Review Article

Title:
Current status of the application of gene editing in pigs

Authors:
Fuminori Tanihara¹,²*, Maki Hirata¹, and Takeshige Otoi¹

¹) Faculty of Bioscience and Bioindustry, Tokushima University, Tokushima 770-8513, Japan.
²) Center for Development of Advanced Medical Technology, Jichi Medical University, Shimotsuke, Tochigi 329-0498, Japan.

Keywords:
ZFN, TALEN, CRISPR/Cas9, gene editing, pig

Running head:
GENE EDITING IN PIGS

* Correspondence:
Fuminori Tanihara, Animal Resource Laboratory, Center for Development of Advanced Medical Technology, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan.
Email: f_tanihara@jichi.ac.jp
Tel.: +81-285-58-7490
Fax: +81-285-44-8629
Abstract

Genetically modified animals, especially rodents, are widely used in biomedical research. However, non-rodent models are required for efficient translational medicine and preclinical studies. Owing to the similarity in the physiological traits of pigs and humans, genetically modified pigs may be a valuable resource for biomedical research. Somatic cell nuclear transfer (SCNT) using genetically modified somatic cells has been the primary method for the generation of genetically modified pigs. However, site-specific gene modification in porcine cells is inefficient and requires laborious and time-consuming processes. Recent improvements in gene-editing systems, such as zinc finger nucleases, transcription activator-like effector nucleases, and the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (CRISPR/Cas) system, represent major advances. The efficient introduction of site-specific modifications into cells via gene editors dramatically reduces the effort and time required to generate genetically modified pigs. Furthermore, gene editors enable direct gene modification during embryogenesis, bypassing the SCNT procedure. The application of gene editors has progressively expanded, and a range of strategies is now available for porcine gene engineering. This review provides an overview of approaches for the generation of genetically modified pigs using gene editors, and highlights the current trends, as well as the limitations, of gene editing in pigs.

Key words: ZFN, TALEN, CRISPR/Cas9, gene editing, pig
1. Introduction

Genetically modified animals, especially rodents, are widely used as biomodels to elucidate animal physiology and disease mechanisms, including human traits and diseases. However, for efficient translational and preclinical studies, additional insights from non-rodent animal models are important [1]. Pigs are similar to humans in several respects, particularly in anatomy and physiology. Additionally, they breed year-round with large litter sizes. As pigs are considered an excellent animal model, gene modification is expected to improve the value of pig resources for biomedical research. The first transgenic pig, reported in 1985 [2], was produced by microinjection of exogenous DNA into the pronuclei of porcine zygotes through a fine glass needle. Subsequently, pronuclear injection has been used to establish genetically modified pig lines [3, 4]. This technique is simple but requires technical proficiency. Furthermore, the low efficiency of generating founder pigs carrying mutations and the random integration of injected DNA into the genome without control of the copy number result in unstable phenotypes, gene silencing, and unpredictable gene expression, thereby limiting the application of such mutant pigs.

In mice, the establishment of embryonic stem cells (ESCs) promoted the development of genetically modified animals owing to the production of chimeras with germline transmission, which represents a significant advance in biomedical research. However, for pigs, stem cell lines, including ESCs, which contribute to the germline, are not available [5]. Since somatic cell nuclear transfer (SCNT) has been established in pigs [6–8], SCNT using genetically modified somatic cells as nuclear donors has been widely chosen as a method for the generation of genetically modified pigs. The correct use of somatic cells carrying the desired mutation, including multiple gene modifications, as nuclear donors virtually ensures that pigs will carry the desired mutations and the appropriate number of copies of the transgene. Furthermore, direct gene editing during embryogenesis often induces genetic mosaicism, which complicates the
phenotypic analysis of founders, whereas SCNT can ensure non-mosaic genotypes in the resulting pigs. These characteristics, which have significant advantages, show that SCNT can be used as a primary method for the generation of genetically modified pigs.

SCNT overcomes the low efficiency and random transmission of gene modifications in delivered piglets that characterize pronuclear microinjection. However, site-specific gene insertion in porcine cells is limited by the low efficiency of homologous recombination (HR) and the sophisticated selection processes within cells following gene modification procedures, necessitating laborious and time-consuming processes [9]. Recently developed precise nuclease-mediated gene editing systems have dramatically improved gene modification in pigs. This review describes the production of genetically modified pigs using gene editors, provides an overview of approaches for the generation of genetically modified pigs using various types of gene editors, and highlights current trends, including the establishment of disease models and research on pig-to-human transplantation, as well as the limitations of gene editing in pigs.

2. Gene editors: improvements in engineered endonucleases

Engineered endonucleases, including artificial nucleases, such as zinc finger nucleases (ZFNs) [10, 11] and transcription activator-like effector nucleases (TALENs) [12], as well as RNA-guided endonucleases, such as the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (CRISPR/Cas) system [13, 14], are major innovations for gene modification in somatic cells, stem cells, and zygotes/embryos of various animal species. These nucleases have precise DNA-binding ability and generate double-strand breaks (DSBs) at the desired genomic locus. DSBs trigger endogenous DNA repair via non-homologous end-joining (NHEJ) or homology-directed repair (HDR) pathways [15]. NHEJ occurs when the ends of a DSB are rejoined without any DNA template to guide this repair. Successfully repaired targeted sequences are repeatedly cut by gene editors, resulting
in the frequent introduction of short DNA insertions/deletions (indels). These indels create
targeted gene knockouts by inducing a frameshift in the codons, which is followed by the
formation of a premature termination codon [12]. HDR relies on donor DNA with homologous
arms from sister chromatids, homologous chromosomes, exogenous DNA templates, or
single-strand donor oligonucleotides (ssODNs), and enables gene knock-in and the introduction
of the desired point mutation. In general, the frequency of HDR is lower than that of NHEJ in
most cell types [16].

ZFNs are gene editors composed of DNA-binding domains (zinc finger proteins) and a
dNA-cutting domain (the chimeric restriction nuclease \textit{FokI}) acting as a heterodimer. Zinc
finger domains recognize specific sequences in genomic DNA, after which \textit{FokI} nuclease and
the zinc finger protein induce DSBs at the targeted position. However, the practical use of ZFNs
in laboratories is hindered by high costs and technical difficulties [17]. TALENs are
conceptually similar to ZFNs. Transcription activator-like effectors (TALEs) are naturally
occurring proteins found in the plant pathogenic bacterial genus \textit{Xanthomonas}. TALENs have a
TALE as the DNA-binding domain and \textit{FokI} as the cleavage domain. The preparation of
TALENs is simpler than that of ZFNs; therefore, they are preferred in laboratory settings for
gene editing.

ZFNs and TALENs are artificial nucleases. In contrast, CRISPR/Cas9 is an RNA-guided
endonuclease that is derived from an adaptive bacterial immune system component [13, 14, 18].
The CRISPR/Cas9 system comprises a guide RNA (gRNA) and Cas9 nuclease. Since the first
practical demonstration of gene editing using the CRISPR/Cas9 system in 2013, the system has
been dramatically improved. Various gRNA/Cas9-related expression plasmids, Cas9 proteins,
tools for gRNA design, and subsequent gRNA order/purchase systems are now available [19].
The system does not require specialized methodology or equipment; this has contributed to its
recent widespread use.
Off-target effects, which are unexpected DNA cleavages caused by the binding of gene editors to unintended genomic sites, are of major concern in gene editing, especially using the CRISPR/Cas9 system; these have limited the research and clinical applications of gene editors [20, 21]. Carey et al. highlighted the frequency of off-target events induced by cytoplasmic microinjection of CRISPR/Cas9 during embryogenesis [22]; they detected off-target cleavage, but concluded that the frequency was low. Zhou et al. also detected off-target events induced by cytoplasmic microinjection of CRISPR/Cas9 [23]. Other off-target events were observed during SCNT-mediated production of gene-edited pigs using a ZFN [24] and CRISPR/Cas9 [25, 26], but mutations were only observed in non-coding regions in two out of the three studies [24, 26]. To the best of our knowledge, off-target events in gene-edited offspring have not been detected in any other study. Choi et al. showed that there was no off-target cleavage in offspring when using whole-genome sequencing [27]. To date, off-target events have not produced any critical problems in gene-edited porcine offspring. Various approaches have been developed to minimize these off-target effects, such as off-target detection by algorithmically designed software and genome-wide assays, the use of cytosine or adenine base editors, prime editing, and the chemical modification of gRNA [19, 28]. Furthermore, Cas9 variants such as Cas9 nickase [29], which cleaves only the target strand (by double nicking), and catalytically dead Cas9 combined with FokI nuclease (FokI-dCas9) [30, 31], reduce off-target events. Variants suggested by structural studies of Cas9, such as Cas9-HF1 [32], evo-Cas9 [33], eSpCas9 [34], and Hypa-Cas9 [35], also improve gene editing efficiency and discrimination against off-target events. The careful design of binding modules or gRNAs and improved application methods will minimize off-target effects in founder generations and reduce the labor required to analyze off-target candidates.

3. Methods for generation of genetically modified pigs using gene editors
The ZFN, TALEN, and CRISPR/Cas9 systems enable efficient gene targeting and the introduction of multiallelic modifications into somatic cells, simplifying the preparation of donor cells for SCNT in pigs. Furthermore, gene editors have enabled the direct modification of genomic DNA in zygotes/embryos using cytoplasmic microinjection and electroporation (Figure 1).

### 3.1. SCNT using gene-edited somatic cells

Gene editors enable the one-step knockout of genes in somatic cells without any marker or exogenous DNA fragments. Such gene-edited cells have accelerated SCNT-mediated production of genetically modified pigs. Gene editors also facilitate multiple gene editing and knock-in of exogenous genes; hence, double- [36–38], triple- [39–41], and quadruple-gene-edited pigs [42] and knock-in pigs [43, 44] have been generated using the SCNT technique. Following appropriate selection of donor cells after gene editing, the delivered piglets carry the desired genotypes. Furthermore, SCNT does not result in mosaicism, which is observed in gene-editor-mediated direct gene modification during embryogenesis, and thus aids in the phenotypic analysis of founder pigs. SCNT is the primary method for generating gene-edited pigs. However, offspring derived from reconstructed embryos often show abnormalities, such as birth defects, abortions, and early postnatal death; this is a limitation of SCNT [45].

### 3.2. Direct introduction of gene editors during embryogenesis

#### 3.2.1. Microinjection of gene editors into zygotes/embryos

The direct introduction of gene editors into the cytoplasm, an alternative to SCNT, simplifies the genetic modification of fertilized zygotes/embryos. Porcine oocytes have high lipid contents; therefore, centrifugation is required to visualize the pronuclei for successful
pronuclear injection at the zygote stage. However, gene editors are generally supplied with nuclear localization signals, making the centrifugation procedure and maneuvering of the glass needle toward the pronuclei unnecessary. Cytoplasmic microinjection-mediated gene-edited pigs have been produced using gene editors in the early stages of their development [46–48]. Microinjection also enables the introduction of large molecules; therefore, microinjection-mediated knock-in pigs can be established [49]. An advantage of microinjection-mediated gene editing is the high viability of the manipulated zygotes/embryos. After the transfer of microinjected zygotes/embryos, the litters obtained from manipulated embryos tend to be larger than those from embryos generated by SCNT [50]. Although the results of embryo transfer depend on the condition of the recipient surrogates and operator skill in embryonic manipulation, the high viability of the zygotes/embryos and resulting piglets reduces labor.

3.2.2. Delivery of CRISPR/Cas9 system via electroporation during embryogenesis

Electroporation-mediated gene editing is a micromanipulation-free method in which large numbers of gene-edited zygotes/embryos can be prepared by introducing gene editors into zygotes. In mice, electroporation is widely used to introduce gene editors [51]. Gene editing via electroporation has also been applied to porcine zygotes [52], with successful gene modification (knockout) [52–55]. Electroporation-mediated gene editing requires no specialized equipment and benefits from a simple process and high zygote viability. However, the introduction of large molecules, including transgenes for knock-in, by electroporation alone is difficult in pigs. Generally, the molecular uptake into cells via electroporation is proportional to the field strength, pulse length, and number of pulses used. Porcine in vitro-fertilized zygotes/embryos are sensitive to electricity, and high voltages are harmful, unlike in mice [52, 56]. Hence, a knock-in system for large transgenes via electroporation has not been established. Further
research focusing on electroporation-mediated gene editing and the proper choice of electroporation and cytoplasmic microinjection techniques (depending on the study purpose and type of mutation) is needed.

4. Recent trends in gene editing in pigs

Gene editors have been used to generate genetically modified pigs. In 2011, fifteen years after the initial report of the concept of ZFNs [10], genetically edited pigs were generated using them [24, 57, 58]. TALEN and the CRISPR/Cas9 system were also applied to generate genetically modified pigs soon after practical gene editing in mammalian cells was demonstrated. The low-density lipoprotein receptor (LDLR)-knockout pigs reported in 2012 were the first to be generated using a TALEN [59]. Using the CRISPR/Cas9 system, Whitworth et al. generated CD163- and CD1D-modified pigs using SCNT and cytoplasmic microinjection to confer disease resistance against porcine reproductive and respiratory syndrome [47]. Recently, gene editors have been utilized extensively for the rapid establishment of valuable engineered pig lines that can be used in human medicine, e.g., as disease models and organ donors.

4.1. Disease models

Pigs are among the best animals for disease models in medical research, which has implications for translational and preclinical research, as they are intermediate between mice and humans in terms of their physiological and anatomical relationships. Selection of the appropriate pig breed or strain, and age is important for the application of surgical and non-surgical procedures typically used in human medicine (e.g., catheterization, heart surgery, and endoscopy). These clinical procedures are particularly difficult or impossible to perform in many other animal models, including rodents, owing to the small size of the species. Various
types of gene-edited pigs have been generated to establish models for intractable diseases (Table 1). Gene editing is expected to accelerate the application of pig lines as disease models.

4.2. Tissue/organ donors for pig-to-human transplantation

Pigs are ideal tissue/organ donors for humans owing to the high similarity of their organs, especially in terms of size and structure. Pig-to-human xenotransplantation is a solution to the shortage of organs for human transplantation. However, xenoantigens cause hyperacute rejection and limit the success of interspecific xenografts. Therefore, genes involved in xenoantigen biosynthesis, such as GGTA1, CMAH, and B4GALNT2, are key targets for improving the outcomes of xenotransplantation. GGTA1 is a major target gene, and its inactivation has been demonstrated using ZFNs [57, 60, 61], TALENs [62–64], and CRISPR/Cas9 [55, 65, 66]. However, for successful xenotransplantation, all major xenoantigens expressed in porcine tissues should be removed. To this end, GGTA1/CMAH double-knockout [37, 67–69] and GGTA1/CMAH/B4GALNT2 triple-knockout pigs [39, 70, 71] have also been generated using SCNT and gene editing. Paris et al. demonstrated that organs derived from ASGR1-deficient pigs exhibit decreased human platelet uptake, which may prevent xenotransplantation-induced thrombocytopenia [72]. Gene editors enable various approaches to regulating immune rejection.

Additional major hurdles for successful xenotransplantation are organ size and the elimination of porcine endogenous retrovirus (PERV). Xeno-organs donated by genetically modified pigs carrying the genetic background of domestic pigs can grow rapidly; this can generate incompatibility with recipients and impair their long-term function after transplantation. Growth hormone receptor (GHR)-deficient pigs with reduced organ size [73, 74] and subsequent GHR/GGTA1 double-knockout pigs expressing the human cluster of differentiation (hCD46) and human thrombomodulin (hTHBD) [75] have been generated by gene editing. This
approach will improve the implementation of xenotransplantation. Furthermore, the risk of PERV transmission to humans after xenotransplantation is a concern [76, 77]. PERVs constitute an integral part of the porcine genome and can be expressed as infectious virus particles. Infection by PERVs in human cells has been observed using in vitro co-culture assays, which demonstrated the possibility of a new epidemic infectious disease induced by xenotransplantation. PERVs are present in various proportions in the whole porcine genome, depending on the pig breed and tissue type, making the inactivation of PERVs a difficult task; however, genome-wide gene editing has the potential to eliminate PERVs from porcine tissues. Gene editing targeting PERVs has been demonstrated using CRISPR/Cas9 [78, 79], and PERV-inactivated pigs have also been generated using SCNT [80]. These hurdles have thus been partially overcome using various gene editing techniques in pigs, improving the feasibility of pig-to-human xenotransplantation.

An alternative strategy for producing functional and transplantable tissues or organs is to build interspecies chimeras at the embryonic level by blastocyst complementation, which involves the injection of human ESCs or human induced pluripotent stem cells into genetically modified porcine embryos lacking the ability to generate specific organs. Missing organs (empty niches) are expected to develop from these injected stem cells, resulting in the generation of organs derived from human cells. A proof-of-concept has been established via the generation of functional pancreases in mouse-rat interspecific chimeras [81]. Matsunari et al. demonstrated the compensation of disabled organogenesis by allogenic blastocyst complementation in pigs by injecting donor blastomeres into gene-edited host embryos [82]. However, at present, the utilization of stem cells for interspecies chimerism is quite limited with respect to pigs [83, 84]. Therefore, further investigations are required.

Figure 2 summarizes recent trends in the number of articles reporting the generation of genetically modified pigs using gene editors available via PubMed. The search terms used were “pig,” “ZFN,” “TALEN,” and “CRISPR”; these picked up studies demonstrating the production of gene-edited pigs/fetuses. The gene editors (ZFN, TALEN, and/or CRISPR-related systems) and methods for generating gene-edited pigs (SCNT, cytoplasmic microinjection, and/or electroporation) used in the studies were investigated. Although the introduction of gene editors during embryogenesis using microinjection or electroporation has an advantage over SCNT with respect to the simplicity of the procedure, the use of SCNT is more common. In this section, we discuss the limitations and future prospects of direct gene editing during embryogenesis.

5.1. Mosaicism

Genetic mosaicism, in which a single individual carries multiple genotypes, contributes to the inability to generate mutant pigs via direct gene editing during embryogenesis [85]. In founder animals, both the direct injection and electroporation-mediated introduction of gene editors into the cytoplasm often induce mosaicism due to the delayed expression of mRNA-related gene editors or remnant activity of the gene editors throughout the cell division process [85–89]. Mosaicism complicates the phenotypic analyses of founders, which require the F1 generation. Owing to the long gestation period and time to reach sexual maturity in pigs, production of the F1 generation involves a tremendous amount of time and cost, seriously limiting research progress. Mosaicism is detected by genotyping (e.g., by the detection of multiple alleles, typically three alleles or more, or extreme deviations in allele frequencies). We investigated previous examples of gene modification during embryogenesis by cytoplasmic microinjection or electroporation using gene editors, including TALENs and ZFNs, and found that mosaicism in gene-edited offspring was reported in 18 out of 23 studies (Table 2).
Mosaicism occurs at various frequencies [85], and the type of gene editor and modification [90, 91], introduction method, animal species, and introduced component of gene editors (e.g., expression plasmid, mRNA, and protein/nuclease) seem to be potential factors.

Appropriate timing of the introduction of gene editors during embryogenesis is considered a key factor in reducing mosaicism [87]. Microinjection of the CRISPR/Cas9 system into the cytoplasm of germinal vesicle-stage oocytes successfully generated non-mosaic genome-edited porcine embryos [92]. Onuma et al. demonstrated that microinjection of the CRISPR/Cas9 system during meiotic maturation preferentially induces heterozygous mutations without mosaicism after germinal vesicle breakdown and chromosome condensation [93]. Conversely, electroporation-mediated gene editing in mature oocytes has demonstrated that the type of egg may influence development after electroporation treatment and the mutation rate in the resulting blastocyst; however, mosaicism is not controlled [94]. A simple approach, the optimization of CRISPR/Cas9 component concentrations, is effective in increasing gene editing efficiency in cytoplasmic microinjection [95] and electroporation [96]. Such strategies will improve gene editing efficiency during embryogenesis. Further optimization of the methods for the application of gene editors in pigs is required.

5.2. Multiple gene editing

Currently, the generation of multiple-gene-edited pigs is an important research goal aimed at a better understanding of complex biological processes and the management of redundancies and compensatory changes in signaling pathways. Gene editors can induce mutations in multiple targeting sites, enabling the one-step generation of double- and triple-knockout pigs via direct introduction into zygotes/embryos. Multiple-gene-edited animals have been generated by cytoplasmic microinjection of CRISPR/Cas9 in mice [97, 98], rats [99], and monkeys [100]. In pigs, the SCNT technique is the primary method, as described above, and there are few reports
of one-step generation of multiple-gene-edited pigs by microinjection or electroporation [101].

As the number of simultaneously targeted genes increases, the risk of insufficient gene knockout, including mosaicism, will also increase. The investigation of *in vitro* electroporation-mediated multiple-gene editing has progressed [102, 103]. The reductions in mosaicism and the optimization of multiple-gene editing efficiency in zygotes/embryos achieved to date are inadequate; highly efficient direct gene modification is expected to be achieved in the near future.

### 5.3. Knock-in during embryogenesis

Knock-in of transgene(s) is a crucial approach for the generation of valuable pigs for experimental research, such as those with knock-in of human complement regulatory proteins (CD46, CD55, CD59, etc.) to reduce complement activity in xenotransplantation [41]. Although most knock-in pigs have been generated by SCNT using gene-edited somatic cells carrying transgenes as donor cells [31, 43, 44, 104–109], cytoplasmic microinjection of gene editors can also be used to successfully generate knock-in pigs [49, 110]. However, knock-in pigs have not been successfully generated by electroporation-mediated methods, because the introduction of large transgenes for knock-in is difficult using electroporation alone in pigs, as described above.

Direct knock-in during embryogenesis using gene editors has a wide range of applications. Although HDR followed by DSBs induced by a gene editor facilitates the generation of knock-in animals, the HDR efficiency and the resulting rate of knock-in events are low [16], in contrast to the high efficiency of Cas9 cleavage. Accordingly, the system needs to be optimized for practical use.

Various issues need to be resolved to achieve electroporation-mediated knock-in of transgenes into zygotes/embryos. Owing to the greater sensitivity of *in vitro*-fertilized porcine zygotes to electricity compared with that of *in vivo*-derived mouse embryos [52, 56], the size of
molecules that can be introduced into zygotes/embryos is limited. To efficiently deliver
knock-in donor DNA into zygotes without mechanical injury, an adeno-associated viral (AAV)
vector has been applied in mice [111] and rats [112] without removing the zona pellucida.
Although AAV vector-mediated gene modification in porcine cells has been adapted to generate
mutant pigs by combining it with SCNT techniques [113], the investigation of gene
modification during embryogenesis via an AAV vector is insufficient. The development of new
and efficient techniques for delivering large molecules into zygotes and embryos is crucial.

5.4. Introduction of point mutations during embryogenesis

A large number of disease-causing single-nucleotide polymorphisms have been identified
in humans. Although post-DSB gene corrections by gene editors often induce random insertions
and deletions at the target locus, the co-introduction of an ssODN as a template enables the
introduction of point mutations in precise positions via the HDR pathway. In the use of gene
editors, challenges related to the establishment of human disease models originating from point
mutations [23, 114–116] and humanized pigs expressing human insulin [117] have been
reported. However, the SCNT technique was used in almost all of these studies [114–117].
Inhibition of NHEJ or enhancement of HDR is crucial for achieving targeted gene knock-ins or
point mutations at precise positions during embryogenesis [118]. At present, the low frequency
of HDRs in porcine zygotes/embryos limits the utilization of this methodology. Despite
progress in trials aimed at enhancing HDR using an NHEJ inhibitor or HDR enhancer in cell
lines and mouse/rabbit embryos [119], studies using porcine zygotes/embryos are required.

The CRISPR/Cas-mediated base editor system, another approach for the introduction of a
point mutation at a precise position without dependence on HDR, generates mutations at a
single-base level [120, 121]. Cytosine base editors convert targeted C–G base pairs to T–A pairs,
and adenine base editors convert targeted A–T pairs into G–C pairs without causing DSBs.
Wang et al. demonstrated base editing in porcine fetal fibroblast cells using a modified base editor system [122], and Xie et al. generated base-edited pigs via cytoplasmic microinjection and SCNT [123]. These studies further support the feasibility of using pigs as human disease models. Although there are some technical limitations, such as insufficient specificity, protospacer adjacent motif (PAM) compatibility concerns, and a narrow active window [124], this technology has the potential to revolutionize gene therapy for genetic diseases and enable the efficient generation of animal models of diseases.

6. Conclusion

Owing to the development of gene-editing technologies, the generation of genetically modified pigs has dramatically expanded. However, some limitations remain. SCNT using gene-edited somatic cells ensures the generation of desired mutations in the resulting pigs, but requires sophisticated techniques. Microinjection- and electroporation-mediated gene editing are simple but limited by insecure knockout/knock-in efficiencies and mosaicism. However, various types of gene editors and their related technologies can be effectively applied to pigs using optimized and appropriate methods for introduction. In the future, gene editors will enable the on-demand preparation of pigs carrying desired mutations, including precise knock-ins.

Conflicts of interest

The authors declare no conflicts of interest.


Retroviruses (PERVs) Pol Genes in Porcine Embryos. *Animals (Basel)* 2019; 702(9).


SRCR5 domain are fully resistant to both PRRSV genotypes while maintaining biological function. *PLoS pathogens* 2017; 13: e1006206.


130. Yu HH, Zhao H, Qing YB, Pan WR, Jia BY, Zhao HY, Huang XX, Wei HJ. Porcine Zygote Injection with Cas9/sgRNA Results in DMD-Modified Pig with Muscle Dystrophy. *Int J Mol Sci* 2016; 17.


X-linked genetic disorders in pigs with ensured fertility. *Proc Natl Acad Sci USA* 2018; **115**: 708-713.


Figure 1. Schematic of major methods for generating genetically modified pigs using gene editors.
Figure 2. Trends in recent reports on generating genetically modified pigs using gene editors.

Number of PubMed articles reporting the generation of genetically modified pigs using gene editors over the last 10 years (2011–2020; search terms: “pig,” “ZFN,” “TALEN,” and “CRISPR”). (A) Total number of articles. (B) Changes in the number of articles per year, including information on gene editors and the method used to generate mutant pigs. SCNT, somatic cell nuclear transfer; CMI, cytoplasmic microinjection into zygotes/embryos; EP, electroporation into zygotes/embryos; CRISPR, clustered regularly interspaced short palindromic repeats; Cas, CRISPR-associated protein; TALEN, transcription activator-like effector nuclease; ZFN, zinc finger nuclease. Studies using multiple gene editors or multiple methods used to generate mutant pigs were classified into each relevant category and were therefore double-counted. Studies using CRISPR/Cas-related methods (e.g., Cas9 nickase and FokI-dCas9) were classified under “CRISPR.”
<table>
<thead>
<tr>
<th>Disease</th>
<th>Gene targeted</th>
<th>Method</th>
<th>Editor</th>
<th>Reference</th>
<th>CT, chromatin transfer; SCNT, somatic cell nuclear transfer; CMI, cytoplasmic microinjection; EP, electroporation.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>DAZL, APC, RUNX3, TP53</td>
<td>CT</td>
<td>TALEN, CRISPR</td>
<td>Tan et al. (2013)</td>
<td>[125]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SCNT</td>
<td>CRISPR</td>
<td>Kang et al. (2016)</td>
<td>[126]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SCNT</td>
<td>TALEN</td>
<td>Shen et al. (2017)</td>
<td>[127]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP</td>
<td>CRISPR</td>
<td>Tanihara et al. (2018)</td>
<td>[53]</td>
</tr>
<tr>
<td>Cardiomyopathy</td>
<td>MYH7, SGCD</td>
<td>SCNT</td>
<td>TALEN</td>
<td>Montag et al. (2018)</td>
<td>[114]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Matsunari et al. (2020)</td>
<td>[128]</td>
</tr>
<tr>
<td>Cryopyrin-associated periodic syndrome</td>
<td>NLRP3</td>
<td>SCNT</td>
<td>CRISPR</td>
<td>Li et al. (2020)</td>
<td>[116]</td>
</tr>
<tr>
<td>Diabetes</td>
<td>INS, SGCD</td>
<td>SCNT</td>
<td>CRISPR</td>
<td>Cho et al. (2018)</td>
<td>[129]</td>
</tr>
<tr>
<td></td>
<td>IAPP</td>
<td>SCNT</td>
<td>CRISPR</td>
<td>Zou et al. (2019)</td>
<td>[107]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP</td>
<td>CRISPR</td>
<td>Tanihara et al. (2020)</td>
<td>[54]</td>
</tr>
<tr>
<td>Duchenne muscular dystrophy</td>
<td>DMD</td>
<td>CMI</td>
<td>CRISPR</td>
<td>Yu et al. (2016)</td>
<td>[130]</td>
</tr>
<tr>
<td>Familial hypercholesterolemia</td>
<td>LDLR, ApoE, LDLR</td>
<td>SCNT</td>
<td>CRISPR</td>
<td>Carlson et al. (2012)</td>
<td>[59]</td>
</tr>
<tr>
<td>Human Waardenburg syndrome</td>
<td>MITF</td>
<td>SCNT, CMI</td>
<td>CRISPR</td>
<td>Wang et al. (2015)</td>
<td>[132]</td>
</tr>
<tr>
<td></td>
<td>MITF</td>
<td>CMI</td>
<td>CRISPR</td>
<td>Hai et al. (2017)</td>
<td>[133]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Chen et al. (2020)</td>
<td>[134]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Yan et al. (2018)</td>
<td>[109]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dorado et al. (2019)</td>
<td>[108]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Quadalqui et al. (2018)</td>
<td>[135]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Umeyama et al. (2016)</td>
<td>[136]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Matsunari et al. (2018)</td>
<td>[137]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Yao et al. (2014)</td>
<td>[138]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Zhou et al. (2015)</td>
<td>[139]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Wang et al. (2016)</td>
<td>[101]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Zhu et al. (2018)</td>
<td>[115]</td>
</tr>
<tr>
<td>Marfan syndrome</td>
<td>F9</td>
<td>SCNT</td>
<td>CRISPR</td>
<td>Chen et al. (2020)</td>
<td>[134]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Huang et al. (2017)</td>
<td>[131]</td>
</tr>
<tr>
<td>Huntington’s disease</td>
<td>HTT</td>
<td>SCNT</td>
<td>CRISPR</td>
<td>Carlson et al. (2012)</td>
<td>[59]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dorado et al. (2019)</td>
<td>[108]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Quadalqui et al. (2018)</td>
<td>[135]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Umeyama et al. (2016)</td>
<td>[136]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Matsunari et al. (2018)</td>
<td>[137]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Yao et al. (2014)</td>
<td>[138]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Zhou et al. (2015)</td>
<td>[139]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Wang et al. (2016)</td>
<td>[101]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Zhu et al. (2018)</td>
<td>[115]</td>
</tr>
<tr>
<td>Phenylnketonuria</td>
<td>PAH</td>
<td>CMI</td>
<td>CRISPR</td>
<td>Koppes et al. (2020)</td>
<td>[140]</td>
</tr>
<tr>
<td>Polycystic kidney disease</td>
<td>PKD1</td>
<td>SCNT</td>
<td>ZFN</td>
<td>He et al. (2015)</td>
<td>[141]</td>
</tr>
<tr>
<td>von Willebrand disease</td>
<td>vWF</td>
<td>CMI</td>
<td>CRISPR</td>
<td>Hai et al. (2014)</td>
<td>[48]</td>
</tr>
<tr>
<td>X-linked severe combined immunodeficiency</td>
<td>IL2RG</td>
<td>SCNT</td>
<td>ZFN</td>
<td>Watanabe et al. (2013)</td>
<td>[142]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SCNT, CMI</td>
<td>CRISPR</td>
<td>Kang et al. (2016)</td>
<td>[143]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CMI</td>
<td>CRISPR</td>
<td>Chen et al. (2019)</td>
<td>[144]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SCNT</td>
<td>CRISPR</td>
<td>Ren et al. (2020)</td>
<td>[145]</td>
</tr>
<tr>
<td>Reference</td>
<td>Method</td>
<td>Gene targeted</td>
<td>Introduced components</td>
<td>Gene-edited/total offspring and fetuses (%)</td>
<td>Mosaic/gene-edited (%)</td>
</tr>
<tr>
<td>--------------------</td>
<td>--------</td>
<td>------------------</td>
<td>--------------------------------------</td>
<td>---------------------------------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>Lillico et al. (2013) [46]</td>
<td>CMI</td>
<td>RELA</td>
<td>mRNA</td>
<td>1/9 (11.1)</td>
<td>0/1 (0)</td>
</tr>
<tr>
<td>Tallen et al. (2013) [46]</td>
<td>CMI</td>
<td>RELA</td>
<td>mRNA</td>
<td>8/39 (20.5)</td>
<td>2/8 (25.0)</td>
</tr>
<tr>
<td>Wang et al. (2016) [146]</td>
<td>CMI</td>
<td>B2M</td>
<td>mRNA</td>
<td>6/7 (85.7)</td>
<td>3/6 (50.0)</td>
</tr>
<tr>
<td>CRISPR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hai et al. (2014)   [48]</td>
<td>CMI</td>
<td>vWF</td>
<td>Cas9 mRNA and gRNA</td>
<td>11/16 (68.8)</td>
<td>2 or more/11 (-)</td>
</tr>
<tr>
<td>Whitworth et al. (2014) [47]</td>
<td>CMI</td>
<td>CD163</td>
<td>Cas9 mRNA and gRNA</td>
<td>4/4 (100)</td>
<td>0/4 (0)</td>
</tr>
<tr>
<td>Wang et al. (2015)  [132]</td>
<td>CMI</td>
<td>MITF</td>
<td>Cas9 mRNA and gRNA</td>
<td>2/2 (100)</td>
<td>0/2 (0)</td>
</tr>
<tr>
<td>Zhou et al. (2016)  [23]</td>
<td>CMI</td>
<td>Sox10 (point mutation)</td>
<td>Cas9 mRNA and gRNA</td>
<td>12/12 (100)</td>
<td>8/12 (66.7)</td>
</tr>
<tr>
<td>Peng et al. (2015)  [49]</td>
<td>CMI</td>
<td>recombinant human serum albumin (knock-in)</td>
<td>Cas9 mRNA and gRNA</td>
<td>16/16 (100)</td>
<td>1/16 (6.25)</td>
</tr>
<tr>
<td>Wang et al. (2016)  [101]</td>
<td>CMI</td>
<td>Parkin, DJ-1, PINK1</td>
<td>Cas9 mRNA and gRNA</td>
<td>2/2 (100)</td>
<td>0/2 (0)</td>
</tr>
<tr>
<td>Petersen et al. (2016) [66]</td>
<td>CMI</td>
<td>GGTA1</td>
<td>Plasmid</td>
<td>11/12 (91.7)</td>
<td>4/11 (36.4)</td>
</tr>
<tr>
<td>Yu et al. (2016)    [130]</td>
<td>CMI</td>
<td>DMD</td>
<td>Cas9 mRNA and gRNA</td>
<td>1/2 (50.0)</td>
<td>1/1 (100)</td>
</tr>
<tr>
<td>Kang et al. (2016)  [143]</td>
<td>CMI</td>
<td>IL2RG</td>
<td>Cas9 mRNA and gRNA</td>
<td>4/6 (66.7)</td>
<td>0/4 (0)</td>
</tr>
<tr>
<td>Park et al. (2017)  [147]</td>
<td>CMI</td>
<td>NANOS2</td>
<td>Cas9 mRNA and gRNA</td>
<td>18/18 (100)</td>
<td>5/18 (27.8)</td>
</tr>
<tr>
<td>Wu et al. (2017)    [148]</td>
<td>CMI</td>
<td>PDX1</td>
<td>Cas9 mRNA and gRNA</td>
<td>3/9 (33.3)</td>
<td>2/3 (66.7)</td>
</tr>
<tr>
<td>Hai et al. (2017)   [133]</td>
<td>CMI</td>
<td>MITF</td>
<td>Cas9 mRNA and gRNA</td>
<td>2/2 (100)</td>
<td>0/2 (0)</td>
</tr>
<tr>
<td>Hinrichs et al. (2018) [73]</td>
<td>CMI</td>
<td>GHR</td>
<td>Cas9 mRNA and gRNA</td>
<td>3/8 (37.5)</td>
<td>0/3 (0)</td>
</tr>
<tr>
<td>Xiang et al. (2018) [149]</td>
<td>CMI</td>
<td>IGF2</td>
<td>Nickase mRNA and gRNA</td>
<td>6/6 (100)</td>
<td>3/6 (50.0)</td>
</tr>
<tr>
<td>Whitworth et al. (2019) [150]</td>
<td>CMI</td>
<td>ANPEP</td>
<td>Cas9 mRNA and gRNA</td>
<td>13/18 (72.2)</td>
<td>3/13 (23.1)</td>
</tr>
<tr>
<td>Tu et al. (2019)    [151]</td>
<td>CMI</td>
<td>CMP-N-glycoylneuraminic acid hydroxylase</td>
<td>Cas9 mRNA and gRNA</td>
<td>5/6 (83.3)</td>
<td>3/5 (60.0)</td>
</tr>
<tr>
<td>Chen et al. (2019)  [144]</td>
<td>CMI</td>
<td>TYR, IL2RG, RAG1</td>
<td>Cas9 mRNA and gRNA</td>
<td>15/16 (93.8)</td>
<td>5/15 (33.3)</td>
</tr>
<tr>
<td>Tanihara et al. (2016) [52]</td>
<td>EP</td>
<td>MSTN</td>
<td>Cas9 protein and gRNA</td>
<td>9/10 (90.0)</td>
<td>4/9 (44.4)</td>
</tr>
<tr>
<td>Tanihara et al. (2018) [53]</td>
<td>EP</td>
<td>TP53</td>
<td>Cas9 protein and gRNA</td>
<td>6/9 (66.7)</td>
<td>4/6 (66.7)</td>
</tr>
<tr>
<td>Tanihara et al. (2020) [54]</td>
<td>EP</td>
<td>PDX</td>
<td>Cas9 protein and gRNA</td>
<td>9/10 (90.0)</td>
<td>4/9 (44.4)</td>
</tr>
<tr>
<td>Tanihara et al. (2020) [55]</td>
<td>EP</td>
<td>GGT A1</td>
<td>Cas9 protein and gRNA</td>
<td>5/6 (83.3)</td>
<td>2/5 (40.0)</td>
</tr>
</tbody>
</table>

CMI, cytoplasmic microinjection; EP, electroporation.
In this table, offspring/fetuses carrying three alleles or more, or extreme deviations in allele frequencies are denoted as mosaic.