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Chapter

Recent Advance in Genome Editing-Based Gene Modification in Pigs

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Abstract

Recently, a series of genome editing technologies including ZFNs, TALENs, and CRISPR/Cas9 systems have enabled gene modification in the endogenous target genes of various organisms including pigs, which are important for agricultural and biomedical research. Owing to its simple application for gene knockout and ease of use, the CRISPR/Cas9 is now in common use worldwide. The most important aspect of this process is the selection of the method used to deliver genome editing components to embryos. In earlier stages, zygote microinjection of these components [single guide RNA (sgRNA) + DNA/mRNA for Cas9] into the cytoplasm and/or nuclei of a zygote has been frequently employed. However, this method is always associated with the generation of mosaic embryos in which genome-edited and unedited cells are mixed together. To avoid this mosaic issue, *in vitro* electroporation of zygotes in the presence of sgRNA mixed with Cas9 protein, referred to as a ribonucleoprotein (RNP), is now in frequent use. This review provides a historical background of the production of genome-edited pigs and also presents current research concerning how genome editing is induced in somatic cell nuclear transfer-derived embryos that have been reconstituted with normal nuclei.

Keywords: genome editing, CRISPR/Cas9, ZFNs, TALENs, pigs, gene modification, microinjection, electroporation, somatic cell nuclear transfer, knock out, knock in, gene-engineered, ribonucleoprotein

1. Introduction

The domestic pig has been widely used as a large animal model in biomedical research, as it is similar to humans with respect to the size of body and internal organs, longevity, anatomy, physiology, and metabolic profile [1]. Modification of the porcine genome is also important for studying the mechanisms underlying genetic disorders, developing therapeutic drugs, and improving pig meat production yields [2, 3]. Over the past three decades, attempts have been made to modify the porcine genome using genetic engineering technology, starting after Gordon et al. [4] first reported DNA microinjection (MI)-based production of transgenic (Tg) mice. Hammer et al. [5] first reported the successful production of Tg piglets using the technique reported by Gordon et al. [4], but attaining this result was more difficult than for rodents, where pronuclei are clearly visible using an optical microscope.

In the case of porcine zygotes, pronuclei are difficult to see due to the presence of high lipid content in the cytoplasm. Researchers must briefly centrifuge zygotes to visualize the pronuclei prior to MI [5], which is labor-intensive and requires skill. Moreover, MI-mediated transgene integration into host chromosomes occurs randomly, which often causes gene silencing [6]. However, for precise and efficient genetic modification in the porcine genome, homologous recombination (HR)-based gene targeting technology may be recommended, which was first developed by Smithies' group in mice [7]. In this case, the use of germline-competent embryonic stem (ES) cells is a prerequisite. These ES cells are first transfected with a targeting vector and then recombinant ES clones showing successful targeting are obtained. This vector usually contains a gene of interest (GOI) to be integrated into the target locus, together with a selection marker gene such a neomycin resistance gene (*neo*), and DNA sequences of appropriate length, termed homology arms (HA), that correspond to the endogenous target gene, are placed at both ends of the DNA containing the GOI and a marker gene. Chimeric mice can be obtained through blastocyst injection with the targeted ES clones, and the resulting chimeric mice would contribute to produce heterozygous mice carrying mutated traits (GOI/selection marker gene) in the target locus [8]. Unfortunately, there are no germline-competent porcine ES cells, despite extensive efforts [9–13]. Thus, to date, production of gene-targeted pigs derived from recombinant porcine ES cells has not yet been successful.

In 1996, scientists at *the Roslin Institute* (Wilmut and colleagues) first succeeded in producing cloned sheep using somatic cell nuclear transfer (SCNT) technology. They used fetal fibroblasts [14] or adult mammary gland-derived fibroblasts [15] as SCNT donors. Notably, prior to these reports, an attempt to produce cloned embryos by the similar technique shown by the *Roslin's* group has been made by Prather et al. [16], who performed transfer of nuclei from two, four and eight-cell embryos to the enucleated oocytes and successfully produced one piglets that had been derived from the four-cell embryo's nucleus. This approach is called "blastomere transplantation," which is basically different from the somatic cell-based SCNT. Generally, fibroblasts used as SCNT donors can proliferate actively *in vitro*, and therefore are considered to be ideal cells for transgenesis or gene targeting. If these engineered fibroblasts are used for SCNT, the resulting cloned embryos and piglets should have the engineered traits in their genome. This possibility was first proven by *the scientists at the Roslin Institute* [17, 18] who successfully produced genetically engineered (GE) cloned sheep through SCNT using GE fetal fibroblasts. Since then, SCNT using GE cells as SCNT donors has been a common approach for production of knockout (KO) or Tg piglets [19, 20]. However, as mentioned below, the efficiency of producing cloned GE piglets is extremely low and the preparation of GE donor cells is laborious and time-consuming [21]. During the past two decades, production of only a few GE (KO) piglets has been reported by traditional approaches [22–27]. Moreover, almost all resulting KO piglets were heterozygous with respect to the KO allele, thus requiring additional tasks such as breeding (likely one or two generations), sequence targeting, or *in vitro* cell cloning to obtain homozygous KO animals [28], which is also laborious, expensive, and time-consuming.

However, this situation drastically changed when new gene-targeting technologies emerged for precisely manipulating mammalian genomes, called "second-generation genome editing." These technologies require the design of site-specific engineered nucleases which can be zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) or clustered regularly interspaced short palindromic repeat-associated protein 9 (CRISPR/Cas9) nucleases, all of which induce a double-stranded break (DSB) at a specific site in the genome. This DSB facilitates genetic modification such as nonhomologous end-joining (NHEJ) and homology directed repair (HDR) [29], as described below. Using these genome-editing systems, many

GE piglets have been produced using SCNT of genome-edited cells, or direct micro-injection of genome-editing components (including engineered endonucleases) into the cytoplasm of zygotes, as described below in more detail.

2. Background of second-generation genome editing

As mentioned above, site-specific engineered nucleases are used in these genome-editing techniques. ZFNs, TALENs, and CRISPR/Cas9 can all bind to DNA and induce DSB, which triggers endogenous DNA repair. If the template DNA is absent, the DSB is repaired via the NHEJ pathway where insertion or deletion of nucleotides (hereinafter called "indels") can happen in the cleaved area. These indels often cause frameshift of the amino acid sequence, leading to the generation of abnormal proteins or formation of a premature stop codon leading to cessation of protein synthesis. If template DNA homologous to the target site is present, it is inserted into the cleaved area via a site-specific HR event which is called HDR. Generally, NHEJ occurs in cells independent of its cell cycle, but HDR occurs primarily in dividing cells [30].

The ZFN technique uses the ZF protein (which binds to the target DNA) and the endonuclease *Fok* I (which cleaves DNA) [31]. ZF protein has several protein motifs capable of recognizing specific sequences of three nucleotides and binding to them. Notably, Urnov et al. [32] first demonstrated that ZFN is effective to induce DNA editing at the endogenous target gene in mammalian cells. Its targeting efficiency was over 18% in the absence of drug selection, which is ~1000-fold higher than that achieved by traditional gene targeting.

The TALEN technique uses proteins, termed transcription activator-like effectors (TALEs), which contain 33–35 amino acid repeats that flank a central DNA binding region (amino acids 12 and 13), and *Fok* I nuclease, as in ZFN, thus the term TALE nucleases (TALENs) [33–35]. Notably, the design and engineering of TALENs is simpler than that of ZFN, and thus can be done faster [35, 36].

CRISPR/Cas9 employs a short (20 bp) RNA sequence called single-guide RNA (sgRNA) which can bind to the specific chromosomal DNA site together with the Cas9 endonuclease [37–40]. Once bound, two independent nuclease domains in Cas9 each cleave one of the DNA strand's three bases upstream of the protospacer adjacent motif (PAM), introducing DSB at the target site of the host chromosome, which is then repaired by NHEJ. This system is different from the other genome editing tools such as ZFNs and TALENs, and thus synthesis of sgRNA is a *prerequisite* for this system. This development dramatically reduced both the complexity and time required for the design and implementation of gene editing.

3. History of GE in pigs

Table 1 lists instances of production of GE piglets with genome editing technology from 2011 to 2018. This section provides a brief explanation on the background of GE pig production.

In 2011, three types of GE piglets were produced using ZFNs from different laboratories. All of these piglets were produced by SCNT using GE cells as a SCNT donor. The first report showing successful production of GE piglets involved the disruption of enhanced green fluorescent protein (*EGFP*) gene in a hemizygous manner. Whyte et al. [41] demonstrated that a ZFN pair efficiently inactivated the expression of *EGFP* that was integrated into the chromosomes of porcine fibroblasts via the NHEJ pathway with an efficiency of ~5%. From this experiment, it

Method	Genome editing tool (mode for gene modification)	Method for gene modification	Outcome	Target gene	References
SCNT	ZFN (indels)	Using adult porcine ear fibroblasts hemizygous for the eGFP transgene	Seven of nine embryos (Day 12) exhibited loss of fluorescence	*eGFP* transgene	[41]
SCNT	ZFN (indels)	Using porcine fibroblasts transfected with ZFN plasmid	Of 10 live piglets delivered, two carried the predicted ZFN-induced mutation; lower expression of both *PPAR-γ1* and *PPAR-γ2* was observed in those clones	*PPARγ*	[42]
SCNT	ZFN (indels)	Using porcine fetal fibroblasts transfected with ZFN plasmid	Of six fetuses, all completely lacked α-Gal epitopes	*GGTA1*	[43]
MI	TALEN (indels)	Cytoplasmic MI of TALEN mRNA toward IVF-derived zygotes	CI of TALEN mRNAs inducing gene KO in up to 75% of embryos; Of the 18 live-born clones, eight contained monoallelic mutations and 10 contained biallelic modifications of the *LDLR* gene	*LDLR*	[44]
MI	ZFN, TALEN (indels)	Cytoplasmic MI of ZFN or TALEN mRNA toward *in vivo* fertilized zygotes	Of 39 piglets produced, eight carried TALEN-derived editing events (21%); of nine piglets produced, one carried an editing event at the ZFN target site (11%)	*NF-kappaB subunit*	[45]
SCNT	TALENs (indels)	Using porcine fetal fibroblasts transfected with TALEN plasmid	Three piglets with biallelic mutations of the *GGTA1* gene exhibited loss of α-Gal epitopes on the surface of cells	*GGTA1*	[46]
SCNT	TALENs (indels/KI)	Using porcine fibroblasts transfected with TALEN mRNA + ssODN	Of eight piglets born from *DAZL*-modified cells, three are still born; of the six piglets from *APC*-modified cells, only one alive	*DAZL*, *APC*	[47]
SCNT	ZFNs (indels)	Using porcine fetal fibroblasts transfected with ZFN mRNA	The resulting *IL2RG* KO pigs completely lacked a thymus and were deficient in T and NK cells, similar to human X-linked SCID patients	*IL2RG*	[48]
SCNT	ZFN (indels)	Using porcine adult liver-derived cells transfected with ZFN plasmid through the two-steps	Four viable and healthy cloned pigs obtained exhibited disruption of the *GGTA1* and the *CMAH* loci	*GGTA1*, *CMAH*	[49]
SCNT	ZFN (KI)	Using porcine fibroblast cells transfected with ZFN plasmid and donor DNA	Successfully produced healthy monoallelic/biallelic CMAH KO pigs	*CMAH*	[50]

Method	Genome editing tool (mode for gene modification)	Method for gene modification	Outcome	Target gene	References
SCNT/MI	CRISPR/Cas9 (KI/indels)	Using porcine fetal fibroblasts transfected with sgRNA-Cas9 plasmids + donor DNA/cytoplasmic MI of Cas9 mRNA/sgRNA toward IVF-derived zygotes	Of the *CD163* recipients, five delivered healthy piglets by cesarean section; 12 of the 13 piglets contained either a biallelic or homozygous deletion of *CD1D*	*eGFP*, *CD163*, *CD1D*	[51]
MI	CRISPR/Cas9 (indels)	Cytoplasmic MI of Cas9 mRNA/sgRNA toward *in vivo* fertilized zygotes	Ten of 16 resulting piglets had indels with an efficiency of 63% and were comprised by cells with monoallelic mutant; they can be a model for von Willebrand disease	*vWF*	[52]
SCNT	CRISPR/Cas9 (indels)	Using porcine fetal fibroblasts transfected with Cas9/sgRNA expression plasmid	A total of three piglets were obtained; fibroblasts from all three animals were negative for class I SLA cell surface expression	*class I MHC*	[53]
SCNT	TALENs (indels)	Using pig fetal fibroblasts transfected with TALEN plasmid	Of 27 live cloned piglets obtained, nine were targeted with biallelic mutations in *RAG1*, three were targeted with biallelic mutations in *RAG2*, and 10 were targeted with a monoallelic mutation in *RAG2*	*RAG1*, *RAG2*	[54]
SCNT	ZFN (indels)	Using pig fetal fibroblasts transfected with ZFN plasmid	Three *GGTA1* null piglets showing loss of α-Gal epitope expression were born	*GGTA1*	[55]
SCNT	CRISPR/Cas9 (indels)	Using pig liver-derived cells transfected with two or three plasmids expressing Cas9 and sgRNA targeting to GGTA1, CMAH, or putative iGb3S genes	Of 10 fetuses obtained, five had mutations in both the *GGTA1* and *CMAH* genes	*GGTA1*, *CMAH*, *putative iGb3S*	[56]
SCNT	ZFNs (indels)	Using pig fetal fibroblasts transfected with ZFN plasmid	The *MSTN*-mutant pigs grew normally, had increased muscle mass with decreased fat accumulation	*MSTN*	[57]
SCNT	TALENs (indels)	Using pig liver-derived cells transfected with TALEN plasmid	Livers from *ASGR1*−/− pigs exhibit decreased human platelet uptake	*ASGR1*	[58]
SCNT	ZFN/TALEN (indels)	Using pig fetal fibroblasts transfected with TALEN or ZFN mRNA	One of the cloned pigs generated GalT/CMAH-double homozygous KO pigs	*CMAH*	[59]
SCNT	CRISPR/Cas9 (KI)	Using pig fetal fibroblasts transfected with targeting donor vector and two expression vectors for sgRNA and Cas9	Highly efficient KI (up to 54%) was achieved after drug selection; one cloned piglet obtained showed correct targeting	*H11*	[60]

Method	Genome editing tool (mode for gene modification)	Method for gene modification	Outcome	Target gene	References
SCNT	CRISPR/Cas9	Using pig fetal fibroblasts transfected with sgRNA, Cas9 expression plasmids	Four cloned double KO piglets showing loss of expression for both *PARK2* and *PINK1* were produced	*TYR*, *PARK2*, *PINK1*	[61]
MI	CRISPR/Cas9 (KI)	Cytoplasmic MI of Cas9 mRNA + sgRNA + ssODN toward *in vivo* fertilized zygotes	Two live-born piglets obtained showed the white coat-color phenotype over its entire body	*MITF*	[62]
MI	CRISPR/Cas9 (KI)	Cytoplasmic MI of Cas9 mRNA + sgRNA + circular vector toward *in vivo* fertilized zygotes	All 16 piglets born were healthy and carried the expected KI allele; the KI allele was successfully transmitted through germline	*Alb*	[63]
MI	CRISPR/Cas9 (indels)	Cytoplasmic MI of Cas9 mRNA and sgRNA toward *in vivo* fertilized zygotes	Bi-allelic modifications of pig *Npc1l1* were achieved at the efficiency as high as 100%	*Npc1l1*	[64]
SCNT	CRISPR/Cas9 (indels)	Using pig fetal fibroblasts transfected with sgRNA-Cas9 encoding vector	Of eight marker-gene-free cloned pigs with biallelic mutations obtained, some showed phenotypes similar to DM	*MSTN*	[65]
SCNT	CRISPR/Cas9 (indels)	Using liver-derived cells transfected with sgRNA-Cas9 encoding vectors	One triple knockout pig was obtained; Cells from this cloned pig exhibited reduced human IgM and IgG binding	*GGTA1*, *CMAH*, *β4GalNT2*	[66]
SCNT	CRISPR/Cas9 (indels)	Using pig fetal fibroblasts transfected with sgRNA-Cas9 encoding vector	Three cloned piglets with biallelic mutation produced showed no antibody-producing B cells	*IgM* J_H	[67]
EP	CRISPR/Cas9 (indels)	Using Cas9 protein and sgRNA (RNP) toward IVF-derived zygotes	The use of gene editing by electroporation of Cas9 protein (GEEP) resulted in highly efficient targeted gene disruption and efficient production of *Myostatin* mutant pigs	*MSTN*	[68]
MI	CRISPR/Cas9 (indels)	Cytoplasmic MI of Cas9 mRNA and sgRNA toward *in vivo* fertilized zygotes	Of two piglets obtained, one piglets exhibited *DMD* phenotype, as exemplified by degenerative and disordered skeletal and cardiac muscle	*DMD*	[69]
SCNT	ZFN (indels)	Using pig fetal fibroblasts transfected with ZFN-encoding mRNA	The heterozygous *FBN1* mutant pigs obtained exhibited abnormal phenotype, which resembles MFS found in humans	*FBN1*	[70]

Method	Genome editing tool (mode for gene modification)	Method for gene modification	Outcome	Target gene	References
SCNT	CRISPR/Cas9 (KI)	Using pig fetal fibroblasts transfected with Cas9-sgRNA expression vector + donor DNA containing Cre/loxP system	Two male live piglets with mono-allelic *MSTN* KO obtained exhibited enhanced myofiber quantity, but the myofiber size remained unaltered	*MSTN*	[71]
SCNT	TALENs (indels)	Using pig fetal fibroblasts transfected with TALEN plasmids	In total, 12 live and two stillborn piglets were collected; all fetuses and piglets exhibited homozygous *GGTA1*-null mutation	*GGTA1*	[72]
SCNT	TALENs (indels)	Using porcine fetal fibroblasts co-transfected with TALEN and hDAF expression plasmids	Six live-born piglets and three stillborn piglets were obtained; the piglets showed eight base mono-allelic mutations of *GGTA1* and *hDAF* expression	*GGTA1*	[73]
SCNT	CRISPR/Cas9 (indels)	Using fetal fibroblast cells transfected with sgRNA-Cas9 encoding vector	Four live *RUNX3* KO piglets with monoallelic mutation showed the lack of RUNX3 protein in their internal organ system	*RUNX3*	[74]
SCNT	TALENs (indels)	Using dermal fibroblasts transfected with TALEN plasmids	*MSTN* KO piglets exhibited a double-muscled phenotype, possessing a higher body weight and longissimus muscle mass measuring 170% that of wild-type piglets, with double the number of muscle fibers	*MSTN*	[75]
SCNT	TALENs/Cas9 (KI)	Using fetal fibroblast cells transfected with Cas9/sgRNA or TALEN vector + ssODN	Of seven cloned piglets, some expressed human insulin	*INS*	[76]
SCNT	CRISPR/Cas9 (KI)	Using fetal fibroblast cells transfected with sgRNA-Cas9 encoding vector + ssODN	One cloned stillborn piglet harbored the orthologous p.C313Y mutation at the *MSTN* locus	*APP*, *LRRK2*, MSTN	[77]
SCNT	TALENs (indels)	Using ear fibroblasts transfected with TALEN vectors	Thirty *GGTA1* biallelic KO piglets were successfully delivered and grew normally.	*GGTA1*	[78]
MI	CRISPR/Cas9 (KI)	Cytoplasmic MI of Cas9 mRNA + sgRNA + ssODN toward *in vivo* fertilized zygotes	Of five piglets delivered alive, three exhibited pigmentary disorders with light-colored iris in eye, which was observed in patients harboring *Sox10* mutations	*Sox10*	[79]
MI	CRISPR/Cas9 (indels)	Cytoplasmic MI of sgRNA-Cas9 encoding vector toward *in vivo* fertilized zygotes	Of six healthy fetuses recovered, four exhibited loss of α-Gal epitope expression, indicating a biallelic KO of *GGTA1*	*GGTA1*	[80]

Method	Genome editing tool (mode for gene modification)	Method for gene modification	Outcome	Target gene	References
MI	CRISPR/Cas9 (indels)	Cytoplasmic MI of Cas9 mRNA + three types of sgRNAs toward *in vivo* fertilized zygotes	Of two live-born piglets delivered, one piglet showed biallelic modification of all three genes, and another showed biallelic modification of the *DJ-1* and *PINK1* genes and monoallelic mutations of *parkin* gene	*Parkin*, *DJ-1*, *PINK1*	[81]
SCNT-MI	CRISPR/Cas9 (indels)	Cytoplasmic MI of RNP toward SCNT embryos	Six fetuses recovered revealed that all fetuses carried biallelic edits for the *GRB10* gene (6/6, 100%)	*GRB10*	[82]
SCNT	CRISPR/Cas9 (indels)	Using kidney fibroblasts transfected with ZFN vectors	Two healthy normal females with *GGTA1*, *CMAH* double KO phenotypes are currently being raised for mating	*GGTA1*, *CMAH*	[83]
MI	CRISPR/Cas9 (indels)	Cytoplasmic MI of Cas9 mRNA + sgRNA toward IVF-derived zygotes	Seventeen live piglets and two stillborn were produced; all had mutations in both genes (no pigs with wild-type sequence)	*RAG2*, *IL2RG*	[84]
MI	CRISPR/Cas9 (indels)	Cytoplasmic MI of Cas9 mRNA + sgRNA toward *in vivo* fertilized zygotes	Eighteen piglets recovered showed either mono- or bi-allelic modifications and no wild-type animals; *NANOS2* KO pigs phenocopied KO mice with male specific germline ablation	*NANOS2*	[85]
SCNT	CRISPR/Cas9 (indels)	Using fetal fibroblasts transfected with sgRNA and Cas9 expression vectors	Six biallelic KO pigs with mutations in *ApoE* and *LDLR* genes were obtained successfully in a single step.	*ApoE*, *LDLR*	[86]
SCNT	TALENs	Using fetal fibroblasts transfected with TALEN plasmid	All six live piglets obtained carried biallelic mutations in the *P53* locus	*P53*	[87]
SCNT	TALEN (indels)	Using fetal fibroblasts transfected with TALEN plasmid	A total of 18 live piglets were obtained; they showed hypermuscular characteristics	*MSTN*	[88]
SCNT	CRISPR/Cas9 (indels)	Using fetal fibroblasts transfected with sgRNA-Cas9 encoding vectors	A total of 37 PERV-inactivated piglets were generated; 15 piglets remain alive	*PERV*	[89]

Method	Genome editing tool (mode for gene modification)	Method for gene modification	Outcome	Target gene	References
SCNT	CRISPR/Cas9 (indels)	Using fetal fibroblasts transfected with sgRNA-Cas9 encoding vectors	Of 26 female piglets delivered, 23 piglets carried mutations in the *MSTN* locus; the bi-allelic KO pigs were viable and exhibited partial double-muscled phenotype	*MSTN*	[90]
SCNT	CRISPR/Cas9 (KI)	Using fetal fibroblasts transfected with Cas9-gRNA plasmid and targeting vector	Twelve male piglets were born and expressed *UCP1* in a tissue-specific manner	*UCP1*	[91]
MI	CRISPR/Cas9 (KI)	Cytoplasmic MI of RNP toward *in vivo* fertilized zygotes	A total of 18 fetuses/born piglets were obtained; successful insertion of pseudo *attP* sites within the *COL1A* locus was observed	*COL1A*	[92]
MI	CRISPR/Cas9 (indels)	Cytoplasmic MI of Cas9 mRNA + sgRNAs toward *in vivo*-derived zygotes	Indels in 92–100% of the embryos analyzed; all resulting 12 piglets had biallelic edits of *TMRPSS2*	*TMPRSS2*	[93]
MI	CRISPR/Cas9 (indels)	Direct pronuclear microinjection of Cas9-gRNA plasmid	Of seven born piglets, one exhibited biallelic KO phenotype and one did monoallelic KO one	*GGTA1*	[94]
MI	CRISPR/Cas9 (indels)	Cytoplasmic MI of Cas9 mRNA and dual sgRNAs toward *in vivo* fertilized zygotes	Of nine fetuses examined, three exhibited bi-allelic mutations at the *PDX1* locus; in those fetuses pancreatic primordium was highly disorganized	*PDX1*	[95]
SCNT (Handmade cloning)	CRISPR/Cas9 (indels)	Using fetal fibroblasts transfected with sgRNA and Cas9 expression vectors	Eleven live bi-allelic *GGTA1*/*CMAH* double KO piglets were obtained with the identical phenotype	*GGTA1*, *CMAH*	[96]
EP	CRISPR/Cas9	Using Cas9 protein and sgRNA (RNP) toward IVF-derived zygotes	Of 11 piglets born, nine survived; six of nine carried mutations in *TP53*; three of the genome-edited pigs (50%) exhibited various tumor phenotypes	*TP53*	[97]
SCNT	CRISPR/Cas9 (KI)	Using fetal fibroblasts transfected with sgRNA- Cas9 plasmid + donor DNA	Three piglets born grew and developed normally; all these piglets had *fat-1* KI at the Rosa26 locus	*Rosa26*	[98]
SCNT	CRISPR/Cas9 (KI)	Using fetal fibroblasts transfected with sgRNA- Cas9 plasmid + donor DNA	Of seven naturally delivered piglets, six showed successful KI; the KI allele was successfully transmitted through germline	*HTT*	[99]

Method	Genome editing tool (mode for gene modification)	Method for gene modification	Outcome	Target gene	References
SCNT	CRISPR/Cas9 (indels)	Using fetal fibroblasts transfected with sgRNA- Cas9 plasmid	Of a total of 17 piglets obtained, 12 appeared healthy; all had mutations at the target locus	*FBXO40*	[100]

Abbreviations: APC, adenomatous polyposis coli; Alb, albumin; GGTA1, α-1,3-galactosyltransferase gene; APP, amyloid precursor protein; ApoE, apolipoprotein E; ASGR1, asialoglycoprotein receptor; β4GalNT2, β1,4-N-acetylgalactosaminyl transferase; class I MHC, class I major histocompatibility complex; CRISPR/Cas9, clustered regulated interspaced short palindromic repeat and CRISPR-associated Cas; COL1A, collagen type I alpha 1 chain; CMAH, cytidine monophosphate-N-acetylneuraminic acid hydroxylase; DAZL, deleted in azoospermia-like; DM, double muscling; DMD, Duchenne muscular dystrophy; PARKIN, E3 ubiquitin ligase PARK2; EP, electroporation; eGFP, enhanced green fluorescent protein; FBXO40, F-box protein 40; FBN1, fibrillin-1; GM, gene-modified; GRB10, growth-hormone receptor binding protein-10; H11, Hipp11; hDAF, human decay-accelerating factor; HTT, huntingtin; indels, insertion or deletion of nucleotides; INS, insulin; IL2RG, interleukin-2 receptor gamma; IVF, in vitro fertilized; iGb3S, isogloboside 3 synthase; IgM J_H, JH region of the pig IgM heavy chain; KI, knock-in; KO, knockout; LRRK2, leucine-rich repeat kinase 2; LDLR, low density lipoprotein receptor; MFS, Marfan syndrome; MI, microinjection; SCNT-MI, microinjection following somatic cell nuclear transfer; MITF, Microphthalmia-associated transcription factor; MSTN, myostatin; NANOS2, nanos C2HC-type zinc finger 2; fat-1, n-3 fatty acid desaturase; Npc1l1, Niemann-Pick C1-Like 1; PDX-1, pancreas duodenum homeobox 1; DJ-1, PARK7; PARK2, parkin; PPARγ, peroxisome proliferator-activated receptor-gamma; PERV, porcine endogenous retrovirus; PINK1, PTEN-induced putative kinase 1; RNP, ribonucleoprotein; RUNX3, Runt-related transcription factor 3; sgRNA, single guide RNA; ssODN, single-stranded DNA oligonucleotides; SCNT, somatic cell nuclear transfer; Sox10, SRY (sex determining region Y)-box 10; TALENs, transcription activator-like effector nucleases; TMPRSS2, transmembrane protease, serine S1, member 2; TYR, tyrosinase; UCP1, uncoupling protein 1; vWF, von Willebrand factor; ZFNs, zinc finger nucleases.

Table 1.
Summary of production of genome-edited pigs.

was found that the endogenous NHEJ pathway is effective for inducing mutation in a porcine target gene. Furthermore, SCNT using GE cells with the mutated allele as a SCNT donor demonstrated that seven of the nine resulting cloned fetuses (at Day 12) stopped expressing EGFP. Yang et al. [42] co-transfected porcine fibroblasts with ZFN and pcDNA3.1 plasmids (providing *neo*) by electroporation (EP), performed SCNT using these GE cells, and finally obtained peroxisome proliferator-activated receptor γ (PPARγ) mono-allelic KO pigs, which are expected to generate a porcine cardiomyopathy model. Hauschild et al. [43] attempted to destroy *GGTA1*, an endogenous gene encoding an enzyme required for production of a xenogeneic antigen called α-Gal epitope, using ZFNs in porcine fetal cells. They found that α-Gal epitope-negative cells with the bi-allelic KO phenotype can be efficiently isolated by FACS, and the resultant GE cells have the potential to make cloned piglets. Importantly, this experiment suggests that it is possible to produce individuals with a bi-allelic KO phenotype with this technology. In other words, bi-allelic KO piglets can be directly created without breeding or subcloning, which contrasts with past instances where only heterozygous KO piglets have been produced through traditional gene targeting. Hauschild et al. [43] also showed that neither off-target cleavage nor integration of the ZFN-coding plasmid occurred.

The successful production of genome-edited piglets with bi-allelic KO genotype obtained after cytoplasmic MI of *in vivo*-derived porcine zygotes using either ZFN or TALEN mRNA was first reported by Lillico et al. [45]. This MI-based production of GE animals was also shown to be successful in mice [101–104].

Hai et al. [52] first demonstrated that GE pigs can be produced using the CRISPR/Cas9 system. They performed cytoplasmic MI with Cas9 mRNA and sgRNA targeted to *von Willebrand factor* gene (*vWF*) to produce a pig *model* for type 1 von Willebrand disease. In this study, 10 of 16 resulting piglets had indels with an efficiency of 63%, and most pigs contained more than two different alleles, suggesting mono-allelic mutants.

Successful knock-in (KI) of a GOI into the target locus was first reported in pigs by Ruan et al. [60] and Peng et al. [63]. Zhou et al. [61] demonstrated the production of SCNT-treated piglets with mutations in multiple genes after a single transfection.

Fischer et al. [83] first succeeded in producing GE pigs by cytoplasmic MI of a Cas9 protein/gRNA complex called a ribonucleoprotein (RNP). Furthermore, GE pigs could be efficiently produced by *in vitro* EP in the presence of RNP [68]. Sheets et al. [82] produced GE fetuses by cytoplasmic MI of RNP into oocytes reconstituted with intact cells using the SCNT technology. By this treatment, they reported highly efficient (100%) generation of bi-allelic modification in the resultant cloned fetuses. The significance of this approach is be that researchers can obtain GE pigs with a defined genetic background, even though the starting oocytes are derived from ovaries obtained from a slaughterhouse.

4. Delivery method

For the production of GE pigs, the choice of delivery method for genome editing components in porcine zygotes is important. As shown in **Table 1**, the methods for the production of GE pigs achieved by delivering genome editing reagents at earlier stages of development can be largely divided into four groups: the first is MI of genome editing reagents (in a form of DNA, mRNA or protein) into zygotes (**Figure 1A**); the second is SCNT using GE cells as the SCNT donor (**Figure 1B**); the third is *in vitro* EP of zygotes in the presence of genome editing reagents (**Figure 1C**); the fourth is MI of genome editing reagents into SCNT-treated

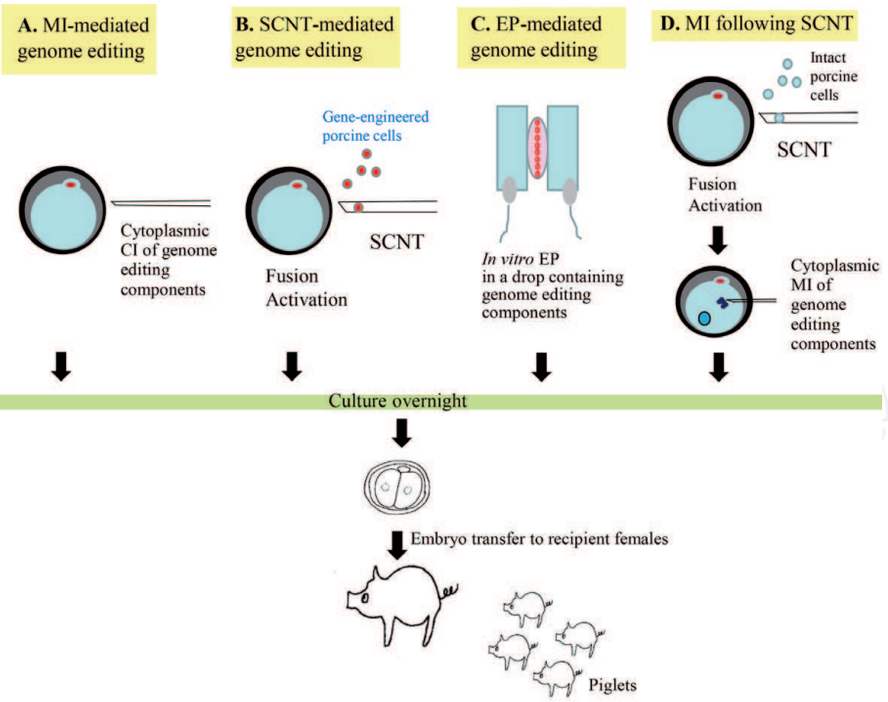

Figure 1.
Several methods to create genome-edited pigs. (A) Microinjection (MI)-based method using zygotes. (B) Somatic cell nuclear transfer (SCNT)-based method using gene-engineered (GE) cells as a SCNT donor. (C) Electroporation (EP)-based method using zygotes. (D) MI-based method using the SCNT-treated embryos.

embryos reconstituted using a normal cell (**Figure 1D**). Furthermore, we will provide a new approach based on *in vitro* EP of the SCNT-treated embryos reconstituted using a normal cell as the fifth group of methods for possible production of GE pigs (**Figure 2A**). In the following sections, each of these methods are described.

4.1 MI

MI is an important tool in the creation of GE piglets. To date, about 30% (17/60) of studies (**Table 1**) have employed this approach. For example, in the case of MI with CRISPR/Cas9-related mRNA, a single cytoplasmic MI of 2–10 pL containing 125 ng/μL Cas9 mRNA and 12.5 ng/μL sgRNA was adopted [62]. Yu et al. [69] employed Cas9 mRNA (20 ng/μL) and sgRNA (10 ng/μL) mixtures for cytoplasmic MI.

Is MI of these components deleterious to the development of porcine zygotes? According to Hai et al. [52], the *in vitro* developmental efficiencies of embryos injected with Cas9 mRNA/sgRNA (~79%) and embryos injected with water (~77%) were both very high and comparable with each other, suggesting that the MI and the Cas9 mRNA/sgRNA had little effect on early embryonic development. On the contrary, Whitworth et al. [51] reported that a higher concentration of sgRNA induces toxicity in porcine embryos. According to Whitworth et al. [51], 10 ng/μL of sgRNA and Cas9 mRNA are recommended.

Selecting appropriate zygotes also appears to be an important factor in the production of GE pigs. For acquisition of viable zygotes, there are at least two methods. One is isolation of zygotes from oviducts of a female that has been inseminated, hereinafter called "*in vivo*-derived zygotes," and the other is acquisition of zygotes by *in vitro* fertilization (IVF) between *in vitro* matured oocytes (derived from the ovaries obtained from the slaughterhouse) and sperm. Generally, it is believed that the *in vivo*-derived zygotes exhibit superior development performance comparing to the IVF-derived zygotes [51, 62]. Indeed, the number of laboratories using

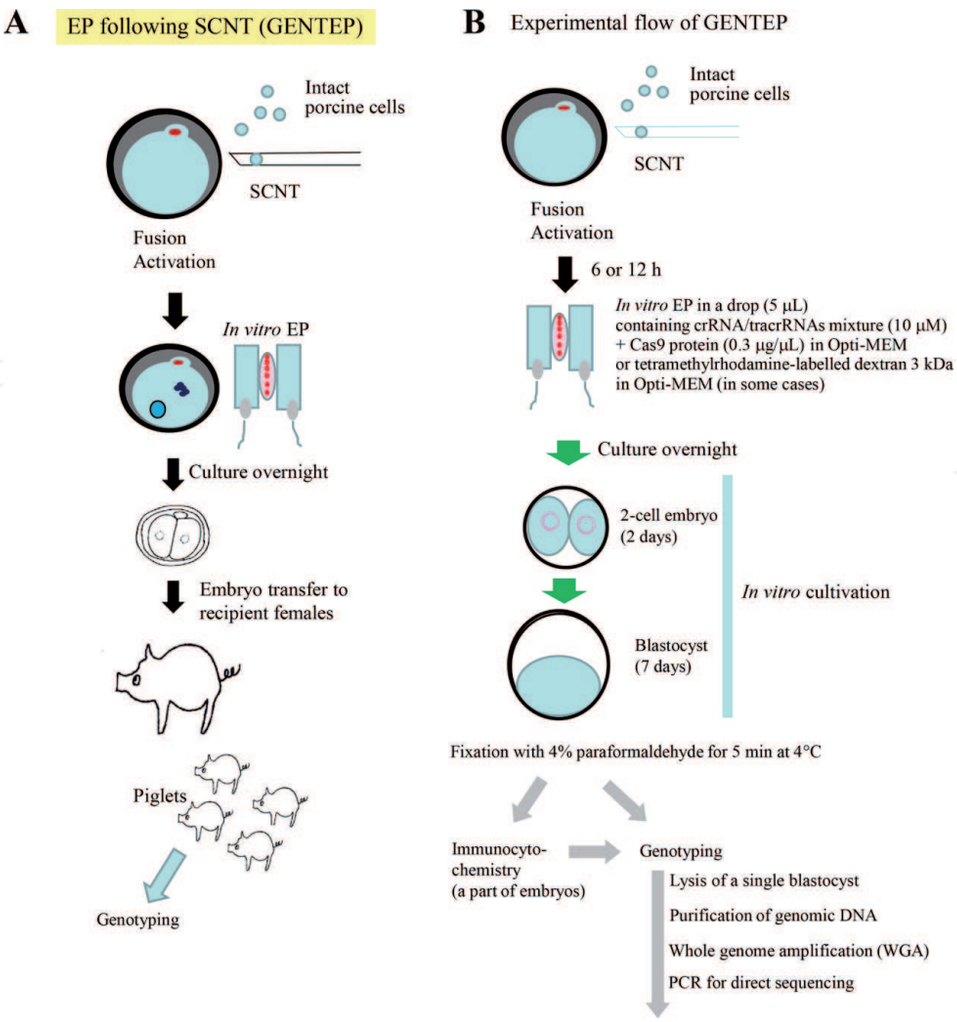

Figure 2.
A new method for production of genome-edited pigs. (A) EP-based method using the SCNT-treated embryos, which is termed "GENTEP." (B) Experimental outline for checking the validity of GENTEP.

IVF-derived zygotes for production of GE pigs is low (~30% (5/17); see **Table 1**). However, acquisition of viable *in vivo*-derived zygotes is laborious and often associated with the sacrifice of pregnant females, which appears to be one of the major goals for improvement in genetic engineering technology using pigs.

The frequent generation of individuals with mosaic genotypes is also a serious problem associated with MI-based GE pig production. Sato et al. [105] demonstrated that cytoplasmic MI of parthenogenetically activated porcine embryos (hereinafter called "parthenotes") with Cas9 mRNA + sgRNA caused frequent mosaicism in the offspring (blastocysts) with cells with mixed genotype, so-called normal wild-type cells and mutated cells, when they were subjected to cytoplasmic MI immediately after oocyte activation. Notably, Carlson et al. [44] suggested that 100% of bovine embryos exhibited fluorescence expression after cytoplasmic MI of *EGFP* mRNA, but only ~40% of porcine embryos did. It is probable that an endogenous system for translation to protein from mRNA may not be sufficiently established in those porcine embryos, especially at the stage immediately after fertilization or zygotic activation. Indeed, Sato et al. [106] demonstrated that this mosaicism can be partially improved when cytoplasmic MI is performed with oocytes 12 h after activation. In contrast, other researchers reported that only 10–20% of MI-treated embryos exhibited mosaicism [51, 84]. Notably, Whitworth et al. [51] performed cytoplasmic MI with Cas9 mRNA + sgRNA toward fertilized oocytes at 14 h post-fertilization. In this context, the use of sgRNA and an RNP instead of Cas9 mRNA

may be the key to solving this issue of mosaicism, as the Cas9 protein is more rapidly translated, folded, and complexed with sgRNAs prior to editing, unlike the Cas9 mRNA [107–109]. For example, in mice, delivering RNPs into zygotes causes rapid genome editing in the target locus, which also maximizes efficiency while minimizing mosaicism [110–112]. Indeed, Sheets et al. [82] demonstrated that after MI with RNP, 100% of piglets produced had the bi-allelic KO genotype.

Interestingly, Petersen et al. [80] demonstrated that cytoplasmic MI of DNA vectors coding for CRISPR/Cas9 targeting the porcine *GGTA1* gene enabled biallelic knockout of *GGTA1* in 7/12 fetuses and piglets (58.3%). As mentioned previously, it is difficult to visualize porcine pronuclei at zygote stage under normal conditions due to high lipid content in the cytoplasm. Researchers therefore must centrifuge them briefly prior to MI. The fact that cytoplasmic MI of DNA vectors can induce genome editing at a target locus may be beneficial for researchers, because preparation of plasmid DNA is easier than that of mRNA, and it is generally more resistant against degradation than mRNA. According to Petersen et al. [80], it currently remains unknown how the circular DNA plasmid translocates from the cytoplasm to the nucleus. They speculate that the SV40 nuclear translocation signal of the CRISPR/Cas9 plasmid could play an important role by facilitating nuclear translocation via association with ubiquitous transcription factors.

4.2 SCNT

SCNT using GE cells as an SCNT donor is another way to produce GE pigs. The merit of this approach is the use of *in vitro* cultivated cells such as fetal fibroblasts to which various genetic engineering techniques (i.e., introduction of multiple KO, KI, and transgenes) can be applied easily. After gene transfer, these cells are subjected to cell selection through drug selection or fluorescence activated cell sorting (FACS) to enrich GE cells as a pure population. Thus, it is highly probable that the resulting SCNT-derived GE founder pigs have a predictable genotype and low rates of mosaicism. Unfortunately, as mentioned previously, the efficiency of SCNT to produce cloned piglets is still very low. Much effort has been focused on improving the low efficiency associated with the SCNT, which includes improvement of the oocyte/zygote culture system and application of chemical reagents to alter the epigenetic status of transferred nuclei. For improving the culture method, researchers have used vitamin C [113], α-tocopherol [114], melatonin [115] or alanyl-glutamine dipeptide (instead of glutamine) [116]. For altering the epigenetic status, researchers have used histone deacetylase inhibitors (HDACi) such as trichostatin A (TSA) [117, 118], valproic acid (VPA) [119–121], scriptaid [122–124], LBH589 (panobinostat) [125], oxamflatin [126], PXD101 (belinostat) [127], quisinostat [128], MGCD0103 [129], or histone methyltransferase inhibitors such as MM-102 [130]. Lin et al. [131] employed tauroursodeoxycholic acid (TUDCA), an inhibitor of endoplasmic reticulum (ER) stress, and demonstrated that TUDCA can enhance the developmental potential of porcine SCNT embryos by attenuating ER stress and reducing apoptosis. Wang et al. [132] demonstrated that administration of siRNA or microRNA-148a, both of which can suppress the function of DNA methyltransferase 1 (*DNMT1*) at a transcriptional level, is effective for enhancing the developmental potential of SCNT embryos. Furthermore, Matoba et al. [133] succeeded in drastically increasing SCNT efficiency by cytoplasmic MI of mRNA coding for histone demethylase (*Kdm4d*) in mice.

4.3 EP

EP is known to be a useful and powerful gene delivery tool enabling transfer of exogenous substances (i.e., DNA) into a cell and was first applied to rat zygotes for

genome editing by Kaneko et al. [134]. Since then, many researchers have successfully induced gene edits by using this technology in mice [135, 136], bovines [137] and pigs [68]. The merit of this technology is that it is simple, rapid and convenient for genome editing in zygotes, compared to the previous MI-based technique. Notably, about 30–50 zygotes can be edited with one pulse of EP. Furthermore, EP only requires a square pulse generator called an electroporator, and not a more expensive micromanipulator system.

As mentioned previously, Tanihara et al. [68] first applied EP to porcine IVF-derived zygotes and produced genome-edited pigs. They used CRISPR/Cas9-based RNP for knock-in of a target gene, and achieved reduced mosaicism and higher efficiency of genome-edited pig production with EP (30 V, square pulse 1.0 ms in duration repeated five times) using an electrode (#LF501PT1-20; BEX Co. Ltd., Tokyo, Japan) connected to a CUY21EDIT II electroporator (BEX Co. Ltd.). Notably, they reported no appreciable reduction in the developmental ability of the EP-treated embryos.

4.4 MI after SCNT

Although direct modification of zygotic genomes provides some advantages, SCNT also provides a significant advantage by permitting the isolation of cells containing precise modifications before the expense of animal production is incurred. As mentioned previously, Sheets et al. [82] successfully produced genome-edited cloned pigs by combining SCNT with CRISPR/Cas9 MI, which is beneficial for researchers as they do not need to manage a founder herd, and can eliminate the need for laborious *in vitro* culture and screening. In this study, all (6/6) of the resultant clone fetuses exhibited 100% bi-allelic modification. Unfortunately, they failed to describe successful production of *live birth piglets*, but it seems that this approach is a powerful tool for GE pig production.

4.5 EP after SCNT

Similar to the approach shown by Sheets et al. [82], we tried to obtain cloned GE piglets through *in vitro* EP in the SCNT-treated embryos, which is called Genome Editing via Nuclear Transfer and subsequent Electroporation or GENTEP (**Figure 2A**). Some results obtained from GENTEP-related experiments are presented below.

SCNT-derived embryos were obtained by inserting fetal fibroblasts derived from microminiature pigs (MMP) [138] into the perivitelline space between enucleated porcine oocytes (derived from ovaries obtained from a slaughterhouse) and zona pellucida, according to the method described by Miyoshi et al. [119] (**Figure 2B**). The resulting SCNT-derived embryos were then subjected to electric activation following electric fusion between an egg and a cell (**Figure 2B**). Six or 12 h after activation, the SCNT-treated embryos were subjected to *in vitro* EP in the presence of RNP targeted to the pig low density lipoprotein receptor (*LDLR*) gene (**Figure 2B**). Parthenotes (~6 h after electric activation) were also used for *in vitro* EP using tetramethylrhodamine-labeled dextran 3 kDa (used as an indicator for successful gene delivery) (as shown in **Figure 3A**) or as controls for immunocytochemistry using anti-LDLR antibody (as shown in **Figure 3B**).

First, we examined whether the *in vitro* EP we used here is effective for successful gene delivery to porcine embryos and does not cause any deleterious effects on their embryonic development, using porcine parthenotes (6 h after activation). The EP procedure was based on the method described by Hashimoto and Takemoto [135]. An electroporation chamber (#LF610P4-4_470; BEX Co. Ltd.), in which two platinum block electrodes were situated with a 1-mm gap between them (**Figure 1C**), was placed under a stereoscopic microscope and connected to

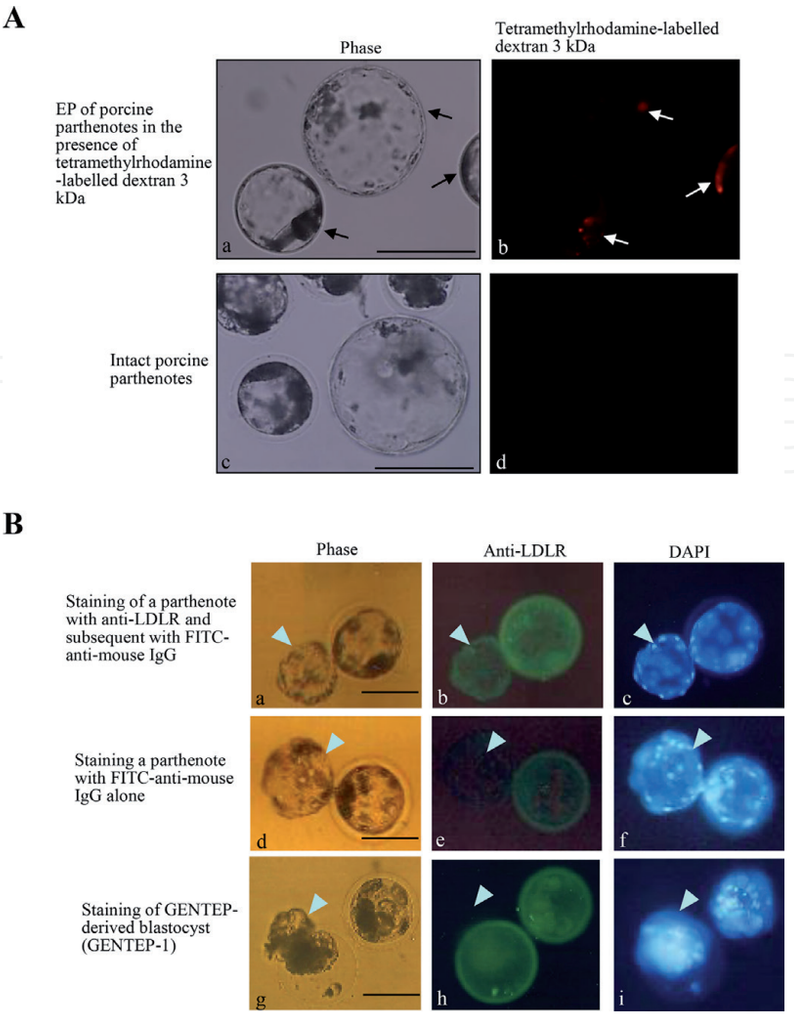

Figure 3.
Validity check of GENTEP. (A) EP of parthenotes (6 h after activation) in the presence of tetramethylrhodamine-labelled dextran 3 kDa. Porcine parthenotes were subjected to in vitro *EP, and then cultured for 7 days up to blastocysts. Note that almost all of the EP-treated blastocysts are fluorescent (arrows in a and b), while intact parthenotes do not fluoresce (c and d). Bar = 100 μm. (B) Staining with anti-LDLR antibody. The intact parthenote (hatched blastocysts) exhibits the reactivity to the antibody (arrowheads in a–c), but not to the second antibody alone (arrowheads in d–f). No reactivity to the antibody was also seen in the GENTEP-derived blastocyst (arrowheads in g–i). Nuclear staining with DAPI was performed after staining with the second antibody. Note that porcine zona pellucida was slightly stained with the second antibody, since it was found to be reactive with the second antibody alone (see d–f). Bar = 100 μm.*

an electric pulse generator (CUY21EDITII Genome Editor™, BEX Co. Ltd.). About 20 parthenotes were placed into a 5-μL drop containing 2 μg/μL tetramethylrhodamine-dextran 3 kDa (#D3307; Thermo Fisher Scientific Inc., Waltham, MA, USA) in Opti-MEM (Invitrogen, Carlsbad, CA, USA) between the electrodes (**Figure 1C**). EP was performed under these conditions: 30 V, square pulses, 1.0 ms in duration at 99 ms intervals, repeated seven times. The EP-treated parthenotes were then cultured for 7 days up to blastocysts to evaluate the *in vitro* developmental rate and uptake of fluorescent dye into the embryos. Approximately 40% of the EP-treated parthenotes developed to the blastocyst stage and ~80% of them exhibited bright tetramethylrhodamine-derived fluorescence [arrows in **Figure 3A(a,b)**]. This result suggests that our EP condition is useful for effective delivery of a foreign substance into porcine embryos and not harmful for their development.

Second, we performed CRISPR/Cas9-based genome editing (targeted to the endogenous *LDLR* gene) with porcine SCNT-treated embryos. We designed sgRNA capable of recognizing a 20 bp sequence spanning the translation initiation codon (ATG) upstream of the protospacer adjacent motif (PAM) sequence (CGG) on the

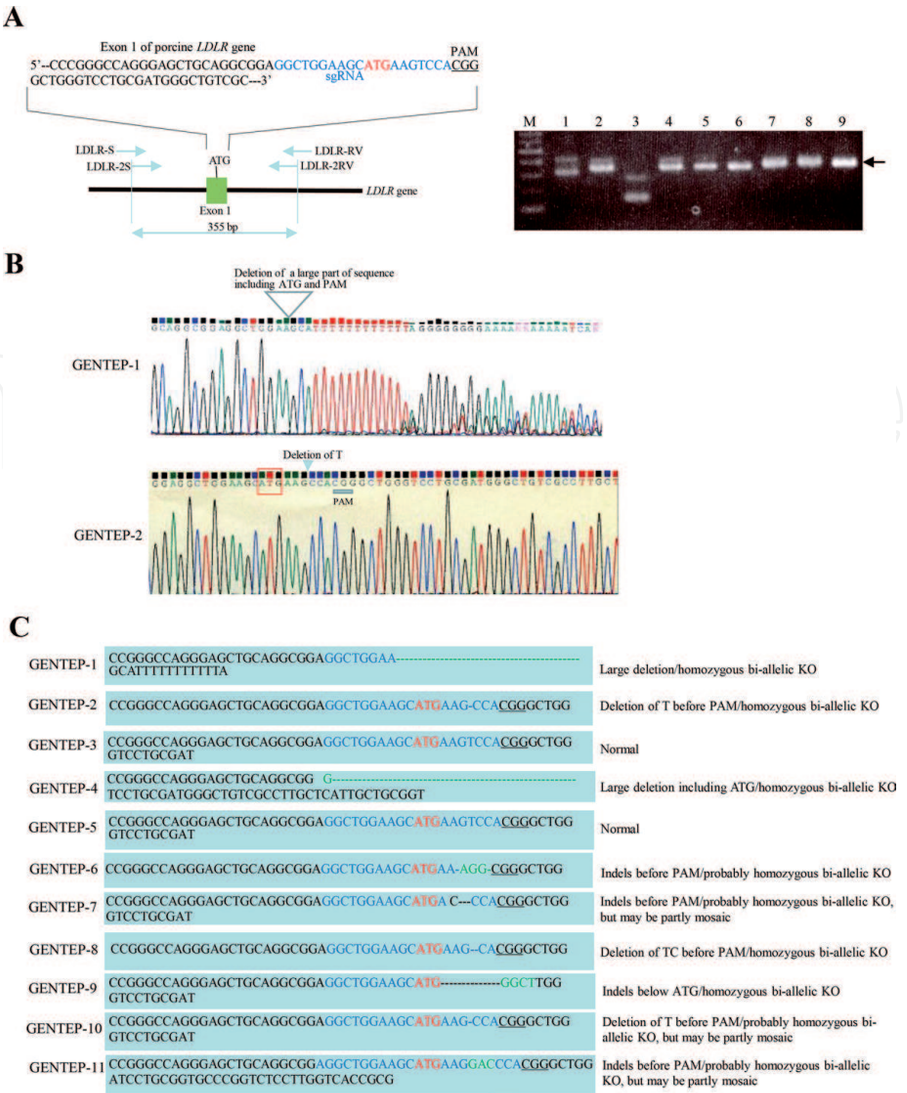

Figure 4.
Molecular biological analysis of the GENTEP-treated embryos (termed GENTEP-1 to -11) at a single embryo level. (A) Structure of porcine LDLR *gene and a target sequence recognized by sgRNA (left panel), and the results of nested PCR (right panel). The target sequence (shown in blue) spanning ATG (shown in red) is located on the first exon of* LDLR*. PAM, protospacer adjacent motif. Primers used for first PCR and nested PCR are shown above the* LDRL*. In the right panel, a part of the nested PCR products (lanes 1–9) loaded onto 2% agarose gel is shown. Arrow indicates the PCR products of 355 bp in size. M, 100-bp ladder markers. (B) Ideogram pattern in the GENTEP-1 and -2 samples obtained after direct sequencing of the nested PCR products using LDLR-2S primer. (C) Various indels found in each GENTEP-treated embryo. ATG is shown in red. Sequence recognized by sgRNA is shown in blue.*

first exon of porcine *LDLR* (left panel of **Figure 4A**). The sgRNA was synthesized by Integrated DNA Technologies, Inc. (IDT; Coralville, Iowa, USA) as Alt-R™ CRISPR crRNA product. The crRNA and tracrRNA (purchased from IDT) were combined for annealing and then mixed with recombinant Cas9 protein (TaKaRa Shuzo Co. Ltd., Shiga, Japan) to form RNP, according to the method of Ohtsuka et al. [139]. The final concentrations of the components in RNP were 30 μM/mL (for crRNA/tracrRNA) and 1 mg/mL (for Cas9 protein). The SCNT-treated embryos 6 or 12 h after activation were transferred to a 5-μL drop (containing RNP in Opti-MEM) and immediately subjected to *in vitro* EP under these conditions: 30 V, square pulses, 1.0 ms in duration at 99 ms intervals (or 0.5 ms in duration at 99.5 ms intervals), both repeated seven times. After EP, the embryos were promptly cultivated in normal medium for 7 days up to blastocysts and then subjected to analysis of molecular biology (possible mutations in the first exon of *LDLR*) and immunocytochemistry (possible loss of LDLR protein synthesis) parameters, as described

Stage at EP after activation of SCNT-treated embryos	EP condition[2]	Total number of SCNT-treated embryos examined	No. of embryos cleaved to the two-cell stage (%)	No. of embryos developed to blastocysts (%)
6 h	0.5	12	8 (66.7)	1 (8.3)
	1.0	12	11 (91.7)	2 (16.7)
12 h	0.5	33	21 (63.6)	5 (15.2)
	1.0	35	23 (65.7)	3 (8.6)

[1]EP in the presence of RNP [10 μM of crRNA/tracrRNA mixture (targeted to LDLR gene) + 0.3 μg/μL of Cas9 protein] is performed on SCNT-treated embryos 6 or 12 h after activation. The EP-treated embryos were then cultured for 7 days to the blastocyst stage for the presence of mutations in the target gene at molecular biological and immunocytochemical levels.
[2]EP was performed under the electric condition of 30 V in voltage, 0.5 ms in length of square pulse with 99.5-ms intervals (0.5) or 1.0 ms in length of square pulse with 99-ms intervals (1.0), and seven times of pulse stimulation using an electroporation chamber (#LF610P4-4_470; BEX Co. Ltd.) connected to an electric pulse generator (CUY21EDITII. Genome Editor™, BEX Co. Ltd.).

Table 2.
Summary of the properties of blastocysts derived from EP[1] toward the SCNT-treated porcine embryos.

in **Figure 2B**. In each group, 8–17% of the EP-treated embryos developed to blastocysts (**Table 2**). These rates appear to be comparable to the yield (24.2%) in experiments performed using intact MMP fetal fibroblasts as SCNT donors [119]. All of the blastocysts obtained were then fixed with 4% paraformaldehyde, and a section of these embryos was subjected to immunocytochemical staining using anti-LDLR antibody (**Figure 2B**). The EP-treated cloned blastocyst (termed GENTEP-1) was unreactive to anti-LDLR (arrows in **Figure 3B(g–i)**). In contrast, a parthenote (blastocyst) exhibited positive reactivity to anti-LDLR (arrow in **Figure 3B(a–c)**). Staining with the second antibody alone failed to react with the antibody (arrow in **Figure 3B(d–f)**). Furthermore, each of these fixed blastocysts was subjected to genomic DNA isolation to examine possible mutations at the individual embryo level (**Figure 2B**). Next, GenomiPhi-based whole genome amplification (WGA) was performed using the isolated genomic DNA as a template, as described previously [140]. PCR was then performed using the WGA products as a PCR template. The primer sets used are LDLR-S (5′-AAACCTCACATTGAAATGCTG-3′)/LDLR-RV (5′-CCTAAACTCTCGCGCCCCCCT-3′) for the first round of PCR and LDLR-2S (5′-CTGCAAATGACTGGGGCCCCG-3′)/LDLR-2RV (5′-CTCCAACCACGTAAGAATGAC-3′) for nested PCR (left panel of **Figure 4A**). Nested PCR using the LDLR-2S/LDLR-2RV primer set yields 355-bp products (left panel of **Figure 4A**). The typical example when the nested PCR products (lanes 1–9) are loaded onto a 2% agarose gel is shown in the right panel of **Figure 4A**. Almost all of the samples tested exhibited 355-bp products, except for lane 3 showing bands of reduced size, suggesting occurrence of a large deletion (probably over 100 bp) around the *LDLR* sequence recognized by sgRNA. In **Figure 4B**, an example of the results obtained from direct sequencing of nested PCR products using LDLR-2S primer is shown. The sample GENTEP-1, which has been shown to exhibit loss of the reactivity to anti-LDLR (see **Figure 3B-g-i**), had a large deletion including a sequence spanning ATG and PAM. Notably, there was no appreciable overlapping in ideograms of the sample, suggesting a homozygous bi-allelic KO phenotype (**Figure 4C**). Subcloning of the PCR products derived from the sample GENTEP-1 into TA cloning vector and subsequent sequencing demonstrated that all six clones obtained exhibited the same sequence as the parental product (data not shown). When the remaining PCR products were sequenced it was found that almost all (82%, 9/11) of the samples exhibited the homozygous bi-allelic KO genotype (**Figure 4C**).

5. Other techniques and factors affecting efficacy of the genome editing system

As shown above, genome editing tools such as ZFNs, TALENs and CRISPR/Cas9 are considered useful in enabling site-specific gene modification in livestock such as pigs. However, there are still several techniques and factors that influence performance which must be addressed. These include the single embryo assay, off-target cutting, multiplexed genome engineering, KI, and Cas9 pigs. In this section, these techniques or factors are described in greater detail.

5.1 Single embryo assay

To increasing the efficiency of genome editing systems, it is important to select suitable sets of ZFNs (or TALENs) or sgRNA (in the case of CRISPR/Cas9). Researchers therefore must check the efficiency of these reagents by introducing them into cultured cells, but at this point it remains unknown whether they will function *in vivo*. Unlike small animals such as mice and rats, large animals have long gestation periods and it is costly to prepare large animal recipients. Therefore, this testing *in vivo* appears to be difficult in larger animals such as pigs. To overcome this issue, a single embryo (blastocyst) assay to evaluate the operability of the genome editing reagents prepared was provided by Wang et al. [62] who later re-validated those sets using porcine parthenotes. To our knowledge, this assay was first developed using mice by Sakurai et al. [141] who reported that it is useful for confirming the fidelity of sgRNAs used.

It may be required to confirm at a molecular level whether the genome-edited embryos have mutations. In this case, WGA has often been employed for amplifying the whole genome of an embryo (blastocyst) using genomic DNA isolated from a single embryo as the DNA template [140, 141], since the blastocyst DNA is often too small to generate a sufficient amount of PCR product. The effectiveness of WGA-based amplification of blastocyst DNA has already been confirmed by ours [142] and others [44]. The resulting products obtained after PCR using WGA-derived DNA as the template are then subjected to direct sequencing for identification of possible mutations in the target gene, as shown in **Figure 4B**.

5.2 Off-target cleavage

Since sgRNA used in the CRISPR/Cas9 system can recognize only a short sequence (20 bp) at the target gene where Cas9 cleaves, other genes with a similar sequence to the sgRNA may be susceptible to Cas9-mediated DNA cleavage, which leads to the occasional generation of off-target cutting [29, 143]. This unintended cutting is considered a serious problem to be resolved.

Several strategies to minimize off-target cutting have been employed including the use of the double nickase mutant form of Cas9, which induces a single-strand break instead of DSB [144]; the use of RNP, whose half-life is shorter than the duration of transcription of plasmid or viral nucleic acids [110, 145]; or the fusion of catalytically inactive Cas9 with *Fok* I nuclease domain (fCas9) to improve the DNA cleavage specificity [146]. Recently, it was reported that Cpf1, a putative Class 2 CRISPR effector, mediates target DNA editing differently from Cas9 [147]. It generates a 5-nucleotide staggered cut with a 5′ overhang, which is particularly advantageous in facilitating an NHEJ-based KI into a genome. Several unique enzymes that can decrease the probability of off-target cleavage have also been produced. For example, two engineered enzymes produced from SpCas9 from *Streptococcus pyogenes* with the goal of enhancing specificity, called eSpCas9 [148] and SpCas9-HF [149], are reported to reduce the probability of mismatched DNA binding. A hybrid

enzyme combining the Cas9-nickase and PmCDA1, an activation-induced cytidine deaminase (AID) ortholog, could perform targeted nucleotide substitution [150]. Furthermore, a CRISPR system using a new Cas-related enzyme called Cas13a that targets RNA has also been recently developed [151].

Notably, in the case of GE pigs and embryos, there have been no reports of off-target mutagenesis as shown by the following papers: [43, 50, 61–64, 69, 74, 77, 78, 80, 86, 100, 105]. This suggests a very low probability of off target-cleavage in GE pigs.

5.3 Multiplexed genome engineering

The CRISPR/Cas9 system can confer multigene KO in one shot of gene delivery [152, 153]. This property is especially beneficial for the purpose of creating disease model animals, as certain types of diseases are known to be caused by multigene defects. Interestingly, Sakurai et al. [154] demonstrated that at least nine endogenous genes can be knocked out simultaneously through a single shot of cytoplasmic MI of 12 sgRNAs together with Cas9 mRNA into murine zygotes. In pigs, Zhou et al. [61] demonstrated successful generation of PARK2 (*parkin*) and PTEN-induced putative kinase 1 (*PINK1*) double-KO pigs through SCNT with GE fetal fibroblasts after co-transfection of Cas9, *PARK2*-sgRNA, and *PINK1*-sgRNA-expressing vectors by electroporation. The percentage of *PARK2*$^{-/-}$/*PINK1*$^{-/-}$ double-KO cells was up to 38.1%. SCNT using these double-KO cells resulted in the birth of 20 cloned piglets. Of these, four piglets developed normally, and both parkin and PINK1 in those individuals were depleted at the protein level. Estrada et al. [66] also succeeded in obtaining one triple-KO cloned piglet with mutations in *GGTA1* (coding for α-1,3-galactosyltransferase), *CMAH* (coding for cytidine monophosphate-*N*-acetylneuraminic acid hydroxylase) and *β4GalNT2* (coding for β1,4-*N*-acetylgalactosaminyl transferase) after SCNT. Wang et al. [81] generated PARK7 (*DJ-1*)/*parkin*/*PINK1* triple-gene modified pigs using the CRISPR/Cas9 system in one step through direct zygote injection of Cas9 mRNA and three types of sgRNAs. According to Wang et al. [81], of two live-born piglets delivered, one piglet showed biallelic modification of all three genes, and another showed biallelic modification of the *DJ-1* and *PINK1* genes and monoallelic mutation of the *parkin* gene.

5.4 KI

As shown in **Table 1**, in 2015 successful KI in pigs was reported by several groups. For example, Wang et al. [62] performed MI with *in vivo* fertilized zygotes (derived from colored pigs) using Cas9 mRNA + sgRNA + single-stranded DNA oligonucleotides (ssODN), targeting microphthalmia-associated transcription factor (MITF), a master regulator gene of melanocyte development, and obtained two live-born piglets showing the white coat color phenotype over its entire body. Peng et al. [63] tried to create KI piglets with a MI approach using a circular vector as donor DNA. They designed an sgRNA targeting the starting codon region (including the adjacent 5′ and ATG) and generated a targeted fragment (donor for HR) with the insert flanked by 1-kb HA on both sides. They performed cytoplasmic MI of Cas9 mRNA + *in vitro* synthesized sgRNA + circular vector containing the targeting fragment, and finally obtained 16 live piglets, all of which were found to carry the expected KI allele. Notably, they confirmed expression of human albumin (Alb) protein generated from the KI allele in the plasma of these cloned pigs. This means that expression of a transgene (human Alb as GOI) is possible under the control of an endogenous promoter system (in this case, Alb promoter).

Ruan et al. [60] demonstrated production of GE pigs with successful KI of GOI into the target *Hipp11* (*H11*) locus, which is considered as "safe harbor" genomic locus that allows gene expression without disrupting internal gene function, like the *Rosa26*

locus. They utilized a positive and negative selection method to insert *GFP* into the *pH 11* locus in pig fetal fibroblast cells by electroporation. The targeting donor vector (4.2 kb in size) contains a reporter cassette with *neo* and GFP genes which are flanked by a 0.8-kb HA to the *H11* locus on each side with the diphtheria toxin A (DTA) gene at the 3′ end. Cells were transfected with the linearized donor vector and two expression vectors for sgRNA (targeted to the *H11* locus) and Cas9. After drug selection, they obtained GE cells with successful KI at the *H11* locus with efficiencies up to 54%. Next, they performed SCNT using these correctly targeted clones, and obtained one cloned piglet which was later confirmed to show correct targeting.

Generally, it is believed that HDR-mediated KI is more difficult than NHEJ-based indels. For example, in proliferating human cells, NHEJ has been reported to repair 75% of DSBs, while HDR repaired the remaining 25% [155]. To enhance the HDR efficiency, several approaches are now being attempted. For examples, co-injection of murine zygotes with a mixture containing Cas9 mRNA, sgRNA, template ssODNs and Scr7 (an inhibitor for DNA ligase IV) significantly improved the efficiency of HDR-mediated insertional mutagenesis [156]. Chu et al. [157] also demonstrated usefulness of Scr7 for abolishing NHEJ activity and increasing HDR in both human and mouse cell lines. However, the function of Scr7 in promoting HDR remains controversial. Some researchers demonstrated that Scr7 failed to increase HDR rates in rabbit embryos [158] and porcine fetal fibroblasts [159]. On the contrary, Li et al. [160] demonstrated that Scr7 promoted HDR efficiency in porcine fetal fibroblasts. The same group also showed that other reagents L755507 (β-3 adrenergic receptor agonist) and resveratrol (small-molecule compound found in grapes) also showed similar effects (promotion of HDR efficiency) in porcine cells.

5.5 Cas9 pigs

As mentioned previously, the current generation of gene-edited pigs has mostly been produced through either MI or SCNT approaches, which are both expensive and time-consuming. In mice, several Tg lines carrying a Cas9-expressing cassette have been created [154, 161, 162]. These Tg mice are thought to be useful animals for direct *in vivo* genome editing experiments, because successful delivery of the expression vectors of sgRNAs alone or RNA itself into selected tissues caused generation of genome-edited tissues. For example, Platt et al. [161] demonstrated that *in vivo* viral administration of Kirsten rat sarcoma viral oncogene homolog (*Kras*), transformation related protein 53 (*Trp53*), and serine/threonine-protein kinase 11 (*Stk11*)-gRNAs to the Cas9-expressing line caused lung carcinomas within a short period. This suggests that if a Cas9-expressing pig is produced, it will provide an easy and efficient way to produce genetic modifications, which should substantially facilitate studying gene functions, modeling human diseases, and promoting agricultural productivity. Based on this concept, Wang et al. [163] first produced Cre-dependent Cas9-expressing pigs to enable efficient *in vivo* genome editing. They first transfected the linear-targeting donor containing Cre-dependent Cas9-expression cassette and TALEN plasmids directed to *Rosa26* locus into porcine fetal fibroblasts and finally selected clones carrying KI cassette. These clones were then used for SCNT to produce cloned GE piglets. They showed that cells isolated from several organs of GE pigs exhibited Cre-induced activation of Cas9 expression. This Cas9 pig line will be used for various studies as indicated above.

6. Conclusion

Because pigs are similar to humans in physiological, anatomical, and genetic aspects, they are now seen as a leading animal model for biomedical research. Recent

advances in genome editing technology have led to accelerated production of GE pigs within a relatively short time period, which is beneficial due to cost savings in propagation of GE animals and maintaining animals for breeding. Production of GE pigs can be largely categorized into two approaches, so-called MI/EP-mediated production of GE zygotes and SCNT using GE cells as the SCNT donor. There are advantages and drawbacks for both these approaches. For example, the former is simpler, more convenient, and cost-effective than the latter. However, the available genetic background is limited. In this context, the latter is beneficial for the flexibility of choosing any type of genetic background, because the genetic background of SCNT-derived cloned pigs is determined by that of donor cells used for SCNT. Unfortunately, the efficiency of SCNT is extremely low at present. MI/EP with SCNT-treated embryos may compensate for these disadvantages associated with MI/EP or SCNT-mediated production of GE piglets, if the efficiency of SCNT is greatly improved in future.

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Conflicts of interest

The founding sponsors had no role in the design of the study, collection, analyses, or interpretation of data, writing of the manuscript, and decision to publish the results.

Author contributions

Masahiro Sato designed the study and drafted the manuscript; Kazuchika Miyoshi and Hiroaki Kawaguchi involved in the GENTEP-related experiment; Emi Inada and Issei Saitoh critically revised the manuscript; Akihide Tanimoto supervised the manuscript.

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