

Advances in gene editing strategies for viral disease resistance in pigs: Knock-out, knock-in, and overexpression approaches

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Abstract

Highly contagious viral pathogens – such as porcine reproductive and respiratory syndrome, classical swine fever, and African swine fever – continue to pose serious threats to the swine industry and cause major global economic losses. Conventional disease control approaches, such as vaccination and biosecurity, are limited by viral heterogeneity and immune escape mechanisms. Gene editing technologies, most notably the CRISPR/Cas9 system, offer an innovative means of introducing innate viral disease resistance or resilience in pigs. The continuous improvement of these tools can provide a sustainable strategy to enhance animal welfare and global food security and reduce reliance on traditional control measures. This review provides an overview of recent advances in the development of genetically engineered pigs with enhanced resistance to major viral diseases, focusing on targeted gene modifications using knock-out, knock-in, and overexpression approaches.

Keywords: ASF, CRISPR/Cas9, CSF, disease-resistant pig, gene editing, PED, PRRS

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Introduction

Pigs play a critical dual role as major agricultural livestock and valuable biomedical models worldwide (Estrada *et al.*, 2015; Niu *et al.*, 2017; Lunney *et al.*, 2021). However, the outbreaks of infectious diseases continue to pose a threat to the stability of the swine industry and consequently to global food security (Islam *et al.*, 2020). Several highly infectious viral diseases, such as African swine fever (ASF), porcine reproductive and respiratory syndrome (PRRS), classical swine fever (CSF), and porcine epidemic diarrhea (PED), are widespread and cause severe morbidity, mortality, and substantial economic losses worldwide (Yuan *et al.*, 2022). For example, PRRS is regarded as the most economically devastating swine disease in North America, Europe, and Asia, resulting in annual losses of hundreds of millions of dollars (Whitworth *et al.*, 2016). ASF, a severe hemorrhagic disease caused by a large DNA virus, causes catastrophic economic losses in endemic regions (Zhang *et al.*, 2021; Yuan *et al.*, 2022). Traditional disease control strategies—such as vaccination and rigorous biosecurity management—are increasingly constrained by several factors, such as the unavailability of effective vaccines and rapid genetic mutation of pathogens (Islam *et al.*, 2020; Tella *et al.*, 2025).

Immunogenomics provides a new conceptual framework for combating recurrent disease challenges in livestock and aims to define the genetic basis of immune responses (Tella *et al.*, 2025). The integration of immunogenomic insights with advanced gene-editing tools, such as Zinc Finger Nucleases (ZFN) (Kim *et al.*, 1996; Miller *et al.*, 2007), Transcription Activator-Like Effector Nuclease (TALEN) (Christian *et al.*, 2010), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR associated proteins 9 (CRISPR/Cas9) (Cong *et al.*, 2013; Mali *et al.*, 2013), has caused a paradigm shift in animal biotechnology. It is now possible to produce animals with heritable resistance through precise modification of host genes that mediate viral replication or susceptibility, thereby providing a durable and sustainable approach to disease control and enhancing animal welfare.

Gene Editing Tools for Generating Viral Disease-resistant Pigs

Gene-editing technologies employ engineered nucleases, which are capable of inducing precise site-specific modifications in the genomic DNA of living organisms (Islam *et al.*, 2020). Compared to classical genetic modifications, which often involve the transfer of genes between species, gene-editing tools enable precise alterations within existing DNA sequences. The principal technology driving the recent surge in gene editing research is the CRISPR/Cas9 system (Cong *et al.*, 2013; Mali *et al.*, 2013; Hsu *et al.*, 2014). The CRISPR/Cas9 system originated from an adaptive antiviral defense mechanism in bacteria (Jinek *et al.*, 2012; Cong *et al.*, 2013; Mali *et al.*, 2013). Compared to previous gene-editing platforms, such as ZFNs (Kim *et al.*, 1996; Miller *et al.*, 2007) and TALEN (Christian *et al.*, 2010), which depend on engineered DNA-binding

proteins for target recognition, CRISPR/Cas9 utilizes a short guide RNA (gRNA) to direct the Cas9 endonuclease to a specific genomic locus. Target recognition requires both sequence complementarity between the gRNA and DNA and the presence of a protospacer adjacent motif, which facilitates precise cleavage by Cas9.

When Cas9 locates its target site, it induces a DNA double-strand break (DSB) (Jinek *et al.*, 2012). Subsequent repair of this break determines the nature of the resulting genetic modifications. In eukaryotic cells, two major DNA repair pathways are primarily involved, as described below (Ran *et al.*, 2013; Chang *et al.*, 2017).

Non-Homologous End Joining (NHEJ): Most eukaryotic cells preferentially utilize this repair pathway, which is error prone. NHEJ often produces small insertions or deletions (indels) at the repair junction (Pannunzio *et al.*, 2018). When these indels occur within the coding sequence of a gene, they can disrupt the reading frame or introduce premature stop codons, resulting in a functional gene knock-out (Shalem *et al.*, 2015).

Homology Directed Repair (HDR): HDR occurs when a donor DNA repair template is supplied alongside the Cas9/gRNA complex (Ran *et al.*, 2013). This pathway enables the precise introduction of specific sequence changes, ranging from single-nucleotide substitutions to accurate insertion of larger gene cassettes (Doudna and Charpentier, 2014).

In addition to CRISPR/Cas9 editing, newer systems include base editors (BEs) (Komor *et al.*, 2016; Gaudelli *et al.*, 2017) and prime editing (Anzalone *et al.*, 2019), which expand the range of gene-editing strategies beyond conventional nuclease-mediated knock-out and HDR-mediated knock-in. Adenine base editors (ABEs), which enable A·T to G·C substitution (Gaudelli *et al.*, 2017), and cytosine base editors, which enable C·G to T·A substitution (Komor *et al.*, 2016), can introduce defined transition mutations without producing double-strand breaks, which may be advantageous when the goal is to alter a critical amino acid in a viral receptor while preserving most physiological functions of the host protein. In pigs, this concept is supported by the CD163 E529G point mutation, which reduced PRRSV proliferation and conferred resistance to HP-PRRSV challenge (Liu *et al.*, 2024). Base editor libraries can be used to explore critical amino acid sites and alter the structure of a viral receptor to preserve the original function of the gene, indicating it as a promising strategy for generating antiviral pigs. Prime editing is theoretically broader because it can install all twelve base substitutions as well as small insertions and deletions without requiring a double-strand break or a donor DNA template (Anzalone *et al.*, 2019). However, prime editing remains less established in pigs than conventional CRISPR/Cas9 and base editing. Current barriers include relatively low editing efficiency, delivery of large editing components, optimization of pegRNA design, embryo mosaicism, and the need for robust validation in founder animals and breeding populations. Therefore, prime editing should be considered a promising but still developing platform for viral disease-resistant pig production.

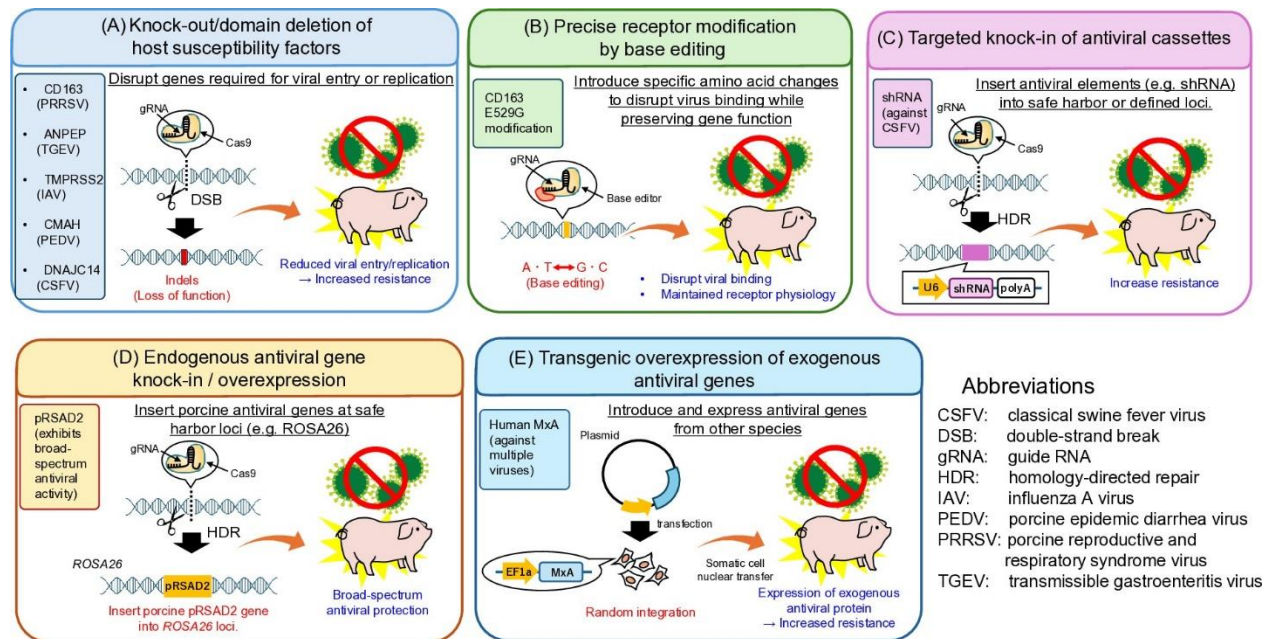


Figure 1 Gene-editing strategies for viral disease resistance in pigs (A) Knock-out/domain deletion of host susceptibility factors, such as *CD163*, *ANPEP*, *TMPRSS2*, *CMAH*, or *DNAJC14*. (B) Precise receptor modification by base editing, such as *CD163* E529G. (C) Targeted knock-in of antiviral cassettes, such as shRNA against CSFV. (D) Endogenous antiviral gene knock-in/overexpression, such as *pRSAD2* at *ROSA26*. (E) Transgenic overexpression of exogenous antiviral genes, such as human *MxA*.

In this review, the terms knock-out, knock-in, overexpression, and transgenesis are used as distinct categories (Fig. 1). Knock-out refers to deletion, disruption, or functional inactivation of an endogenous host gene or domain. Knock-in refers to targeted insertion or precise replacement of a DNA sequence at a defined genomic locus, including insertion of expression cassettes into safe-harbor loci such as *ROSA26*. Overexpression refers to increased expression of an endogenous or introduced gene, either by targeted knock-in or by transgenic integration. Transgenesis refers to the stable introduction of exogenous DNA, especially when the inserted sequence originates from another species or is integrated randomly. Thus, the approaches described below, including shRNA cassette insertion (Xie *et al.*, 2018; Lu *et al.*, 2019), *pRSAD2* knock-in (Xie *et al.*, 2020), *CD28* knock-in (Huang *et al.*, 2017), and human *MxA* overexpression (Zhao *et al.*, 2016), represent related but mechanistically and regulatorily different strategies.

Techniques for Generating Gene-edited Pigs

The generation of genetically modified pigs requires the efficient delivery of gene-editing components, such as Cas9 mRNA/protein, plasmids, and gRNAs, into germline or somatic cells destined for nuclear transfer (Tanihara *et al.*, 2021). Two principal strategies are commonly employed: (1) modification of somatic cells followed by somatic cell nuclear transfer (SCNT) for embryo reconstruction, and (2) direct mechanical delivery into zygotes or early embryos.

Somatic cell nuclear transfer: SCNT is a fundamental technique for generating genetically edited livestock, especially large animals such as pigs (Betthauser *et al.*, 2000; Onishi *et al.*, 2000; Polejaeva *et al.*, 2000). In the absence of established embryonic stem cell lines in pigs

and other large animals, gene-targeting strategies based on embryonic stem cells, as used in small experimental rodents, such as mice, are not applicable. SCNT enables researchers to introduce precise genetic modifications into donor cells in vitro before generating cloned embryos. The general procedure includes: (1) somatic cell preparation and editing, (2) cell selection and genotyping, (3) nuclear transfer, and (4) activation and embryo transfer.

Porcine fetal fibroblasts (PFFs) are commonly used as donor cells. Gene-editing tools and donor plasmids (for knock-in or precise knock-out via HDR) are introduced into these cells through transfection or electroporation. After confirmation of successful gene modification in the selected cell colonies via PCR and Sanger sequencing, nuclei from the verified gene-edited PFF clones are transferred into enucleated oocytes. The reconstructed embryos are subsequently activated (e.g., using electrical pulses) and transferred to surrogate gilts for gestation.

Direct injection of gene editors into zygotes/embryos:

Pronuclear and cytoplasmic microinjections are used to introduce gene-editing components, such as Cas9 mRNA, Cas9 protein, and guide RNA, directly into fertilized one-cell embryos (Hai *et al.*, 2014; Whitworth *et al.*, 2014). The major advantage of these methods is the rapid generation of gene-edited offspring as they bypass the complex SCNT procedure. Typically, microinjections are performed in the pronucleus or cytoplasm of the zygote. Although microinjection is widely used to generate gene-edited animals across species, direct gene editing during embryogenesis often induces genetic mosaicism, which complicates the phenotypic analysis of founders. However, SCNT can ensure non-mosaic genotypes in the resulting pigs; therefore, SCNT continues to be the predominant method for generating genetically modified pigs.

Electroporation-mediated delivery of gene-edited tools into zygotes/embryos: Electroporation has become a widely adopted technique for gene editing in early embryos because it eliminates the need for micromanipulation procedures, such as enucleation, cell injection, or microinjection, which are required in SCNT. This technique has been extensively employed in mice (Sato *et al.*, 2020). In pigs, the introduction of the CRISPR/Cas9 system and gene editing via electroporation into fertilized zygotes has been established (Tanihara *et al.*, 2016), and several gene-knock-out pigs have been successfully produced to date. This electroporation-based approach is technically simple and causes minimal physical damage to embryos, thereby allowing high survival rates when the parameters are properly optimized. Furthermore, recent studies combined electroporation with adeno-associated virus (AAV) vectors to demonstrate successful gene knock-in in mouse embryos (Mizuno *et al.*, 2018). It is anticipated that electroporation in combination with AAV vectors will enable efficient gene knock-in in pigs. Conversely, similar to the microinjection method, electroporation-mediated gene editing in zygotes can lead to mosaicism, which is a potential limitation of this method. Therefore, careful selection and application of SCNT, microinjection, or electroporation should be performed based on specific experimental objectives.

Generation of Viral Disease-resistant Pigs Using the Gene Knock-out Strategy

Gene knock-out strategies involve the deletion or disruption of specific host genes that are essential for viral entry, replication, or pathogenesis. This approach has proven to be highly effective in generating pigs that are resistant to several major viral pathogens (Table 1).

PRRS virus resistance: The PRRS virus is dependent on the host receptor CD163 for entry into target cells, making the CD163 gene a key target for genetic modification (Whitworth *et al.*, 2016; Chen *et al.*, 2019; Wang *et al.*, 2019; Liu *et al.*, 2024).

Complete CD163 knock-out: Pigs that completely lack functional CD163 have been successfully generated using CRISPR/Cas9 technology (Whitworth *et al.*, 2016). When challenged with the virulent PRRS virus isolate NVSL 97-7895, the knock-out pigs exhibited complete resistance. They showed no detectable clinical signs, such as fever or respiratory distress, and neither viremia nor antibody response was observed during the 35-day study period. Histopathology revealed that the lung of knock-out pigs was normal compared to that of wild-type controls, with severe interstitial edema and mononuclear cell infiltration.

Targeting of the SRCR5 domain (Exon 7 of CD163 gene): The CD163 protein contains nine scavenger receptor cysteine-rich (SRCR) domains, among which SRCR domain 5 (SRCR5), encoded by exon 7, is essential for PRRS viral infection (Chen *et al.*, 2019; Wang *et al.*, 2019). The targeting of this specific domain aims to minimize the potential disruption of other

physiological functions of CD163, including its role in hemoglobin-haptoglobin (Hb-Hp) complex clearance.

1) Exon 7 deletion (CD163E7D): Pigs generated via deletion of exon 7 using CRISPR/Cas9 and SCNT displayed resistance to the Highly Pathogenic PRRS virus (HP-PRRSV, strain JXA1) (Wang *et al.*, 2019). All CD163E7D pigs survived the viral challenge, but only 66.6% of wild-type pigs survived (two of six wild-type pigs died). The CD163E7D pigs exhibited milder clinical symptoms, a later onset of fever, and a substantially decreased viral load in the blood. Additionally, exon 7 deletion did not impair the physiological functions of CD163, including red blood cell parameters and serum haptoglobin concentrations.

2) Exon 7 Substitution (CD163Mut/Mut pigs): In another approach, the porcine CD163 exon 7 was substituted with the corresponding exon from human CD163-like 1 (hCD163L1) (Chen *et al.*, 2019). This modification significantly inhibited HP-PRRSV replication in vitro and conferred protection in vivo against a lethal HP-PRRSV (strain JXA1) challenge. CD163Mut/Mut porcine alveolar macrophages (PAMs) strongly suppressed PRRS viral replication by blocking viral uncoating and genome release. In vivo, three of the four CD163Mut/Mut pigs survived, showing significantly reduced viral loads, rapid recovery, and robust resistance to highly pathogenic strains.

3) Point Mutation (E529G): Using base-editing technology (NG-ABE8e), a specific single-base mutation (E529G) was introduced into CD163 (Liu *et al.*, 2024). This modification effectively limited PRRS viral proliferation in vitro and conferred resistance to HP-PRRSV challenge in vivo.

Classical swine fever virus resistance: CSF virus is a highly contagious pathogen with high mortality in pigs (Xie *et al.*, 2018; Yuan *et al.*, 2022). Considerable efforts have been directed toward vaccination-based strategies for the control of CSF, and in 2025, significant progress in infection resistance at the whole-animal level was reported. Crooke *et al.* (2025) reported the successful application of CRISPR/Cas9 gene editing in conferring complete resistance against classical pestiviruses, such as CSF virus in pigs (Crooke *et al.*, 2025). Previous in vitro studies have established that the host molecular chaperone DNAJC14 is a core component that is essential for the replication cycle of classical pestiviruses (Yi *et al.*, 2011; Isken *et al.*, 2019). To test this finding in vivo, researchers used CRISPR/Cas9 to edit the DNAJC14 gene in pig zygotes to produce F1 animals that were homozygous for the targeted alterations. The edited pigs appeared healthy with no obvious phenotypic deficits. When young adult-edited pigs were intranasally challenged with a virulent CSF virus strain (Alfort/187), they showed complete resistance to infection. Unlike the control animals, which developed viremia and anti-CSF virus antibodies, no CSF viral RNA was detected in the blood or tissues of the edited pigs, and they remained healthy. These results indicate that DNAJC14 is required for non-cytopathic classical pestivirus replication in vivo and that targeted DNAJC14 editing can confer strong protection against CSFV under controlled laboratory challenge conditions. However,

the current in vivo evidence should be interpreted cautiously because complete protection was demonstrated in a small laboratory challenge involving four edited pigs and four controls. This places the technology at an experimental proof-of-concept stage. Larger cohorts, independent replication, challenge with diverse CSFV strains, long-term health and reproductive assessment, and evaluation under production conditions will be required before practical deployment can be considered.

Influenza A virus (IAV) resistance: The pathogenicity of IAV depends on the activation of hemagglutinin protein by host proteases. Transmembrane protease serine 2 (TMPRSS2) is a key host enzyme responsible for cleaving and activating mammalian IAVs in the airways. Using CRISPR/Cas9, homozygous TMPRSS2 knock-out pigs were generated (Ciacci Zanella *et al.*, 2023; Kwon *et al.*, 2024). When challenged with swine-adapted H1N1 (CA04) or H3N2 (TX98) strains, these knock-out pigs exhibited markedly altered viral replication dynamics and reduced disease severity. Specifically, TMPRSS2 knock-out pigs showed less pronounced macroscopic and microscopic lung lesions, significantly lower viral loads in respiratory tissues (including nasal turbinates, lungs, and bronchoalveolar lavage fluid), and decreased nasal viral shedding compared to their wild-type littermates. These findings indicate that TMPRSS2 plays a critical role in IAV replication, particularly in the upper respiratory tract of pigs.

Transmissible gastroenteritis (TGE) virus resistance: Pigs have developed resistance to the TGE virus through targeted gene editing of the viral host receptor (Whitworth *et al.*, 2019). TGE virus is an alphacoronavirus, and it is a highly contagious and lethal pathogen that causes severe diarrhea and dehydration, often resulting in nearly 100% mortality among newborn piglets and substantial economic losses worldwide. The primary cellular receptor mediating TGE viral infection is porcine aminopeptidase N (pAPN), also known as ANPEP, which is abundantly expressed in small intestinal epithelial cells. Using CRISPR/Cas9 technology, researchers have successfully generated pAPN knock-out pigs by editing the ANPEP gene. These ANPEP-knock-out pigs exhibited complete resistance to TGE viral infection. Following the viral challenge, knock-out pigs showed no detectable TGE virus nucleic acid in their feces and failed to produce TGE virus-specific antibodies, indicating that ANPEP expression is indispensable for TGE virus infection in pigs.

To achieve simultaneous resistance to multiple viral pathogens, researchers have targeted two distinct host receptors: CD163 for the PRRS virus and pAPN for the TGE virus (Xu *et al.*, 2020). Pigs with a double knock-out (DKO) of both CD163 and ANPEP exhibited dual resistance to PRRS virus (genotype 2) and TGE virus, along with reduced susceptibility to porcine deltacoronavirus (PDCoV). Notably, these DKO pigs maintained normal growth and reproductive performance, comparable to those of wild-type controls.

Table 1 Strategies to confer viral disease resistance in pigs and the corresponding methodological approaches.

Target Disease	Target gene	Methods	Outcome	Reference
Porcine Reproductive and Respiratory Syndrome (PRRS)	CD163	Knock-out	Complete resistance to PRRS	Whitworth et al. (2016)
		Deletion of exon 7	Resistance to highly pathogenic PRRS	Wang et al. (2019)
		Substitution of exon 7	Resistance to highly pathogenic PRRS	Chen et al. (2019)
	CD28	Introduction of point mutation	Resistance to highly pathogenic PRRS	Liu et al. (2024)
Classical Swine Fever (CSF)	DNAJC14	Over expression	Enhanced immune responses to highly pathogenic PRRS	Huang et al. (2017)
	Antiviral RNA Interference (shRNA)	Knock-out	Resistance to CSF	Crooke et al. (2025)
		Knock-in	Attenuated clinical symptoms, reduced viremia, and improved survival rates	Xie et al. (2018)
Influenza A Virus (IAV)	TMPRSS2	Knock-in	Inhibited replication of the CSF virus	Lu et al. (2019)
		Knock-out	Reduced disease severity	Xie et al. (2020)
Transmissible Gastroenteritis (TGE)	ANPEP	Knock-out	Complete resistance to TGE	Ciacci Zanella et al. (2023)
Porcine Epidemic Diarrhea (PED)	CMAH	Knock-out	Reduced susceptibility to PED virus infection	Kwon et al. (2024)
African Swine Fever (ASF)	RELA	amino acid substitution	A modest delay in the onset of clinical signs	Whitworth et al. (2019)
				Xu et al. (2020)
				Tu et al. (2019)
				Lillico et al. (2016)
				McCleary et al. (2020)

Porcine epidemic diarrhea virus resistance: PED causes severe and often fatal diarrhea in neonatal piglets. Gene editing has been applied to target host factors that influence susceptibility, specifically the presence of N-glycolylneuraminic acid (NGNA), a sialic acid derivative associated with viral attachment. The CMP-N-glycolylneuraminic acid hydroxylase (CMAH), which influences NGNA synthesis, was disrupted using CRISPR/Cas9 (Tu *et al.*, 2019). CMAH knock-out piglets exhibited reduced susceptibility to PED viral infection, particularly during early life, indicating the potential for genetic modification as a preventive strategy. However, complete resistance to PEDV has not yet been achieved in pigs.

Gene Knock-in or Transgenic Overexpression Strategies for Viral Disease Resistance

In addition to knock-out or precise modification of host susceptibility factors, antiviral resistance can be enhanced by targeted insertion or increased expression of antiviral molecules (Table 1). These approaches include RNA interference cassettes that suppress viral replication, targeted knock-in of endogenous antiviral genes, immune-modulating gene knock-in, and transgenic expression of exogenous antiviral genes. These categories should be interpreted separately because they differ in mechanism, genomic structure, heritability, regulatory classification, and biosafety considerations.

Classical swine fever virus resistance: CRISPR/Cas9-based receptor knock-out strategy has been used to achieve complete resistance to CSF in pigs; however, substantial efforts have been directed toward the induction of CSF resistance in pigs using a variety of alternative approaches. Strategies to confer resistance have primarily focused on gene knock-in or overexpression of antiviral factors as well as on RNA interference (RNAi)-based approaches that inhibit viral replication and spread.

Antiviral RNA interference (shRNA) knock-in: RNAi employs short hairpin RNAs (shRNAs) to suppress the expression of viral genes (Xie *et al.*, 2018; Lu *et al.*, 2019). Using CRISPR/Cas9-mediated homologous recombination, researchers have achieved site-specific integration of shRNA expression cassettes (shRNA-C3 and shRNA-C6), which target the CSF virus genome, into the porcine ROSA26 locus. Gene-modified pigs that express these shRNAs exhibit marked resistance to lethal CSF viral challenge. Compared with non-transgenic controls, challenged gene-modified pigs showed significantly attenuated clinical symptoms, reduced viremia, and improved survival rates. This RNAi-based knock-in approach provided stable and heritable antiviral protection. Furthermore, this strategy has been reported to achieve simultaneous defense against multiple viral infections, such as the targeting of the Foot and Mouth Disease virus gene and the PRRS virus gene using shRNAs (Oh *et al.*, 2018).

Broad-spectrum antiviral gene overexpression via knock-in (pRSAD2): The porcine RSAD2 gene (pRSAD2, also known as Viperin) exhibits broad-spectrum

antiviral activity. Using CRISPR/Cas9, researchers have achieved precise knock-in and overexpression of pRSAD2 at the ROSA26 locus (Xie *et al.*, 2020). Fibroblasts derived from pRSAD2-knock-in pigs effectively inhibited the replication of the CSF virus Shimen strain *in vitro*. These findings demonstrate the feasibility of enhancing innate antiviral defenses through targeted integration of host immune genes, providing a promising strategy for developing pigs with broad-spectrum viral resistance. A comprehensive demonstration of disease resistance in pRSAD2-knock-in pigs, including controlled infection studies, is highly desirable to validate their translational potential.

Overexpression of Human MxA via random transgenesis: Although not established through gene editing, transgenic pigs have been developed to overexpress the antiviral human MxA protein, a dynamin-like GTPase that inhibits the replication of various RNA viruses (Zhao *et al.*, 2016). Cells derived from these MxA-transgenic pigs, including tail, kidney, and umbilical cord fibroblasts, strongly suppressed CSF virus replication *in vitro*. These findings indicate that MxA confers effective early protection against CSF viral infection and highlight its potential as a transgene for enhancing innate antiviral defense in pigs. Moreover, insights gained from such random-integration overexpression approaches can be readily applied to future viral disease-resistance strategies that employ site-specific knock-in techniques using gene-editing tools such as CRISPR/Cas9.

Enhanced immune response to improve PRRS virus resistance via CD28 knock-in/overexpression: Compared to the strategies that block viral entry to confer resistance to PRRS virus, an alternative approach focuses on the enhancement of innate and adaptive immune responses of the host. This method aims to strengthen antiviral defense mechanisms through targeted overexpression of immune-modulating genes. A study showed that T-cell-mediated immunity was enhanced by overexpressing the CD28 co-stimulatory molecule in transgenic Landrace pigs. This was achieved through CRISPR/Cas9-mediated homologous recombination of knock-in CD28 at the ROSA26 locus, followed by SCNT (Huang *et al.*, 2017). After vaccination and challenge with the HP-PRRSV vaccine strain JXA1-R, CD28-transgenic pigs exhibited elevated virus-specific antibody titers and increased interferon- γ secretion from peripheral blood lymphocytes compared with wild-type controls. These enhanced immune responses correlated with reduced body weight loss, milder fever, and lower serum viral loads during PRRS viral infection.

Attempts to Control African Swine Fever

Efforts to control ASF have been challenging due to the biological complexity of the ASF virus. One area of investigation has been the incorporation of natural resistance traits from warthogs (*Phacochoerus* spp.), which act as natural reservoir hosts for the ASF virus and do not develop the lethal disease phenotype observed in domestic pigs. Attempts have been made

to modulate ASF susceptibility by gene-editing-based substitution of RELA, although the introduction of ASF resistance into pigs has not been successful thus far (Lillico *et al.*, 2016; McCleary *et al.*, 2020). In this study, domestic pigs were generated by replacing portions of the RELA protein sequence with the corresponding warthog-derived residues using ZFN to produce either two or three amino acid substitutions. RELA, a key subunit of the NF- κ B transcription factor complex, plays a central role in immune regulation (Thomson *et al.*, 1980; Palgrave *et al.*, 2011). Following intranasal ASF virus challenge, pigs harboring the two amino acid substitutions showed no detectable differences in infection parameters compared to wild-type controls. Pigs carrying all three warthog-specific substitutions were not fully resistant but exhibited a modest delay in the onset of clinical signs and, in some cases, reduced the level of viral DNA in the blood and nasal secretions. These results indicate that these specific amino acid substitutions in RELA alone are insufficient to confer meaningful ASF resistance, underscoring the complexity of host-virus interactions in ASF and the need for a deeper understanding of the molecular determinants of resistance.

Regulatory, Biosafety, and Biological Considerations

The regulatory and biosafety implications of viral disease-resistant pigs depend strongly on the type of genetic alteration. Foreign-DNA-free edits, such as small deletions, domain deletions, or point mutations in endogenous genes, may resemble genetic variants that could arise naturally or through conventional breeding. In contrast, transgenic pigs expressing exogenous genes or synthetic expression cassettes raise additional questions related to inserted DNA sequence, expression pattern, insertion site, stability, food and feed safety, environmental exposure, and traceability. Therefore, these categories should not be discussed as a single regulatory entity.

For knock-out-based resistance, biological safety must be evaluated at both the pathogen-resistance and host-physiology levels. Host factors such as CD163 are not only viral entry factors but also macrophage-associated proteins with roles in hemoglobin-haptoglobin clearance and inflammatory regulation (Kristiansen *et al.*, 2001; Etzerodt and Moestrup, 2013). Complete gene disruption could therefore have broader effects than domain-specific editing or point mutation. Although several studies have reported normal growth, blood parameters, and reproductive performance in edited pigs (Burkard *et al.*, 2018; Wang *et al.*, 2019; Xu *et al.*, 2020), these observations should be confirmed across genetic backgrounds, ages, production environments, and pathogen-exposure conditions. Particular attention should be paid to macrophage function, immune responses to unrelated pathogens, animal fitness, welfare, and reproductive performance.

Another important issue is the durability of resistance. Receptor-based editing can produce strong resistance when a virus depends on a single essential host factor, but RNA viruses can evolve rapidly. For example, PRRSV has high genetic diversity, and viral

attachment and entry involve interactions between viral glycoproteins and host molecules. Although CD163 is considered essential for productive PRRSV infection, strain variation, accessory entry factors, or future viral adaptation could influence the durability of resistance. Therefore, edited pigs should be challenged with diverse contemporary isolates, and surveillance should be conducted for viral variants capable of partial escape. In the longer term, multiplex or polygenic strategies that combine receptor editing, precise amino-acid modification, and immune modulation may provide more durable resistance than single-gene strategies, but such combinations must be balanced against possible effects on normal physiology.

Conclusion

Gene editing technology, particularly the CRISPR/Cas9 system, has fundamentally transformed the capacity to engineer viral disease resistance in pigs. The studies summarized here demonstrate that precise genetic modifications can confer specific, heritable protection against some of the most economically significant viral pathogens affecting the global swine industry. Despite these remarkable advances, several challenges remain to be addressed. The generation of live, challenge-tested pigs resistant to complex viruses, such as the ASF virus, is still limited by insufficient understanding of key host factors. Moreover, the successful commercialization of gene-edited livestock depends on the establishment of appropriate regulatory frameworks and achievement of broad public acceptance.

A practical roadmap for viral disease-resistant pig development can be divided into short-, mid-, and long-term priorities. In the short term, the most mature route is receptor or receptor-domain editing for pathogens with well-defined host factors, especially PRRSV-like models. These studies should emphasize precise edits that preserve host-gene function, validation across viral genotypes, and assessment of animal health and reproduction. In the mid-term, multiplex editing and immune-modulating knock-in approaches should be developed to broaden protection against multiple viruses or against genetically diverse strains. Such approaches require careful phenotyping to avoid unintended impairment of growth, fertility, or immune balance. In the long term, complex diseases such as ASF will require systems-level identification of host determinants, integration of genome editing with genomic selection, and development of polygenic resistance strategies. Translation into the swine industry will also require internationally compatible regulatory pathways, transparent biosafety evaluation, public communication, and breeding programs that maintain productivity and welfare.

In summary, gene editing is a powerful and sustainable strategy for improving livestock health and welfare. Although the development of resilient herds that can thrive with reduced reliance on intensive preventive measures is made possible by ongoing advancement in this field, researchers need to employ these technologies responsibly via rigorous biosafety management, appropriate regulations, and public

education to prevent unintended environmental release. These advances promise a more secure and sustainable future for global pig production.

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