**ORIGINAL ARTICLE**

# Zona pellucida treatment before CRISPR/Cas9-mediated genome editing of porcine zygotes

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**Abstract**

**Background:** Increasing the permeability of the zona pellucida (ZP) of oocytes before CRISPR/Cas9 electroporation may improve the efficiency of gene editing; however, the effects of this approach on subsequent developmental processes are unclear. In this study, the effects of ZP treatment before electroporation on embryonic development and gene editing in porcine embryos were evaluated.

**Methods:** The ZP of zygotes was weakened or removed by exposure to 0.5% actinase E, followed by electroporation of the Cas9 protein with guide RNA targeting *GGTA1*.

**Results:** The blastocyst formation rate of ZP-free zygotes after electroporation was significantly lower ($p < 0.05$) than that of ZP-intact zygotes. The mutation rate in blastocysts from ZP-weakened zygotes was similar to that in ZP-intact zygotes, whereas ZP removal increased the mutation rate. The mutation efficiency in blastocysts from electroporated zygotes did not differ among ZP treatment groups.

**Conclusions:** Our results indicate that weakening the ZP does not affect the developmental competence, mutation rate, or mutation efficiency of electroporated zygotes, whereas ZP removal has a detrimental effect on embryonic development but may increase the mutation rate.

**KEYWORDS**
CRISPR, electroporation, genome editing, porcine zygote, zona pellucida

## 1 | INTRODUCTION

Pigs are considered one of the best animals for generating models of human diseases and for providing organs for xenotransplantation because their physiological and genetic backgrounds are similar to those of humans (Abkowitz et al., 1995; Meurens et al., 2012). Recently, genome editing technologies, such as the clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) system, have allowed us to efficiently generate genetically engineered pigs in a short period of time (Tanihara et al., 2016; Wang et al., 2015). We established the gene editing by electroporation of Cas9 protein (GEEP) method for the highly efficient disruption of a targeted gene by electroporation (Tanihara et al., 2016). This is a simple application of the CRISPR/Cas9 system in which the Cas9 protein and guide RNA (gRNA) are introduced into in vitro-fertilised zygotes by electroporation. The GEEP method does not require complex techniques associated with micromanipulation (such as microinjection) for the introduction of the CRISPR/Cas9 system into zygotes.

Electroporation-based protocols must confer high levels of gene editing while minimising damage to embryos. Therefore, optimizing electroporation conditions with respect to embryonic development and gene editing are critical. We have previously demonstrated that the mutation frequency in targeted genes and embryonic development are influenced by various electroporation parameters, such as the duration, number, polarity, and voltage of pulses (Nishio et al., 2018; Tanihara et al., 2016). Moreover, the concentration of Cas9 affects the mutation rate and gene editing efficiency in porcine embryos (Le et al., 2020). The efficiency of electroporation-based protocols for a variety of simple editing schemes is well-established; however, the application of this approach to more complex genome engineering, such as the insertion of fluorescent tags or conditional alleles, remains unclear. Therefore, complex editing schemes may require alternative approaches, such as the microinjection of macromolecules, and the zona pellucida (ZP) surrounding the plasma membrane of an oocyte may therefore present a barrier preventing the access of CRISPR/Cas9 components to the zygote by electroporation. In mice, the pre-treatment of zygotes with Tyrode's acidic solution has been used to increase the permeability of the ZP for large RNA molecules during electroporation (Peng et al., 2012; Qin et al., 2015). However, the ZP is important for embryonic development, wherein the developmental potential of ZP-free embryos is often lower than that of ZP-intact embryos. Information on suitable conditions of the ZP for the introduction of the CRISPR/Cas9 system into porcine embryos by electroporation is limited.

In the present study, we investigated whether ZP treatment before electroporation influences embryonic development and the targeted mutation efficiency in porcine embryos from zygotes that were edited via the GEEP method, using gRNA targeting the *GGTA1* gene.

## 2 | MATERIALS AND METHODS

No live animals were used in this study; accordingly, ethical approval was not required. All procedures were approved by the Animal Research Committee of Tokushima University.

## 3 | OOCYTE COLLECTION, IN VITRO MATURATION, AND FERTILISATION

Oocyte collection, in vitro maturation, fertilisation, and embryo culture were carried out as described previously (Nishio et al., 2018). Pig ovaries were obtained from prepubertal, crossbreed gilts (Landrace × Large White × Duroc breeds) at a local slaughterhouse. Cumulus–oocyte complexes (COCs) were collected from 3 to 6 mm diameter follicles on the ovarian surface by the slicing method. Approximately 50 COCs were cultured in 500 $\mu$l of maturation medium consisting of tissue culture medium 199 (TCM 199) with Earle's salts (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% (v/v) porcine follicular fluid, 0.6 mM cysteine (Sigma-Aldrich, St. Louis, MO, USA), 50 $\mu$M $\beta$-mercaptoethanol (Wako Pure Chemical Industries Ltd.,

Osaka, Japan), 50 $\mu$M sodium pyruvate (Sigma-Aldrich), 2 mg/ml D-sorbitol (Wako Pure Chemical Industries Ltd.), 10 IU/ml equine chorionic gonadotropin (Kyoritu Seiyaku, Tokyo, Japan), 10 IU/ml human chorionic gonadotropin (Kyoritu Seiyaku), and 50 $\mu$g/ml gentamicin (Sigma-Aldrich), covered with mineral oil (Sigma-Aldrich) for 22 h in 4-well dishes (Nunc A/S, Roskilde, Denmark). The COCs were transferred into a maturation medium without hormones and then cultured for an additional 22 h. The COCs were incubated at 39°C in a humidified incubator containing 5% $CO_2$.

For in vitro fertilisation (IVF), frozen-thawed boar spermatozoa (Landrace breed) were transferred into 6 ml of porcine fertilisation medium (PFM; Research Institute for Functional Peptides Co., Yamagata, Japan) and washed by centrifugation at 500 × $g$ for 5 min. The pelleted spermatozoa were resuspended in PFM and adjusted to 5 × $10^6$ cells/ml. The matured oocytes were then transferred to the sperm-containing PFM and co-incubated in a humidified incubator containing 5% $CO_2$, 5% $O_2$, and 90% $N_2$ for 5 h at 39°C. After co-incubation, the putative zygotes were denuded from the cumulus cells and the attached spermatozoa by mechanical pipetting, transferred to porcine zygote medium (PZM-5; Research Institute for the Functional Peptides Co.), and then cultured for 7 h until electroporation treatment. After 7 h of culture, zygotes were randomly assigned to each experimental group.

## 3.1 | Zona pellucida treatment

Before electroporation, the ZP of zygotes (1-cell stage) was weakened or removed by actinase E treatment (Figure 1a–c). Briefly, denuded zygotes were exposed to 0.5% (w/v) actinase E (Kaken-Seiyaku Co., Tokyo, Japan) in Dulbecco's PBS (Thermo Fisher Scientific) for 3–15 s and then transferred to PZM-5 to stop the activity of actinase E. Some zygotes were freed completely from their ZP by gentle pipetting. The ZP-weakened or ZP-free zygotes were incubated at 39°C in a humidified incubator containing 5% $CO_2$, 5% $O_2$, and 90% $N_2$ for 1 h before electroporation.

## 3.2 | Electroporation

Electroporation was performed as described previously (Tanihara et al., 2016). Briefly, the electrode (LF501PT1-20; BEX Co. Ltd., Tokyo, Japan) was connected to a CUY21EDIT II electroporator (BEX Co. Ltd.) and placed under a stereoscopic microscope. The zygotes with or without ZP treatment were washed with Opti-MEM I solution (Thermo Fisher Scientific) and placed in a line in the electrode gap on the chamber slide filled with 10 $\mu$l of nuclease-free duplex buffer (Integrated DNA Technologies (IDT), Coralville, IA, USA) with 100 ng/$\mu$l gRNA (Alt-R CRISPR crRNAs and tracrRNA, chemically modified and length-optimised variants of the native guide RNA purchased from IDT) targeting *GGTA1* and 100 ng/$\mu$l Cas9 protein (Guide-it Recombinant Cas9; Takara Bio, Shiga, Japan). A gRNA targeting *GGTA1* (5′-GGATTAAACCAGTCCACTAG-3′) resulting in a high biallelic mutation rate in our previous
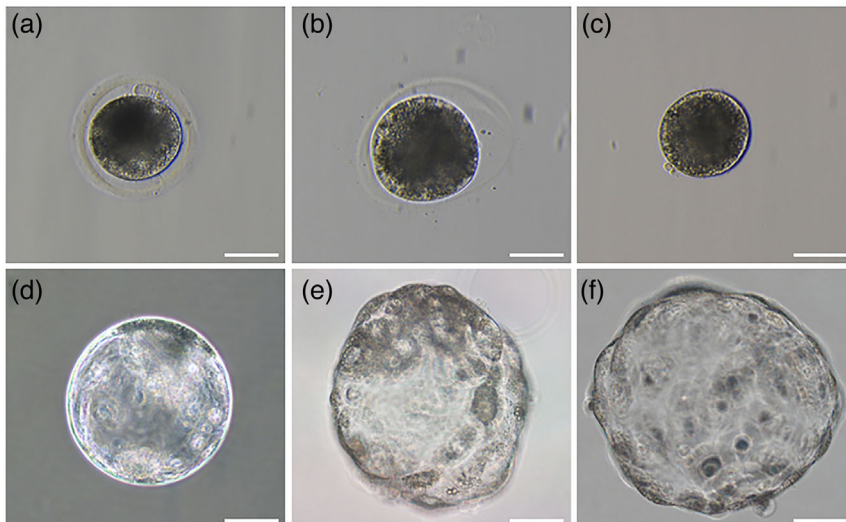
**FIGURE 1** Representative images of porcine zygotes with intact (a), weakened (b), and free (c) zona pellucida, and blastocysts derived from zygotes with intact (d), weakened (e), and free (f) zona pellucida. Blastocysts derived from zygotes with weakened zona pellucida lost the zona pellucida during embryonic development. Scale bar = 50 $\mu$m

study was used for electroporation treatment (Tanihara et al., 2020).

After electroporation with five 1-msec pulses at 25 V, the zygotes with or without ZP treatment were washed with PZM-5 and cultured in 4-well dishes (~35 embryos per well) for 3 days. The embryos were subsequently incubated in porcine blastocyst medium (PBM; Research Institute for the Functional Peptides Co.) for 4 days for evaluations of blastocyst formation and for blastocyst genotyping (Figure 1d–f). As a control, some zygotes were cultured in PZM-5 and PBM for 7 days without electroporation. Zygotes and embryos were incubated at 39°C in a humidified incubator containing 5% $CO_2$, 5% $O_2$, and 90% $N_2$.

### 3.3 | Analysis of targeted gene sequences in embryos

Targeted genes in individual blastocysts were analysed as described previously (Tanihara et al., 2020). Briefly, genomic DNA was isolated from individual blastocysts by boiling in a 50 mM NaOH solution at 98°C for 10 min and then neutralised with 100 mM Tris HCl. After neutralisation, the genomic regions flanking the gRNA target sequences were amplified by polymerase chain reaction (PCR) using the following primer pair: *GGTA1*, 5′-AAAAGGGGAGCACTGAACCT-3′ (forward) and 5′-ATCCGGACCCTGTTTTAAGG-3′ (reverse). The PCR products were separated by agarose gel electrophoresis and purified using a Fast Gene Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan). The PCR products were directly sequenced by Sanger sequencing using the BigDye Terminator Cycle Sequencing Kit version 3.1 (Thermo Fisher Scientific K.K., Tokyo, Japan) and an ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The tracking of indels by decomposition (TIDE) bioinformatics package was used to determine the genotype of each blastocyst (http://shinyapps.datacurators.nl/tide/) (Brinkman et al., 2018). Blastocysts that did not carry wild-type (WT) sequences were classified as having biallelic mutations. Blastocysts that carried more than one type of mutation in addition to the WT sequence were classified as mosaics. Blastocysts that carried only

the WT sequence were classified as WT (Figure 2). The mutation rate was defined as the ratio of the number of blastocysts carrying a mutation to the total number of sequenced blastocysts. The mutation efficiency was defined as the proportion of indel mutation events in blastocysts carrying biallelic or mosaic mutations.

### 3.4 | Statistical analysis

Data for embryonic development were evaluated by an analysis of variance (ANOVA) followed by Fisher's protected least significant difference tests using STATVIEW (Abacus Concepts, Inc., Berkeley, CA, USA). All percentages were subjected to arcsine transformation before ANOVA. The percentages of mutations within all sequenced blastocysts were analysed by a chi-squared test with Yates' correction. Differences with a $p$-value of ≤0.05 were regarded as significant.

## 4 | RESULTS

The blastocyst formation rates of the electroporated zygotes were significantly lower ($p < 0.05$) than those of the control zygotes, irrespective of ZP treatment (Table 1). Moreover, the blastocyst formation rate of the ZP-free zygotes after electroporation was significantly lower ($p < 0.05$) than that of ZP-intact zygotes. In contrast, the mutation rate obtained by sequencing target sites in *GGTA1* in blastocysts from ZP-free zygotes was significantly higher ($p < 0.05$) than that in blastocysts from ZP-intact zygotes. The biallelic mutation rates in the examined blastocysts and the mutation efficiency in the mutant blastocysts did not differ among the ZP treatment groups.

## 5 | DISCUSSION

Electroporation of zygotes is an effective alternative to microinjection for CRISPR/Cas9-mediated genome editing in pigs.

**FIGURE 2** Representative results of Sanger sequencing of blastocysts formed after electroporation with Cas9 protein and gRNAs. Dotted lines indicate the predicted Cas9 cleavage sites. Blue nucleotides indicate the target sequences, and red nucleotides indicate the protospacer adjacent motif (PAM) sequences. Biallelic, bi-allelic mutation; mosaic: mosaic mutation; WT, wild-type
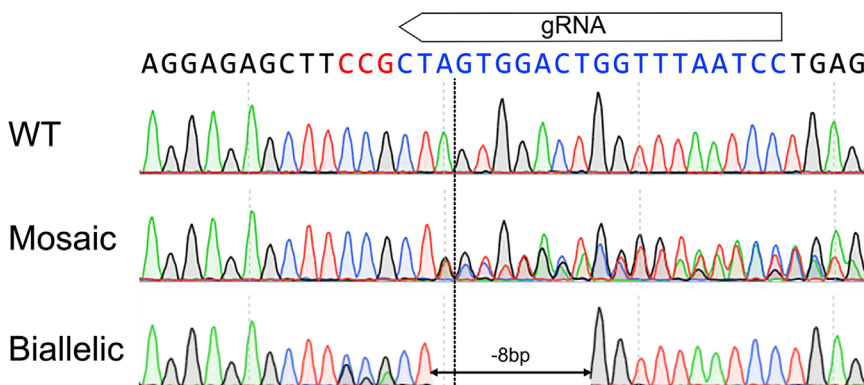


**TABLE 1** Effects of zona pellucida (ZP) treatment before electroporation with the clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) system on the development of zygotes and the mutation rate in blastocysts[†]

| Zygote[††] | No. of zygotes examined | No. (mean ± SEM) of embryos | | No. of blastocysts examined | No. (mean) of blastocysts | | Total mutation efficiency (mean ± SEM)[¶] |
| | | Cleaved | Developed to blastocysts | | Mutant[‡] | Biallelic mutant[§] | |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Control | 199 | 170 (85.4 ± 2.3) | 34 (17.1 ± 1.9)[a] | – | – | – | – |
| ZP-intact | 208 | 171 (82.2 ± 3.0) | 23 (11.1 ± 2.4)[b] | 20 | 7 (35.0)[a] | 2 (10.0) | 69.6 ± 8.4 |
| ZP-weakened | 199 | 163 (81.4 ± 5.0) | 16 (8.2 ± 1.7)[b,c] | 12 | 7 (58.3)[a,b] | 2 (16.7) | 71.9 ± 8.0 |
| ZP-free | 203 | 175 (85.9 ± 4.1) | 6 (3.0 ± 0.9)[c] | 6 | 6 (100)[b] | 3 (50.0) | 88.9 ± 6.7 |

[†]Four replicate trials were carried out.

[††]Electroporation was performed by five 1-msec pulses at 25 V. As a control, zygotes with an intact ZP were cultured without electroporation.

[‡]Proportions were calculated by dividing the number of mutant blastocysts by the total number of sequenced blastocysts.

[§]Proportions were calculated by dividing the number of biallelic mutant blastocysts by the total number of sequenced blastocysts.

[¶]Mean proportions represent the proportion of indel mutation events in mutant blastocysts determined by the TIDE bioinformatics package.

[a-c]Values with different superscript letters in the same column are significantly different ($p < 0.05$).

Electroporation is a rapid procedure and can be applied to many zygotes simultaneously, enabling high-throughput production. However, the successful CRISPR-mediated generation of large genomic integrations by electroporation into zygotes has been limited by the inability of large molecules to reach the nucleus (Remy et al., 2017). Insufficient permeability of the nuclear membrane may also be responsible for low transgene integration upon electroporation. Moreover, in 1-cell-stage embryos, the ZP makes the introduction of macromolecules difficult. To overcome this physical barrier, the weakening of the ZP before electroporation has been performed to increase permeability for efficient introduction (Peng et al., 2012; Qin et al., 2015). To date, enzymatic digestion with pronase for ZP removal has been applied to oocytes and embryos in domestic and laboratory species, including cattle, pigs, and sheep (Li et al., 2013; Peura & Vajta, 2003). It has been suggested that ZP removal by pronase improves the quality of porcine parthenogenetic embryos by accelerating the speed of embryonic development and decreasing the number of apoptotic cells in blastocysts (Li et al., 2013). In the present study, therefore, we used actinase E (a synonym of pronase) for the ZP treatment. We found that ZP removal had a detrimental effect on the blastocyst formation rates of electroporated zygotes; however, the blastocyst formation rate was similar in ZP-weakened and ZP-intact zygotes. When the quality of blastocysts from the electroporated zygotes with and without the ZP treatment was examined (number of examined embryos in each group, 9–10), there were no differences in the total cell numbers of embryos among the groups (83.4–121.8 cells). However, the apoptotic rate of blastocysts from ZP-weakened zygotes (7.0%) was significantly higher ($p < 0.05$) than that of blastocysts from ZP-free zygotes (2.9%) but was similar to that from ZP-free zygotes (6.5%) (data not shown). These results indicate that the ZP treatment may affect embryonic development or quality. It has been reported that, in mice, the weakening of the ZP by acidic Tyrode's solution does not compromise the survival of the electroporated embryos and improves access to embryos by CRISPR/Cas9 reagents, resulting in a high targeting efficiency (Peng et al., 2012; Qin et al., 2015). Accordingly, under identical electric pulses, reagents can enter the weakened ZP more easily. However, our results showed that the mutation rate in blastocysts from ZP-weakened zygotes was similar to that in blastocysts from ZP-intact zygotes, whereas the removal of the ZP increased the mutation rate in blastocysts. There were no differences in the biallelic mutation rates in blastocysts and the mutation efficiency in mutant blastocysts among the electroporation

groups; however, half of the embryos from ZP-free zygotes had biallelic mutations. Previous studies have suggested that the weakening of the ZP before electroporation is necessary for gene editing using Cas9 mRNA of about 4,500 nucleotides length but does not substantially improve the uptake of small molecules, like morpholinos of only 25 nucleotides in length (Peng et al., 2012; Qin et al., 2015). These studies are consistent with our results for mutation rates and efficiencies, demonstrating that the weakening of the ZP does not increase the mutation rate in blastocysts from zygotes electroporated with the Cas9 protein (160 kDa), which is much smaller than Cas9 mRNA (~1500 kDa). Although electroporation variation, such as a higher voltage, may enhance the mutation efficiency in blastocysts from ZP-weakened zygotes, our results indicated that ZP removal increased the mutation rate in embryos and biallelic mutation rates.

In conclusion, the weakening of the ZP did not affect the developmental competence of electroporated zygotes and did not improve the mutation rate or mutation efficiency in blastocysts. In contrast, ZP removal had a detrimental effect on embryonic development but increased the mutation rate of embryos and biallelic mutations.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. All the animals involved in this study received humane care in compliance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council. All procedures were approved by the Animal Research Committee of Tokushima University.

## AUTHOR CONTRIBUTIONS

*Writing – original draft preparation and investigation*: Zhao Namula. *Conceptualisation and writing – review & editing*: Maki Hirata. *Investigation*: Quynh Anh Le, Qingyi Lin, and Koki Takebayashi. *Conceptualisation and writing – review & editing*: Naoaki Yoshimura. *Investigation, formal analysis, and writing – review & editing*: Fuminori Tanihara. *Methodology and writing – review & editing*: Chommanart Thongkittidilok. *Conceptualisation, project administration, and writing – review & editing*: Takeshige Otoi.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

## PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/vms3.659

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