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—Review—

Current status of the application of gene editing in pigs

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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: Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (CRISPR/Cas), Gene editing, Pig, Transcription activator-like effector nuclease (TALEN), Zinc finger nuclease (ZFN)

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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

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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.

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 *FokI*) acting as a heterodimer. Zinc finger domains recognize specific sequences in genomic DNA, after which *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 Xanthomonas. TALENs have a TALE as the DNA-binding domain and *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.

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 (Fig. 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,

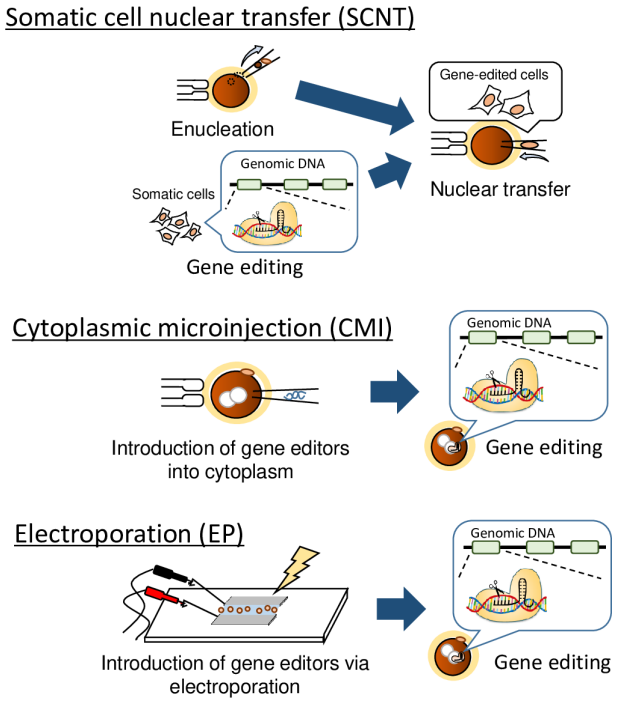

Fig. 1. Schematic of major methods for generating genetically modified pigs using gene editors.

and early postnatal death; this is a limitation of SCNT [45].

Direct introduction of gene editors during embryogenesis

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.

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.

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.

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.

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

Table 1. Gene-edited pigs to establish models for intractable diseases

Disease	Gene targeted	Method	Editor	Reference	
Cancer	DAZL, APC	CT	TALEN, CRISPR	Tan *et al*. (2013)	[125]
	RUNX3	SCNT	CRISPR	Kang *et al*. (2016)	[126]
	TP53	SCNT	TALEN	Shen *et al*. (2017)	[127]
	TP53	EP	CRISPR	Tanihara *et al*. (2018)	[53]
Cardiomyopathy	MYH7	SCNT	TALEN	Montag *et al*. (2018)	[114]
	SGCD	SCNT	TALEN	Matsunari *et al*. (2020)	[128]
Cryopyrin-associated periodic syndrome	NLRP3	SCNT	CRISPR	Li *et al*. (2020)	[116]
Diabetes	INS	SCNT	CRISPR	Cho *et al*. (2018)	[129]
	IAPP	SCNT	CRISPR	Zou *et al*. (2019)	[107]
	PDX1	EP	CRISPR	Tanihara *et al*. (2020)	[54]
Duchenne muscular dystrophy	DMD	CMI	CRISPR	Yu *et al*. (2016)	[130]
Familial hypercholesterolemia	LDLR	SCNT	TALEN	Carlson *et al*. (2012)	[59]
	ApoE, LDLR	SCNT	CRISPR	Huang *et al*. (2017)	[131]
Human Waardenburg syndrome	MITF	SCNT, CMI	CRISPR	Wang *et al*. (2015)	[132]
	MITF	CMI	CRISPR	Hai *et al*. (2017)	[133]
Hemophilia B	F9	SCNT	CRISPR	Chen *et al*. (2020)	[134]
Huntington's disease	HTT	SCNT	CRISPR	Yan *et al*. (2018)	[109]
Hutchinson–Gilford progeria syndrome	NLRP3	SCNT	CRISPR	Dorado *et al*. (2019)	[108]
Leigh syndrome	SURF1	SCNT	TALEN, CRISPR	Quadalti *et al*. (2018)	[135]
Marfan syndrome	FBN1	SCNT	ZFN	Umeyama *et al*. (2016)	[136]
Ornithine transcarbamylase deficiency	OTC	SCNT	TALEN	Matsunari *et al*. (2018)	[137]
Parkinson's disease	GGTA1, Parkin, DJ-1	SCNT	TALEN	Yao *et al*. (2014)	[138]
	TYR, PINK1, PARK2	SCNT	CRISPR	Zhou *et al*. (2015)	[139]
	Parkin, DJ-1, PINK1	CMI	CRISPR	Wang *et al*. (2016)	[101]
	SCNA	SCNT	CRISPR	Zhu *et al*. (2018)	[115]
Phenylketonuria	PAH	CMI	CRISPR	Koppes *et al*. (2020)	[140]
Polycystic kidney disease	PKD1	SCNT	ZFN	He *et al*. (2015)	[141]
von Willebrand disease	vWF	CMI	CRISPR	Hai *et al*. (2014)	[48]
X-linked severe combined immunodeficiency	IL2RG	SCNT	ZFN	Watanabe *et al*. (2013)	[142]
	IL2RG	SCNT, CMI	CRISPR	Kang *et al*. (2016)	[143]
	IL2RG	CMI	CRISPR	Chen *et al*. (2019)	[144]
	IL2RG	SCNT	CRISPR	Ren *et al*. (2020)	[145]

CT, chromatin transfer; SCNT, somatic cell nuclear transfer; CMI, cytoplasmic microinjection; EP, electroporation.

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.

Current Status and Future Prospects of Gene-edited Pigs

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.

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

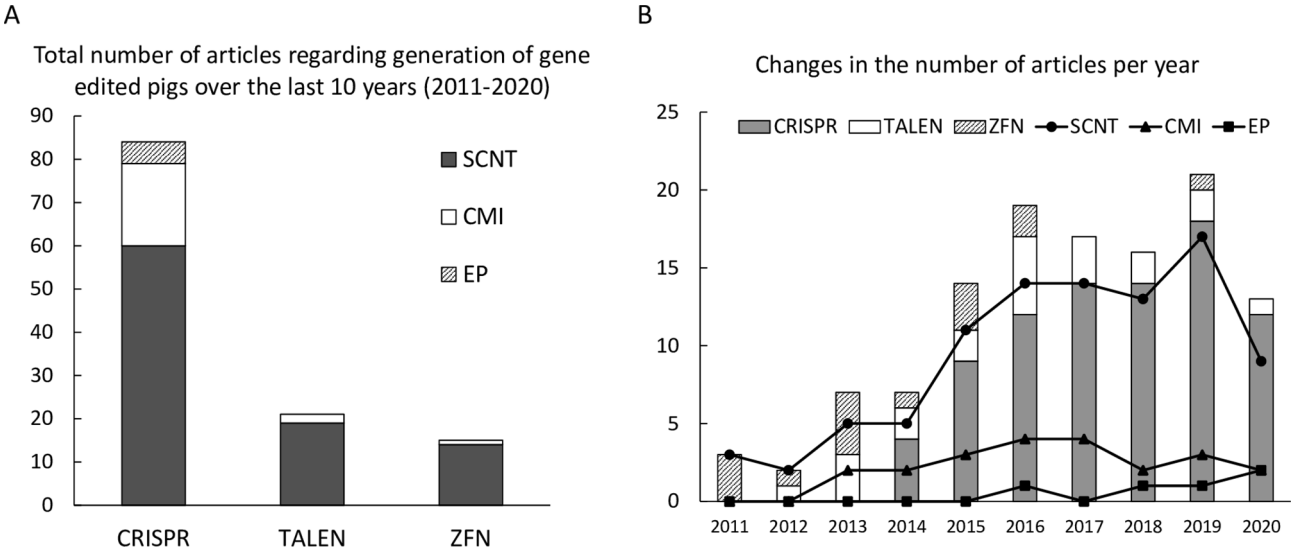

Fig. 2. Trends in recent reports on generating genetically modified pigs using gene editors. Number of PubMed articles reporting the generation of genetically modified pigs using gene editors over the last 10 years (2011–2020; search terms: "pig," "ZFN," "TALEN," and "CRISPR"). (A) Total number of articles. (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 2. Gene editing efficiency and mosaicism of resulting offspring/fetuses in studies using cytoplasmic microinjection- or electroporation-mediated gene editing

Reference		Method	Gene targeted	Introduced components	Gene-edited/ total offspring and fetuses (%)	Mosaic/gene-edited (%)
ZFN						
Lillico *et al*. (2013)	[46]	CMI	RELA	mRNA	1/9 (11.1)	0/1 (0)
TALEN						
Lillico *et al*. (2013)	[46]	CMI	RELA	mRNA	8/39 (20.5)	2/8 (25.0)
Wang *et al*. (2016)	[146]	CMI	B2M	mRNA	6/7 (85.7)	3/6 (50.0)
CRISPR						
Hai *et al*. (2014)	[48]	CMI	vWF	Cas9 mRNA and gRNA	11/16 (68.8)	2 or more/11 (-)
Whitworth *et al*. (2014)	[47]	CMI	CD163	Cas9 mRNA and gRNA	4/4 (100)	0/4 (0)
			CD1D	Cas9 mRNA and gRNA	4/4 (100)	1/4 (25.0)
Wang *et al*. (2015)	[132]	CMI	MITF	Cas9 mRNA and gRNA	2/2 (100)	0/2 (0)
Zhou *et al*. (2016)	[23]	CMI	Sox10 (point mutation)	Cas9 mRNA and gRNA	12/12 (100)	8/12 (66.7)
Peng *et al*. (2015)	[49]	CMI	recombinant human serum albumin (knock-in)	Cas9 mRNA and gRNA	16/16 (100)	1/16 (6.25)
Wang *et al*. (2016)	[101]	CMI	Parkin, DJ-1, PINK1	Cas9 mRNA and gRNA	2/2 (100)	0/2 (0)
Petersen *et al*. (2016)	[66]	CMI	GGTA1	Plasmid	11/12 (91.7)	4/11 (36.4)
Yu *et al*. (2016)	[130]	CMI	DMD	Cas9 mRNA and gRNA	1/2 (50.0)	1/1 (100)
Kang *et al*. (2016)	[143]	CMI	IL2RG	Cas9 mRNA and gRNA	4/6 (66.7)	0/4 (0)
Park *et al*. (2017)	[147]	CMI	NANOS2	Cas9 mRNA and gRNA	18/18 (100)	5/18 (27.8)
Wu *et al*. (2017)	[148]	CMI	PDX1	Cas9 mRNA and gRNA	3/9 (33.3)	2/3 (66.7)
Hai *et al*. (2017)	[133]	CMI	MITF	Cas9 mRNA and gRNA	2/2 (100)	0/2 (0)
Hinrichs *et al*. (2018)	[73]	CMI	GHR	Cas9 mRNA and gRNA	3/8 (37.5)	0/3 (0)
Xiang *et al*. (2018)	[149]	CMI	IGF2	Nickase mRNA and gRNA	6/6 (100)	3/6 (50.0)
Whitworth *et al*. (2019)	[150]	CMI	ANPEP	Cas9 mRNA and gRNA	13/18 (72.2)	3/13 (23.1)
Tu *et al*. (2019)	[151]	CMI	CMP-N-glycolylneuraminic acid hydroxylase	Cas9 mRNA and gRNA	5/6 (83.3)	3/5 (60.0)
Chen *et al*. (2019)	[144]	CMI	TYR, IL2RG, RAG1	Cas9 mRNA and gRNA	15/16 (93.8)	5/15 (33.3)
Tanihara *et al*. (2016)	[52]	EP	MSTN	Cas9 protein and gRNA	9/10 (90.0)	4/9 (44.4)
Tanihara *et al*. (2018)	[53]	EP	TP53	Cas9 protein and gRNA	6/9 (66.7)	4/6 (66.7)
Tanihara *et al*. (2020)	[54]	EP	PDX	Cas9 protein and gRNA	9/10 (90.0)	4/9 (44.4)
Tanihara *et al*. (2020)	[55]	EP	GGTA1	Cas9 protein and gRNA	5/6 (83.3)	2/5 (40.0)

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.

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.

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.

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.

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.

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.

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