9	Cytotoxicity; difficult for *in vivo* use	[94], [95]
Hydrodynamic delivery	Virus-free; easy-to-use; low-cost	Non-specific; tissue-invasive	[89], [96], [97]
Microinjection	Highly specific and reproducible	Time-consuming; suitable for *in vitro* applications; low-throughput	[94], [87]
Polymer nanoparticles	Safe; low-cost; simple manipulation; greater encapsulation capability	Low delivery efficiency	[94], [92]
Gold nanoparticles	Membrane-fusion-like delivery	Nonspecific inflammatory response; potential toxicity	[89], [98]
iTOP	Use for the delivery of Cas9 protein and sgRNA	Need to master sophisticated operating skills	[84], [89]
Nanoclews	Virus-free	Need to modify the template DNA	[99]

Table 2.
Comparison of different delivery methods for CRISPR/Cas9 system.

sgRNA, and CRISPR/Cas9 plasmid. Different CRISPR/Cas9 formats cooperate with special transport vehicle to complete the transportation task for gene-editing elements. Some research studies indicate that CRISPR/Cas9 ribonucleoprotein (RNP) delivery seems to exceed gene delivery as it provides multiple function advantages: short-term delivery, no insertional mutagenesis, minimal immunogenicity, and low off-target effect [87]. As previously mentioned, viral vectors usually have their own limitations to be overcome compared to nonviral vectors. However, nonviral vectors are generally used for *in vitro* genome editing studies due to their biological incompatibility or cytotoxicity [95]. Recently, developing efficient and biocompatible nonviral vectors (e.g., liposome and nanocarrier) has just emerged, and achievements have been made. For example, a low cytotoxic cationic polymer has been proven to mediate efficient CRISPR/Cas9 plasmid delivery for genome editing [92]. In addition, a research article presented that lipid-based Cas9 mRNA delivery has lower off-target effects than lentivirus-packaged Cas9 mRNA transportation [104]. Generally speaking, the packaging modes and delivery tools are two biggest factors that affect efficiency of the CRISPR/Cas9 system apart from this system itself. In order to describe the possible challenges for delivering the CRISPR/Cas9 system and the strategies used to overcome these challenges, we form a table to illustrate in detail (**Table 3**) and further to promote much research applications appropriately.

Challenges	Delivery methods	Strategies	Text refs
Off-target effects	Both in viral and nonviral vectors; using plasmid-based system	Engineering high specificity Cas9 protein; optimizing sgRNA design; proper selection of targeting site	[105], [94]
Packaging challenges	AAV (~4.7kpb), adenovirus, lentivirus (~10kpb)	Nonviral vectors have no packaging limitation, easy to prepare, and low in cost	[87], [106]
Insertional mutagenesis	AAV, adenovirus, lentivirus, retrovirus	Using Cas9–RNP for delivering; improved specificity	[87], [93], [107]
Mosaic genotypes	Microinjection	Stimulating the HDR pathway; use of Cas9 nickase	[108]
Immunogenicity	AAV, adenovirus, lentivirus, retrovirus	Using nonviral vectors to lower immunogenicity	[87], [95], [109]
Editing efficiency (transfection efficiency)	Nonviral vectors (not including electroporation)	Need to be further optimized; combination of viral vectors and nonviral vectors	[16], [95]
Systemic delivery	Viral and nonviral vectors	Difficult to achieve through nonviral vectors; tailoring new carriers	[16], [87]
Targeted delivery	Nonviral vectors	Viral vectors provide tissue tropism	[110]

Table 3.
Challenges for delivering the CRISPR/Cas9 system and the strategies that respond to these challenges.

4. Discussion

CRISPR/Cas9 technology is not only simple and easy to perform, but also has significantly improved performances for mutational studies, which has accelerated the application of the CRISPR/Cas9 toolkit [68, 111]. However, there are still some limitations and difficulties in the use of the CRISPR/Cas9 system for pig research.

1. The CRISPR/Cas_9 system itself is not flawless, and its off-site concerns vary in different biological species [112, 113]. In addition, if the design and construction of sgRNA are not ideal, off-target editing of the genomic DNA can easily occur. With more available datasets of CRISPR/Cas9, more newfangled tools for designing sgRNA will be developed to lower the off-target effects.

2. In pigs, complex traits associated with multiple genes enhance the difficulties of using CRISPR/Cas_9 to simultaneously and precisely edit and program DNA in the porcine genome.

3. Complex environmental factors including water sources and feed qualities, as well as animal husbandry production methods, as a range of external stimuli, could collaboratively affect CRISPR/Cas_9-derived pigs in the long-term.

4. Strategies and timing for delivering CRISPR/Cas_9 systems need to be optimized to control the ratio of HDR to NHEJ in order to enhance the efficiency of homology-directed recombination (HDR)-mediated precise gene modification [105].

5. Cytotoxicity produced by the CRISPR/Cas_9 system and toxic response to CRISPR/Cas_9 in mammalian cells has become an issue that must be taken into account. Recently, there have been reports that DSBs induced by Cas_9 triggered a P53-dependent toxic response that reduced the editing efficiency when applying the CRISPR/Cas_9 system to human programmed cells [114, 115]. Corresponding studies on pigs have not yet been undertaken, but the human studies provide some useful lessons for the development of pig research on genome editing.

6. Using the resulting fetuses or newborns edited by CRISPR/Cas_9 for screening of effective clones is time-consuming and laborious [80]. Probably, the method of T7E1 assay for detecting insertion/deletion (INDEL) mutations in blastocysts could help researchers to save time and money [80].

5. Conclusion

Over the past few years, genome-editing technology clearly allows scientists to produce genetically engineered pigs that are healthier to consume and more resistant to diseases in an efficient way. Nowadays, the use of the CRISPR/Ca9 technique on pigs in immunity, autoimmunity, obesity, aging, etc. is increasingly expanding and showing advantages over the conventional methods. In addition, another version of CRISPR named CRISPR/Cpf1 was discovered in microbes, which further expanded the CISPR toolkit, and holds promise to be applied in pig research. CRISPR/Ca9-modified pigs are providing a better perspective for understanding various aspects of pig biology and are paving the way for advancing the fields of basic biology, translational medicine, biomedicine, and drug development.

Acknowledgements

We would like to thank Professor XF Qi (Jinan University, China), Professor Edouard C. Nice (Monash University, Australia), and Professor Mark Baker (Macquarie University, Australia) for providing assistance and guidance in this project. We specially acknowledge Professor Edouard C. Nice for revising this article.

Conflict of interest

None declared.

Fundings

This work was collectively supported by grants from the Guangdong Innovative and Entrepreneurial Research Team Program (2014ZT05S136), National Key Research and Development Plan (2016YFD0500600), Guangdong Provincial Science and Technology Plan Project (2017B020207004), and Fundamental Research Funds for the Central Universities (21618309).

Acronyms and abbreviations

AAV	adeno-associated virus
APC	adenomatous polyposis coli

ASFV	African swine fever virus
CD163	cysteine-rich domain 163
CRISDA	CRISPR/Cas9-triggered nicking endonuclease-mediated strand displacement amplification
CSFV	classical swine fever virus
gRNAs	guided RNAs
HD	Huntington's disease
HDR	homology-directed recombination
ICSI	intracytoplasmic sperm injection
IGF2	insulin-like growth factor 2
LDLR	low-density lipoprotein receptor
MT/hGH	metallothionein-I/human growth hormone
MSTN	myostatin
PAM	protospacer adjacent motif
PERVs	porcine endogenous retroviruses
PFFs	porcine fetal fibroblasts
PRV	pseudorabies virus
PSCs	pluripotent stem cells
PRRSV	porcine reproductive and respiratory syndrome virus
RNP	ribonucleoprotein
SCNT	somatic cell nuclear transfer
SpCas9	*Streptococcus pyogenes* Cas9
SMG	selectable marker gene
SMGT	sperm-mediated gene transfer
SRSRs	short regularly spaced repeats
T7E1	T7 endonuclease 1
UCP1	uncoupling protein 1
ZAL	zwitterionic amino lipid

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