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In vitro Electroporation in the Presence of CRISPR/Cas9 Reagents as a Safe and Effective Method for Producing Biallelic Knock-Out Porcine Embryos

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Abstract

The production of genetically modified (GM) pigs is considered valuable in biomedical research for the development of model animals for various diseases and pigs with resistance against viral infection. The porcine genome may be modified using several methods, such as somatic cell nuclear transfer (SCNT) using GM cells as the SCNT donor, direct injection of the transgene or the genome editing components (GEC) into fertilized eggs referred to as zygotes, the *in vitro* electroporation (EP) of the zygotes in the presence of GECs, viral infection using retroviruses, injection of the GECs into the SCNT-treated embryos, and the *in vitro* EP of the SCNT-treated embryos in the presence of GECs. In our previous study, we administered a cytoplasmic injection of CRISPR/Cas9-based GEC into parthenogenetically-activated porcine

oocytes (referred to as parthenotes) and observed that these oocytes comprised a mixture of genome-edited and genome-unedited cells, referred to as the "mosaic". In contrast, when *in vitro* EP of the SCNT-treated embryos in the presence of GEC was performed, bi-allelic knock out (KO) of the target gene was detected in most oocytes (82%; 9/11). The production of bi-allelic KO piglets is particularly beneficial for investigating GM domestic animals as it does not require further breeding trials to obtain bi-allelic KO individuals, which would otherwise be a time-consuming and laborious task. In this context, the present study was aimed to confirm the efficiency of *in vitro* EP in producing bi-allelic KO porcine embryos without multiple breeding trials, for which parthenotes were subjected to EP in the presence of a ribonucleoprotein containing Cas9 protein and single-guide RNA (targeted toward *GGTA1*). The treated embryos were cultured until they transformed into blastocysts. The genomic DNA isolated from these blastocysts was used for molecular biology analysis to detect the possible insertion and deletion of sequences (indels) at the *GGTA1* locus. Among the 32 blastocysts obtained, 21 (66%) were observed to be the bi-allelic KO ones. The remaining embryos either had a normal phenotype (25%; 8/32) or mosaic mutations (9%; 3/32). These findings confirm the efficiency of *in vitro* EP in producing bi-allelic KO porcine embryos.

Keywords
Electroporation; α-1,3-galactosyltransferase; CRISPR/Cas9; ribonucleoprotein; single guide RNA; mosaicism; Bi-allelic KO; indels; porcine parthenotes

1. Introduction

Recently, a series of nuclease-based genome-editing tools and technologies have been developed, including zinc-finger nuclease (ZFN), transcription activator-like effector nucleases (TALEN), and clustered regularly-interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas)9 (CRISPR/Cas9), etc., which enable targeted and efficient modification of various eukaryotic species, including mammals [1, 2]. The CRISPR/Cas9-based genome editing requires a guide RNA (gRNA) that would bind to the specific chromosomal DNA site together with Cas9 endonuclease [3-6]. Once bound, each of the two independent nuclease domains in Cas9 cleaves a DNA strand three bases upstream of the protospacer adjacent motif (PAM) and introduces double-strand breaks (DSBs) at the host chromosome target site, which are later repaired through non-homologous end-joining (NHEJ). The NHEJ-based repair process introduces nucleotide insertions or deletions (indel mutations), which may lead to the formation of premature termination (stop) codons, thereby causing protein expression failure via nonsense-mediated mRNA decay, a translation-dependent surveillance mechanism in eukaryotes [7]. The simplicity and the convenience of gRNA designing have enabled the widespread application of CRISPR/Cas9 as a powerful tool for producing genetically modified (GM) organisms [1, 2].

Currently, several methods are available for the production of GM embryos and piglets, such as 1) microinjection of nucleic acids (NAs) into the pronuclei of zygotes [8]; 2) somatic cell nuclear transfer (SCNT) from a GM donor cell into the enucleated porcine oocytes [9-11]; 3) microinjection of nucleic acids (NAs) into SCNT-treated embryos [12]; 3) *in vitro* electroporation (EP) of porcine zygotes in the presence of NAs [13]; and 5) *in vitro* EP of the SCNT-treated embryos in the presence

of NAs [14]. According to Sato et al. [14], in the case of the 61 genome-edited piglets produced since 2013, most of these experiments were focused either on using SCNT-based production of GM piglets or on zygote microinjection [14], while the *in vitro* EP-based production of GM piglets was used by only a few laboratories [13, 15-20]. This is in contrast to the case of genome-edited mice. For instance, since Kaneko et al. [21] first reported the successful use of this technology in 2014, several genome-edited mice have been produced by numerous laboratories worldwide [22-34]. Tanihara et al. [13] pioneered in demonstrating *in vitro* EP as a promising tool for producing GM piglets with high efficiency. For instance, when *in vitro* EP (30 V, square pulses, 1.0 ms in duration, and repeated 5 times) using an electrode (LF501PT1-20; BEX, Tokyo, Japan) and CUY21EDIT II electroporator (BEX) in the presence of genome editing components (GECs) [Cas9 protein (50 ng/μL) along with a single guide RNA (sgRNA) (200 ng/μL)] was performed for *in vitro* fertilized (IVF) porcine eggs (13 h after IVF), successful genome editing was observed in 67% (10/15) of the treated embryos (blastocysts) [13]. The authors named this process "gene editing by electroporation of Cas9 protein (GEEP)". Since then, several genome-edited pigs have been produced using this GEEP technique, including the TP53 (which encodes p53)-mutant pigs [15], CD163 [a putative fusion receptor for the virus causing the porcine reproductive and respiratory syndrome (PRRS)]-mutant pigs [16], and myostatin-mutant pigs [20].

When producing genetically engineered domestic animals, the production of mutations (or transgene insertion) in both alleles of the gene is important as it enables evaluating the results of the phenotypic alteration caused by the gene alteration in an early stage of the investigation. In the event of the GM animals developing mutations (or transgene insertion) only in one allele, obtaining homozygous knock out (KO) animals is time-consuming (at least >0.5 years in the case of pigs) and cumbersome [35]. In mice, *in vitro* EP is beneficial for producing animals with bi-allelic KO phenotypes with relatively high efficiency, as the GECs are incorporated into the embryo with lesser bias [34]. This is in contrast with the findings of our previous work, in which a cytoplasmic injection administered to obtain genome-edited porcine embryos resulted in frequent mosaic mutations in the target locus [36]. Unfortunately, there is little information regarding the efficiency of the embryos with bi-allelic KO phenotype when the porcine zygotes or IVF-derived oocytes are subjected to *in vitro* EP in the presence of GECs.

In the present study, it was investigated whether *in vitro* EP would also be beneficial for the acquisition of embryos with bi-allelic KO phenotypes in the case when porcine parthenotes are used as an alternative to zygotes. Parthenotes were selected as it is convenient to obtain ovaries carrying the oocytes from a slaughterhouse without any additional cost, and the resulting *in vitro* activated oocytes (referred to as "parthenotes") mimic the development of zygotes, at least up to the early gestational stage [37]. In order to induce efficient genome editing in a target locus, Cas9 protein/sgRNA complex [referred to as ribonucleoprotein (RNP)] targeted toward *GGTA1*, a gene encoding α-1,3-galactosyltransferase (α-GalT), was used as it allows rapid genome editing without leaving any traits (GECs) in the target chromosomes [38]. Whether the porcine embryos have bi-allelic KO phenotype is easily detectable using the molecular biology analysis of the porcine genome DNA isolated from a single blastocyst.

2. Materials and Methods

2.1 Preparation of porcine parthenotes

The porcine parthenotes were produced using the methods described in our previous studies [39]**.** Briefly, the ovaries collected from prepubertal gilts at a local slaughterhouse were transported to the laboratory, and the cumulus oocyte complexes (COCs) were extracted from the antral follicles with 2-mm to 5-mm diameter using an 18-gauge needle (Terumo, Tokyo, Japan) fixed to a 5-mL disposable syringe (Nipro, Osaka, Japan). The COCs were washed three times with HEPES (Nacalai Tesque, Kyoto, Japan)-buffered Tyrode's lactate-pyruvate-polyvinyl alcohol (PVA; Sigma-Aldrich Co., Saint Louis, MO, USA) (HEPES-TLP-PVA). Next, approximately 40-50 COCs were transferred to 200 µL of the maturation medium (90% (*v*/*v*) TCM-199 containing Earle's salts (Gibco BRL, Grand Island, NY, USA) and supplemented with 0.91 mM sodium pyruvate (Sigma-Aldrich Co.), 3.05 mM D-glucose (Wako Pure Chemical, Osaka, Japan), 0.57 mM cysteine hydrochloride hydrate (Sigma-Aldrich Co.), 10 ng/mL epidermal growth factor (Sigma-Aldrich Co.), 10 IU/mL eCG (Aska Pharmaceutical Co., Tokyo, Japan), 10 IU/mL hCG (Aska Pharmaceutical Co.), 100 µg/mL amikacin sulfate (Meiji Seika, Tokyo, Japan), 0.1% (*w*/*v*) PVA, and 10% (*v*/*v*) pig follicular fluid covered with paraffin oil (Nacalai Tesque) in a 35-mm dish (#1008; Becton Dickinson, Franklin Lakes, NJ, USA), and then pre-equilibrated overnight at 38.5 °C in 5% CO_2 atmosphere. After 42 to 44 h of maturation, the cumulus cells were removed by pipetting with 0.1% (*w*/*v*) hyaluronidase (Sigma-Aldrich Co.), and the oocytes with polar bodies were selected for further experiments.

The parthenote production was performed by placing the denuded oocytes (20-40) between two wire electrodes that were 1 mm apart in an activation medium [250.3 mM sorbitol, 0.5 mM $Ca(CH_3COO)_2$, 0.5 mM $Mg(CH_3COO)_2$, and 0.1% bovine serum albumin (BSA)] [40] and then inducing activation with one direct current pulse of 100 V/mm for 50 µs using an LF101 Fusion Machine (Nepa Gene Co., Chiba, Japan).

2.2 Preparation of RNP

The gRNA (also referred to as #3 gRNA; [41]) capable of recognizing a 20 bp sequence spanning the translation initiation codon (ATG) upstream of the protospacer adjacent motif (PAM) sequence (AGG) on the 4th exon of porcine *GGTA1* (Figure 1C) was designed. The gRNA that ranked first in the CHOPCHOP analysis (https://chopchop.cbu.uib.no/), which is one of the most widely used web tools for CRISPR-based genome editing, was selected. Furthermore, our preliminary study already confirmed that there was no off-target induction in the parthenotes (blastocysts) microinjected with a mixture of CRISPR/Cas9-related GECs [36].

The Integrated DNA Technologies, Inc. (IDT; Coralville, Iowa, USA) synthesized the gRNA as an Alt-R™ CRISPR crRNA product. The crRNA and tracrRNA (purchased from IDT) were combined for annealing to generate the sgRNA, which was followed by the addition of recombinant Cas9 protein (TaKaRa Bio, Inc., Ohtsu, Japan) to form the RNP, using the methods reported by Ohtsuka et al. [42]. The resulting RNP contained the humanized Cas9 protein (50 ng/µL) and the sgRNA (200 ng/µL), as described by a previous report by Tanihara et al. [13].

2.3 In vitro EP

The EP was performed using the method described by Hashimoto and Takemoto [22]. An electroporation chamber (#LF610P4-4_470; BEX Co. Ltd.) containing two platinum block electrodes situated at a distance of 1 mm from each other (Figure 1A) was placed under a stereoscopic microscope and then connected to an electric pulse generator CUY21EDITII Genome Editor™ (BEX Co. Ltd.). Approximately 30 parthenotes (6 h after activation) were added to a 5-μL drop of Opti-MEM (Invitrogen, Carlsbad, CA, USA) containing 2 μg/μL tetramethylrhodamine-dextran 3 kDa (#D3307; Thermo Fisher Scientific Inc., Waltham, MA, USA) placed between the electrodes (Figure 1A). The EP was performed under the following conditions: 30 V, square pulses, 1.0 ms in duration, at 99 ms intervals, repeated seven times (only 4 times in certain cases). The EP-treated parthenotes were cultured in the PZM-3 (mPZM-3) medium [43] at 38.5 °C under 5% CO_2:5% O_2:90% N_2 atmosphere for 2 days until the 2-cell stage was reached, following, which they were subjected to the evaluation of the uptake of fluorescent dye by the embryos.

In order to perform the EP with GECs, 10-20 parthenotes (6 h after activation) were added to a 5-μL drop of Opti-MEM containing RNP and immediately subjected to *in vitro* EP under the conditions similar to those used for transfection with tetramethylrhodamine-dextran (3 kDa). After the EP, the embryos were cultivated in the PZM-3 medium for 7 days until blastocyst formation and then subjected to molecular biology analysis (to detect the possible mutations in the 4th exon of *GGTA1*) as described below.

2.4 Isolation of Single Blastocysts and Genomic DNA for Molecular Biology Analysis

Genomic DNA isolated from a single blastocyst was analyzed for possible mutations at the individual-embryo level. Briefly, the single blastocyst was transferred to a drop (1 μL) of Ca^{2+} and Mg^{2+}-free Dulbecco's modified phosphate-buffered saline (PBS) in a 0.5-mL PCR tube (#PCR-05-C; AxyGen Scientific, Inc., Union City, CA, USA) using a mouth-controlled micropipette.

Genomic DNA was extracted from this single blastocyte by adding 20 μL of lysis buffer (0.125 μg/mL of proteinase K, 0.125 μg/mL of Pronase E, 0.32 M sucrose, 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 1% (v/v) Triton X-100) to the drop containing the blastocyst followed by incubation at 37 °C for 2-3 days and then phenol/chloroform extraction. The resultant supernatant was subjected to ethanol-precipitation of DNA using a GenTLE® Precipitation Carrier (#9094; TaKaRa Bio, Inc.). The precipitated DNA was dissolved in 20 μL of sterile water and then stored at 4 °C until use.

In order to amplify the total genomic DNA, WGA was performed using the illustra GenomiPhi V2 DNA Amplification Kit (#25-6600-31; GE Health Care Japan, Tokyo, Japan) as described in a previous report [44]. Briefly, 2 μL of the extracted genomic DNA was mixed with 8 μL of the reaction buffer containing 20 μL enzyme, followed by overnight incubation at 30 °C.

The resulting WGA products (2 μL) were subjected to the 1st round of PCR using the Ex-S (5'-GCAAATTAAGGTAGAACGCA-3') and Ex-RV (5'-GCTGCCCCTGAGCCACAACG-3') primer set (Figure 1C), in a reaction volume of 20 μL and under the PCR conditions described in a previous report [44]. Subsequently, 2 μL of the 1st PCR products were subjected to nested PCR, performed using Ex4-2S (5'-CTCCTTAGCGCTCGTTGGCT-3') and Ex4-2RV (5'- GCAACTCTCTGGAATGCTTT-3') primer set (Figure 1C), in a reaction volume of 20 μL and under the same PCR conditions as used in the 1st PCR round. The resultant product was ~350 bp in length, as determined by electrophoresis performed by running 1 μL of the PCR product in 2% agarose gel to assess the band size. The remaining volume of

the PCR product was subjected to purification using a NucleoSpin® Gel and PCR Clean-up (#U0609A; TaKaRa Bio, Inc.).

The sequencing was performed by subjecting 4 μL of the purified solution containing the nested PCR products, ~350 bp in length, to direct sequencing using the Ex4-2S primer, while a few samples of the nested PCR products were sub-cloned into the TA cloning vector pCR2.1 (Invitrogen) and the resulting clones were sequenced using the Ex4-2S primer. The direct sequencing of the PCR products was performed using a custom DNA sequencing service (Eurofins Genomics K.K., Tokyo, Japan).

3.5 Fluorescence Observation

The electroporated embryos were examined for tetramethylrhodamine-derived red fluorescence under a fluorescence microscope (BX60; Olympus, Tokyo, Japan) using DM600 filters (BP545-580 and BA6101F; Olympus). The micrographs were obtained using a digital camera (FUJIX HC-300/OL; Fuji Film, Tokyo, Japan) attached to the fluorescence microscope, and the images were printed using Mitsubishi digital color printer (CP700DSA; Mitsubishi, Tokyo, Japan).

3. Results and Discussion

In order to allow the parthenogenetic development of a porcine oocyte, the oocytes were isolated from the ovary and subjected to electric activation [45] for 12 h, following which the embryos were subjected to *in vitro* EP. In order to determine whether the *in vitro* EP system employed in the present study was effective in the transfection of porcine parthenotes as well, first, EP was performed in the presence of 2 μg/μL of tetramethylrhodamine-labeled dextran 3 kDa, which is used frequently as the indicator of successful gene delivery in certain laboratories [21,42,44]. The parthenotes (10-20) were added to a drop of placed between electrodes and then electroporated under the following conditions: 30 V, square pulses, 1.0 ms in duration, at 99 ms intervals, repeated seven times (Figure 1A). Among 31 parthenotes, 26 (84%) exhibited red fluorescence in their cytoplasm (indicated by arrows in Figure 1B-a and 1B-b) when the ~4-cell stage embryos were examined 2 days after the *in vitro* EP. No appreciable fluorescence was noted in the rest of these embryos (demarcated by an arrowhead in Figure 1B-a and 1B-b), indicating a failure in the uptake of dextran. Furthermore, all the untreated intact parthenotes were non-fluorescent (Figure 1B-c and 1B-d). These results indicated the significance of the *in vitro* EP system used in the present study.

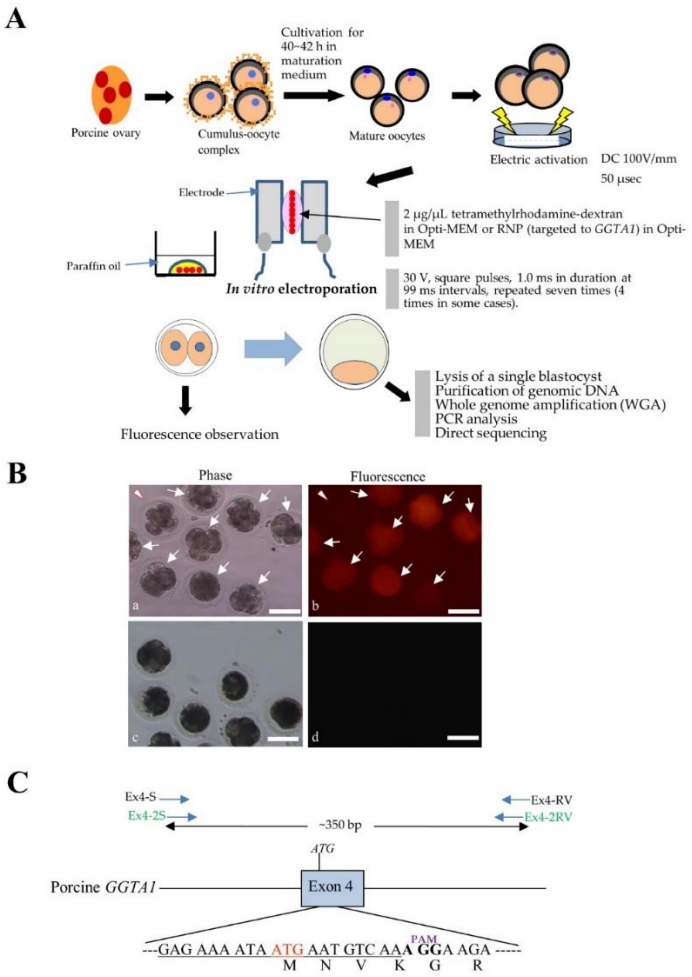

Figure 1 *In vitro* electroporation (EP) is beneficial for the acquisition of genome-edited porcine parthenotes. **A.** Schema for the *in vitro* EP of porcine parthenotes in the presence of tetramethylrhodamine-labeled dextran 3 kDa or ribonucleoprotein (RNP). The ovaries collected from a slaughterhouse were used for isolating the cumulus-oocyte-complex, which was further processed to obtain mature oocytes. After electric activation, these mature oocytes (parthenotes) were subjected to *in vitro* EP. The treated parthenotes were cultured until blastocyst formation in a drop of -3 medium under paraffin oil. The embryos at the 2~4 cell stage were examined for fluorescence under a fluorescence microscope. Single blastocysts were used for isolating the genomic DNA and the subsequent molecular biology analysis. **B.** Fluorescence in the 2~4 cell-stage embryos after the *in vitro* EP in the presence of tetramethylrhodamine-labeled dextran 3 kDa (a,b) and that in the untreated embryos (c,d). Interestingly, over 80% of the parthenotes exhibited fluorescence (indicated by arrows in a,b), while the remaining embryos exhibited no fluorescence (indicated by arrowhead in a,b). The untreated embryos did not exhibit any overt signs of fluorescence (c,d). Scale bar: 100 μm. **C.** Structure of exon 4 of porcine *GGTA1*. The sequence recognized by the sgRNA is underlined. ATG indicates the translation initiation site, and the amino acid sequence is depicted below the nucleotide sequence and the primer sets used (Ex4-S/Ex4-RV for the 1st PCR and Ex4-2S/Ex4-2RV for the nested PCR) are depicted above the sequence. AGG (shown in bold) indicates the PAM site.

Next, it was investigated whether effective genome-editing could be conducted on the endogenous *GGTA1* via the electroporation of porcine parthenotes in the presence of RNP targeted toward *GGTA1*. The RNP contained the humanized Cas9 protein (50 ng/μL) and the sgRNA (200 ng/μL) targeted toward the 20-bp sequence immediately upstream of PAM on the exon 4 of *GGTA1* (Figure 1C). Approximately 10-20 parthenotes were added to a drop of placed between electrodes and then electroporated under the conditions stated above. In this case, the *in vitro* EP was performed under the electric conditions of 30 V and 1.0 ms in the length of a square pulse with 99-ms intervals (1.0). Pulse stimulation was repeated either 4 or 7 times. The treated embryos were subsequently transferred to the PZM-3 medium [43], which allowed for *in vitro* development for seven days until the blastocyst stage. The rate of development of the parthenotes to the 2-cell stage was 68% (34/50) when the pulse was administered 7 times. A similar rate (70%; 35/50) was observed when the pulse stimulation was repeated 4 times (Table 1). In both the groups, the developmental rates for the parthenotes that reached the blastocyst stage ranged between 30% (15/50) and 38% (19/50), which were slightly lower than the developmental rate observed for the untreated parthenotes (51%; 46/91) (Table 1).

Table 1 Summary of the developmental rates to blastocyst stage after EP[1] of porcine parthenotes.

Times of pulse stimulation[2]	No. of experiments	Total number of parthenotes examined	No. of embryos cleaved to the 2-cell stage (%)	No. of embryos developed to blastocysts (%)
-	3	91	73 (80)	46 (51)
4	2	50	35 (70)	19 (38)
7	2	50	34 (68)	15 (30)

[1]EP in the presence of ribonucleoprotein (RNP) is performed on the porcine parthenotes 6 h after the activation. The EP-treated embryos were subsequently cultured for seven days to the blastocyst stage and analyzed for the presence of mutations in the target gene at the molecular biology level.

[2]EP was performed under the electric conditions of 30 V in voltage, 1.0 ms in the length of a square pulse, with 99-ms intervals (1.0), and pulse stimulation repeated 4 or 7 times using an electroporation chamber (#LF610P4-4_470; BEX Co. Ltd.) connected to an electric pulse generator (CUY21EDIT II Genome Editor™, BEX Co. Ltd.). As the control, intact parthenotes without *in vitro* EP were cultivated until the blastocyst stage.

In order to determine the degree of genome editing at a molecular biology level, each blastocyst was subjected to genomic DNA isolation, followed by whole genome amplification (WGA) and the subsequent PCR of a region spanning the target sequence (Figure 1C). Among the 34 blastocysts obtained, two blastocysts exhibited failed target region amplification. Therefore, it was decided to directly sequence the remaining 32 PCR products using the primer Ex4-2S as a nested sense primer (refer to Figure 1C; Materials and Methods section). Consequently, 21 samples (66%) were observed to have bi-allelic KO phenotypes, as evidenced by no significant overlapping in the electrophoretograms of these samples (Table S1). A typical example of the results obtained from the direct sequencing of the nested PCR products using the Ex4-2S primer is presented in Figure 2-

b (for embryo #2) and 2-c (for embryo #6), which depict the samples with nucleotide insertion and nucleotide deletion, respectively. The bi-allelic mutation in these samples was confirmed by sub-cloning the PCR products into pTA cloning vectors. All the obtained sub-clones (6 clones examined for each embryo) exhibited the same sequence as their respective parental products (Table S2).

The remaining embryos either had a normal phenotype (22%; 7/32; Figure 2-a; exemplified by embryo #5; Suppl. Table 1) or mosaic mutations (9%; 3/32; Figure 2-d for embryo #22; Suppl. Table 1). In the case of the mosaic mutations identified in embryo #22, the sequence recognized by the sgRNA was overlapped and the nucleotide(s) were often displayed as "N", indicating a mixture of edited (in the form of bi-allelic or mono-allelic KO mutation) and unedited cells (indicated by arrowheads in sample #22 depicted in Figure 2-d; samples #1, #22, and #32 in Table S1). In order to analyze the results of the DNA sequence analysis for samples #1, #22, and #32 in further detail, the PCR products were sub-cloned into pTA cloning vectors. Sample #1 was observed to have a normal sequence (11%, 1/9), 18-bp deletion (including ATG) (78%, 7/9), and replaced nucleotide (A to C) immediately below the PAM (11%, 1/9) (Table S2), suggesting that this sample had mosaic mutations (including multiple KO alleles). Samples #22 and #32 exhibited phenotypes similar to that of sample #1 (Table S2).

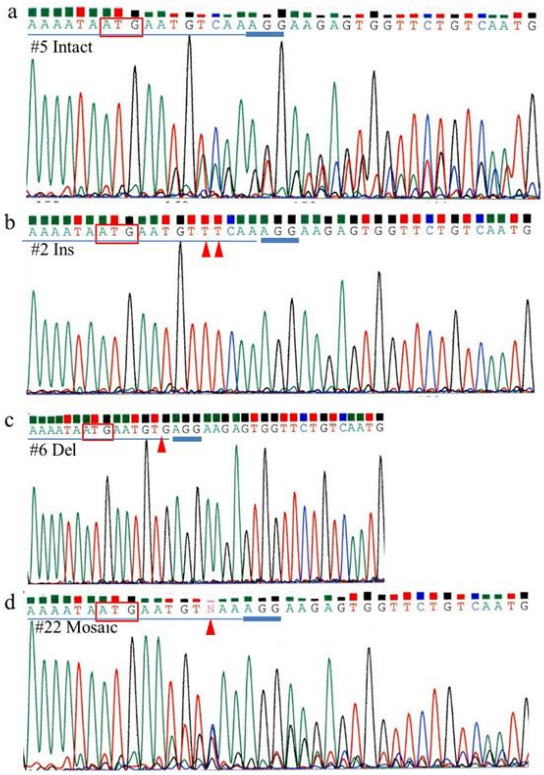

Figure 2 Ideogram pattern in the single blastocyst samples (presented in Suppl. Table 1) obtained after the direct sequencing of the nested PCR products using the Ex4-2S primer. a. Intact blastocyst (#5). b. Blastocyst (#2) exhibiting insertion (Ins). c. Blastocyst (#6) exhibiting deletion (Del). d. Blastocyst (#22) exhibiting mosaic mutation. ATG is depicted in red. The sequence recognized by the sgRNA is depicted with blue underlining. The PAM site is depicted with a bold blue line. The mutations are indicated by red arrowheads under the nucleotide sequence.

In this study, *in vitro* EP was applied to obtain porcine embryos having a bi-allelic knockout (KO) phenotype with high efficiency. When the RNP targeted toward *GGTA1* was used for the porcine parthenotes, 66% of the resulting blastocysts exhibited a bi-allelic KO phenotype, while the remaining embryos either had a normal phenotype (25%) or mosaic mutations (9%).

It is important to understand the reason for the generation of mosaic embryos after transfection with CRISPR/Cas9 components. Several studies have reported that the zygote-derived embryos injected with exogenous materials are often edited in a mosaic pattern [36, 42, 46, 47]. As mentioned earlier, mosaic embryos comprise a combination of edited and unedited cells. This event first occurs when a zygote splits into a 2-cell embryo. One of the blastomeres receives sufficient amounts of GECs, and is, therefore, susceptible to gene editing via the system used in the present study, while the other blastomere might have only a few GECs that may not be sufficient to initiate genome editing at the target locus. This is particularly noticeable when low concentrations of GECs are microinjected cytoplasmically, which may hinder the formation of the sgRNA/Cas9 complex and extend over an entire embryo, thereby leading to mosaicism [36]. Notably, Tanihara et al. [48] investigated the effects of the different concentrations of the CRISPR/Cas9 components on the genetic mosaicism in cytoplasmically microinjected porcine embryos and reported that the ratio of the number of blastocysts that carried mutations to the total number of blastocysts examined in one group (injection at 100 ng/μL of each Cas9 protein and gRNA) was significantly higher than that in the other group (injection at 20 ng/μL of each Cas9 protein and gRNA). Furthermore, no blastocysts with bi-allelic mutations were present in the latter group, while 16.7% of the blastocysts in the former group carried a bi-allelic mutation. In contrast, when *in vitro* EP was used, sufficient amounts of GECs were incorporated throughout the oocyte cytoplasm, leading to unbiased localization of the GECs upon cleavage into the 2-cell stage in mice [34]. A similar observation was reported when *in vitro* EP was performed in a solution containing tetramethylrhodamine-labeled dextran 3 kDa (arrows in Figure 1B-a,b). Moreover, the present study revealed that a high efficiency (66%; 21/32) of obtaining the parthenotes with bi-allelic KO phenotypes could be achieved with the use of *in vitro* EP. These findings suggest that *in vitro* EP enables the unbiased incorporation of relatively higher amounts of GECs into the porcine parthenotes, which may, in turn, contribute to reducing genetic mosaicism.

Yamashita et al. [49] demonstrated the usefulness of three-prime repair exonuclease 2 (Trex2), an exonuclease that improves the gene-editing efficiency, as a reagent for suppressing mosaicism. The authors observed that the co-delivery of murine *Trex2* mRNA and GECs *via* EP into porcine zygotes resulted in the generation of non-mosaic or mosaic mutant blastocysts with the efficiencies of 29% and 71%, respectively. In contrast, the delivery of GECs alone resulted in the generation of non-mosaic or mosaic mutant blastocysts with efficiencies of 6% and 93%, respectively. Therefore, this reagent could be beneficial, particularly when the *in vitro* EP occasionally results in poor performance in terms of inducing bi-allelic KO phenotype in the porcine embryos.

In regard to the effect of the *in vitro* EP on the development of porcine embryos, it was observed that there was a slight decrease in the development into blastocysts with the use of *in vitro* EP (30%-38% vs. 51%) (Table 1). Notably, Tanihara et al. [13] reported that *in vitro* EP may not significantly affect the blastocyst formation rates [23% for control embryos vs. 18%-19% for the embryos electroporated in the presence of Cas9 mRNA + sgRNA or Cas9 protein + sgRNA]. These findings indicate the safety of using *in vitro* EP for the production of viable piglets.

The off-target effects caused by the CRISPR/Cas9 system are concerning. This issue was analyzed prior to attempting the elimination of *GGTA1* using the CRISPR/Cas9 system, and it was discovered that there were no off-target mutations in the candidate genes, which are theoretically prone to be genome edited[36].

Another interesting question would be whether the *in vitro* EP of porcine zygotes in the presence of genome editing components could be targeted toward two or more loci, as the CRISPR/Cas9-based genome editing system enables targeting multiple loci in a single shot of transfection [50, 51]. Such attempts have been successful with porcine fibroblasts that were used as donor cells for the somatic cell nuclear transfer (SCNT)-mediated production of genome-edited piglets [52-55] and porcine zygotes [56]. Notably, Hirata et al. [56] investigated whether *in vitro* EP could be applied for one-step multiplex CRISPR/Cas9-based genome editing targeting the interleukin-2 receptor gamma (*IL2RG*) and growth hormone receptor (*GHR*) genes in porcine embryos and reported success in obtaining double bi-allelic mutations for both genes in ~25% of the embryos (blastocysts). Furthermore, no significant differences in embryo development rates were detected between the control embryos and the embryos subjected to *in vitro* EP. This demonstrated the feasibility of using *in vitro* EP to transfer multiple guide RNAs and Cas9 to porcine zygotes, thereby enabling the double bi-allelic mutation of multiple genes along with favorable embryo survival.

Unfortunately, the *in vitro* EP-based technology for obtaining genome-edited piglets continues to be applied scarcely, with only a few laboratories worldwide reporting success in producing genome-edited porcine embryos and piglets using this technology [13-20, 56]. Tanihara and Otoi successfully knocked-out the genes encoding myostatin (*MSTN*) [13,20], porcine endogenous retroviruses (PERV) polymerase (*pol*) [18], *TP53* (which encodes p53) [15], cluster of differentiation 163 (*CD163*) [16], and pancreas duodenum homeobox 1 (*PDX-1*) [17] by using *in vitro* EP in porcine embryos. For instance, when *TP53* was targeted, all the tested blastocysts (15) exhibited mutations in the *TP53* target region, while approximately 45% of the blastocysts carried bi-allelic KO mutations [15]. Notably, in our previous study concerning the *in vitro* EP of SCNT-treated embryos in the presence of the RNP targeted toward the low-density lipoprotein receptor *(LDLR*) gene, almost all (82%, 9/11) of the resulting embryos (blastocysts) exhibited the bi-allelic KO genotype [14]. Taken together, these findings suggest that the *in vitro* EP-based genome-editing technology used in the present study is safe and efficient for the production of porcine embryos with bi-allelic mutated phenotypes.

Author Contributions

M.S conceived and designed the study, drafted the manuscript, performed the molecular analysis of parthenotes (blastocysts), and revised the manuscript. H.J. performed the experiments, especially acquisition of parthenotes, *in vitro* EP and embryo cultivation. E.A. and K.M. critically revised the manuscript.

Competing Interests

The authors have declared that no competing interests exist.

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