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One-step genome editing of porcine zygotes through the electroporation of a CRISPR/Cas9 system with two guide RNAs

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Abstract

In the present study, we investigated whether electroporation could be used for one-step multiplex CRISPR/Cas9-based genome editing, targeting *IL2RG* and *GHR* in porcine embryos. First, we evaluated and selected guide RNAs (gRNAs) by analyzing blastocyst formation rates and genome editing efficiency. This was performed in embryos electroporated with one of three different gRNAs targeting *IL2RG* or one of two gRNAs targeting *GHR*. No significant differences in embryo development rates were found between control embryos and those subjected to electroporation, irrespective of the target gene. Two gRNAs targeting *IL2RG* (nos. 2 and 3) contributed to an increased biallelic mutation rate in porcine blastocysts compared with gRNA no. 1. There were no significant differences in the mutation rates between the two gRNAs targeting *GHR*. In our next experiment, the mutation efficiency and the development of embryos simultaneously electroporated with gRNAs targeting *IL2RG* and *GHR* were investigated. Similar embryo development rates were observed between embryos electroporated with two gRNAs and control embryos. When *IL2RG*-targeting gRNA no. 2 was used with *GHR*-targeting gRNAs no. 1 or no. 2, a significantly higher double biallelic mutation rate was observed than with *IL2RG*-targeting gRNA no. 3. In conclusion, we demonstrate the feasibility of using electroporation to transfer multiple gRNAs and Cas9 into porcine zygotes, enabling the double biallelic mutation of multiple genes with favorable embryo survival.

Keywords *IL2RG* · *GHR* · CRISPR/Cas9 · Electroporation · Pig

Introduction

Domestic pigs have been shown to be an effective model organism for biomedical research, as their background physiology and genetic heterogeneity are closely related to those of humans (Abkowitz *et al*. 1995; Meurens *et al*. 2012). Pigs are considered to have a high reproductive performance, as heat cycles start as early as 5 mo old. They also have a short generation interval and a high number of offspring per litter (Wolf *et al*. 2000; Meurens *et al*. 2012). Owing to these advantages and the ease of genome manipulation, domestic pigs have recently become an important animal model in genetic engineering studies (Staunstrup *et al*. 2012; Umeyama *et al*. 2012; Wu *et al*. 2013; Chou *et al*. 2014). To date, a number of genome editing systems, including engineered endonucleases like the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system, have been used to generate genetically modified animals (Zhou *et al*. 2015; Tanihara *et al*. 2016; Tanihara *et al*. 2018). In previous studies, we have successfully demonstrated the direct editing of single genes by delivering the CRISPR/Cas9 system via electroporation, to generate various types of mutations in porcine blastocysts (Hirata *et al*. 2019a; Tanihara *et al*. 2019).

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However, to increase the feasibility and effectiveness of a porcine genome editing system, there remains a need for a simple method of one-step multigene targeting.

The co-expression of multiple guide RNA (gRNAs) in the CRISPR/Cas9 system can enhance genome editing techniques by precisely disrupting the genomic DNA at multiple sites simultaneously (Cao *et al*. 2016; Minkenberg *et al*. 2017). Several methods to efficiently express multiple gRNAs in target organisms have been employed, including the injection of multiple in vitro-prepared gRNA molecules to target two different genes in cynomolgus monkey (*Ppar-γ* and *Rag1*), mouse (*Tet1* and *Tet2*), and pig (*Rag2* and *IL2RG*) zygotes (Wang *et al*. 2013; Niu *et al*. 2014; Lei *et al*. 2016) and the electroporation of a single plasmid to deliver multiple gRNAs targeting five different genes (*pikA*, *pikB*, *pikC*, *pikF*, and *pikG*) into the simple amoeboid eukaryote *Dictyostelium discoideum* (Sekine *et al*. 2018). Compared with rodents (Colucci *et al*. 1999), it is costly and time consuming to generate pigs with multiple edited genes through the breeding of established single-gene knockout animals, because of their long gestation period and extended time to reach puberty. We considered that this multiplex CRISPR/Cas9-based genome editing and electroporation method would significantly improve the efficiency of the system, shortening the process duration and reducing the cost of the overall system. Recently, we have reported the evaluation of simultaneous multiplex gene editing via electroporation in the porcine embryos (Hirata *et al*. 2020). However, we obtained blastocysts in which only two of the four target genes were edited, and biallelic mutation was detected in only one target gene. Therefore, technical improvements are required to achieve the simultaneous knockout of multiple target genes using electroporation.

Pigs with severe combined immunodeficiency (SCID) have been a useful model in regenerative medicine, xenotransplantation, and cancer cell transplantation owing to similarities in the pathophysiology of their immune system compared with that of humans (Kang *et al*. 2016). Deletion or mutation of the interleukin-2 receptor gamma (*IL2RG*) gene is associated with the development of SCID in pigs; therefore, several studies have generated *IL2RG* knockout pigs as models for studies related to this condition (Suzuki *et al*. 2012; Watanabe *et al*. 2013; Kang *et al*. 2016). In addition, mutations in the growth hormone receptor (*GHR*) gene can result in Laron syndrome, causing a slow growth rate and small body size (Yu *et al*. 2018); this provides many advantages for their use in laboratories, such as the ease of handling, and the need for small rearing spaces and low doses of test substances. However, the generation of *IL2RG/GHR* knockout pigs is technically challenging because of the two targets for disruption. To clarify the possibility and efficiency of a one-step multiplex CRISPR/Cas9-based genome editing protocol using electroporation, in this study, we examined the multiplex gene editing of porcine zygotes by introducing various combinations of gRNAs targeting *IL2RG* and *GHR* at distinct regions.

Materials and methods

Oocyte collection, in vitro maturation, and fertilization Pig ovaries were obtained from prepubertal gilts (Landrace × Large White × Duroc breeds) at a local slaughterhouse and were transported to the laboratory within 1 h in physiological saline at 30°C. Ovaries were washed three times with a prewarmed physiological saline solution supplemented with 100 IU/ml penicillin G potassium (Meiji, Tokyo, Japan) and 0.1 mg/ml streptomycin sulfate (Meiji). Follicles with diameters of 3–6 mm on the ovarian surface were sliced on a sterilized dish using a surgical blade, and cumulus-oocyte complexes (COCs) were visualized and collected under a stereomicroscope. Approximately 50 COCs were cultured in 500 μl of maturation medium consisting of tissue culture medium 199 with Earle's salts (TCM 199; Thermo Fisher Scientific, Waltham, MA) supplemented with 10% (v/v) porcine follicular fluid, 0.6 mM cysteine (Sigma-Aldrich, St. Louis, MO), 50 μM β-mercaptoethanol (Wako Pure Chemical Industries Ltd., Osaka, Japan), 50 μM sodium pyruvate (Sigma-Aldrich), 2 mg/ml D-sorbitol (Wako Pure Chemical Industries Ltd.), 10 IU/ml equine chorionic gonadotropin (Asuka Pharmaceutical, Tokyo, Japan), 10 IU/ml human chorionic gonadotropin (Nippon Zenyaku Kogyo, Fukushima, Japan), and 50 μg/ml gentamicin (Sigma-Aldrich). They were then covered with mineral oil (Sigma-Aldrich) and incubated for 22 h in 4-well dishes (Nunc A/S, Roskilde, Denmark). The COCs were then transferred into a maturation medium without hormones and incubated for an additional 22 h at 39°C in a humidified incubator containing 5% CO_2.

The matured oocytes were subjected to in vitro fertilization as described previously (Nguyen *et al*. 2017). Briefly, freeze-thawed ejaculated spermatozoa were transferred into 5 ml of porcine fertilization medium (PFM; Research Institute for the 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 1×10^6 cells/ml. Then, approximately 50 oocytes were transferred to 500 μl of sperm-containing PFM, covered with mineral oil in 4-well dishes, and co-incubated for 5 h at 39°C in a humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2. 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 cultured for 7 h until electroporation.

Electroporation Electroporation was performed as described previously (Tanihara *et al*. 2016). Briefly, an electrode (LF501PT1-20; BEX, Tokyo, Japan) was connected to a CUY21EDIT II electroporator (BEX) and was set under a stereoscopic microscope. The inseminated zygotes were washed with Opti-MEM I solution (Thermo Fisher

Scientific) and placed in a line between the electrode gap in a chamber slide filled with 10 μl of Nuclease-Free Duplex Buffer (Integrated DNA Technologies (IDT), Coralville, IA) containing 100 ng/μl gRNA (Alt-R CRISPR crRNAs and tracrRNA, chemically modified and length-optimized variants of the native guide RNAs purchased from IDT) and 100 ng/μl Cas9 protein (Guide-it Recombinant Cas9; Takara Bio, Shiga, Japan). The gRNAs were designed using the CRISPR direct web tool (https://crispr.dbcls.jp/) (Naito *et al.* 2015). To minimize off-target effects, the 12 nucleotides at the 3′ end of the designed gRNAs had no sequence matches in the pig genome other than the target regions of *IL2RG* and *GHR,* as determined using the COSMID web tool (https://crispr.bme.gatech.edu/) (Cradick *et al.* 2014).

After electroporation (five square pulses at 25 V, pulse length (Pd on): 1.00 ms, pulse interval (Pd off): 99.0 ms), the zygotes were washed with PZM-5 and were cultured for 3 d. The embryos were subsequently incubated in porcine blastocyst medium (PBM; Research Institute for the Functional Peptides Co.) for 4 d to evaluate their blastocyst formation ability and for blastocyst genotyping. As a control, some of the inseminated zygotes were cultured in PZM-5 and PBM for 7 d 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.

Analysis of the targeted genes after electroporation Genomic DNA was isolated from the blastocysts by boiling in 50 mM NaOH solution. After neutralization, the genomic regions flanking the gRNA target sequences were amplified by polymerase chain reaction (PCR) using the following primers: for *IL2RG*, 5′-CCCAGGTTCCTGACACAGTC-3′ (forward) and 5′-GAGGCAAAGGGAAGACATGA-3′ (reverse); for *GHR*, 5′-CCCACCGGAAGTAGCATTTA-3′ (forward) and 5′-ACAACACTCCCGGAAACATC-3′ (reverse). The PCR products were resolved by agarose gel electrophoresis and extracted, and the targeted genomic regions were directly sequenced. Sanger sequencing was performed using the BigDye Terminator Cycle Sequencing Kit ver. 3.1 (Thermo Fisher Scientific) and an ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA). The Tracking of Indels by Decomposition (TIDE) bioinformatics package (Brinkman *et al.* 2014) was used to determine the genotypes of blastocysts. Blastocysts were classified as having biallelic mutations (carrying no wild-type (WT) sequences), mosaics (carrying more than one type of mutation and the WT sequence), or WT (carrying only the WT sequence).

Experimental design *Experiment 1: generation of mutant blastocysts and confirmation of the gene-targeting efficiency of the gRNAs*

Three different gRNAs were designed to target *IL2RG* (gRNAs no. 1 to no. 3) and two targeted *GHR* (gRNAs no. 1 and no. 2; Table 1 and Fig. 1). Each gRNA, along with the Cas9 protein, was introduced into in vitro-fertilized zygotes by electroporation. The blastocyst formation rate in the electroporated embryos and the efficiency of genome editing in the resulting blastocysts were evaluated. The biallelic mutation rate was defined as the ratio of blastocysts with biallelic mutation to total examined blastocysts. The total mutation rate was defined as the ratio of blastocysts carrying biallelic mutation or mosaic mutations to total examined blastocysts.

Experiment 2: generation of IL2RG/GHR mutant blastocysts

Two gRNAs (no. 2 and no. 3) that exhibited high efficiencies in targeting *IL2RG* in Experiment 1 were used in Experiment 2. To generate embryos with both *IL2RG* and *GHR* mutations, a combination of one gRNA (no. 2 or no. 3) targeting *IL2RG* and one gRNA (no. 1 or no. 2) targeting *GHR* was introduced into in vitro-fertilized zygotes by electroporation. Again, we examined the blastocyst formation rate of embryos produced by frozen-thawed spermatozoa that were different from Experiment 1 and evaluated the efficiency of genome editing in the resulting blastocysts. Using Sanger sequencing and TIDE analysis, the blastocysts were classified as having double biallelic mutations (carrying biallelic mutations in both *IL2RG* and *GHR*), double mutations (carrying biallelic or mosaic mutations in both target genes but excluding blastocysts with double biallelic mutations), a single mutation (carrying a biallelic or mosaic mutation in either *IL2RG* or *GHR*), or as WT.

Statistical analysis The percentage of embryos that developed to the blastocyst stage was subjected to arcsine transformation. The transformed data were evaluated using analysis of variance, followed by protected Fisher's least significant difference tests. The analysis was performed using StatView software (Abacus Concepts, Berkeley, CA). The percentages of mutated blastocysts were analyzed using chi-squared tests with Yates' correction. Differences with probability values (p) < 0.05 were considered statistically significant.

Results

We examined the effects of the gRNAs targeting each gene on embryonic development (Figs. 2 and 3). There were no differences in the rates of blastocyst formation among the gRNAs targeting either *IL2RG* or *GHR* (Figs. 2*a* and 3*a*). Moreover, the blastocyst formation rates of embryos subjected to electroporation treatment were similar to those of control embryos without the electroporation treatment, irrespective of the target gene. For gRNAs that targeted *IL2RG*, the gRNA sequences influenced the biallelic mutation rates but not the total mutation rates (Fig. 2*b*). *IL2RG* gRNAs no. 2 and no. 3 had higher biallelic mutation rates. There were no significant

Table 1. Sequences of the gRNAs targeting *IL2RG* or *GHR*

Target gene	No.	Target sequence	PAM	Strand
IL2RG	No. 1	CCACTGTGCGTGAGGACCTT	CGG	Antisense
	No. 2	CCGAAGGTCCTCACGCACAG	TGG	Sense
	No. 3	AGGAATAAGAGGGATTTGAC	TGG	Antisense
GHR	No. 1	GCTTCTGTTGACCTTGGCAG	TGG	Sense
	No. 2	ACTTGAGCCTGCCACTGCCA	AGG	Antisense

differences in the biallelic and total mutation rates between the two gRNAs targeting *GHR* (Fig. 3*b*).

When evaluating the development of zygotes electroporated simultaneously with two gRNAs targeting different genes, we observed that electroporation did not influence blastocyst formation rates (Fig. 4*a*). Next, we evaluated the mutation rates by sequencing each target site in the resulting blastocysts (Fig. 5) and observed that embryos derived from zygotes electroporated simultaneously with gRNA no. 2 targeting *IL2RG* and either of the gRNAs targeting *GHR* showed higher rates of double biallelic mutations and double mutations (Fig. 4*b*). However, the gRNA sequences and target genes did not influence the total mutation rates in the resulting blastocysts.

Discussion

The porcine SCID model is a valuable tool to study the pathogenesis of human diseases and xenogeneic transplantation (Suzuki *et al*. 2012; Lei *et al*. 2016). The combined disruption of certain genes, such as *IL2RG* and *GHR*, can result in smaller pigs with severe immunocompromised status, which could be ideal model organisms for SCID-related diseases (Kang *et al*. 2016; Watanabe *et al*. 2013). Although transgenic porcine models of SCID have been successfully produced using microinjection (Lei *et al*. 2016), limitations of this method include high embryo lethality (Ivics *et al*. 2014) and a requirement for advanced technical skills (Iqbal *et al*. 2009). Several studies have investigated whether electroporation could be an alternative method for the production of transgenic models, thanks to its easy application, relatively small workload, reduced requirement for equipment training, and increased embryo survival rates (O'Meara *et al*. 2011; Chen *et al*. 2016; Nakagawa *et al*. 2018). Therefore, in the present study, we investigated whether electroporation could be similarly applied in one-step multiplex CRISPR/Cas9-based genome editing of porcine embryos, more specifically targeting *IL2RG* and *GHR*.

In Experiment 1, we first evaluated multiple gRNAs that targeted *IL2RG* and *GHR*, analyzing blastocyst formation rates and genome editing efficiencies in the resulting blastocysts. Different gRNA sequences had no apparent effects on blastocyst formation, which did not correspond to the findings of our previous studies (Hirata *et al*. 2019b; Tanihara *et al*. 2019), in which the development of porcine embryos edited using the CRISPR/Cas9 system was affected by gRNA sequence used. Conversely, we also recently reported that the gRNA sequence did not affect the blastocyst formation rate in the genome editing of target genes including *GHR* (Hirata *et al*. 2020). These results suggested that the effect of genome editing on the developmental competence of porcine embryos may differ depending on the target gene or gRNA sequence. The mutation rates and efficiencies obtained in the present study demonstrated that good gRNA design is a key enabling highly efficient gene targeting using the CRISPR/Cas9 system. As reported in previous studies, the sequence features of

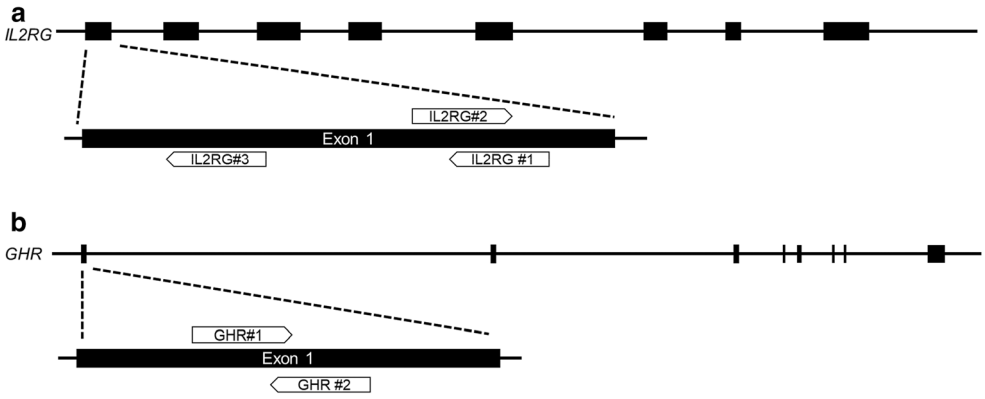

Figure 1. Genomic structures of *IL2RG* (*a*) and *GHR* (*b*) and gRNAs targeting their first exons.

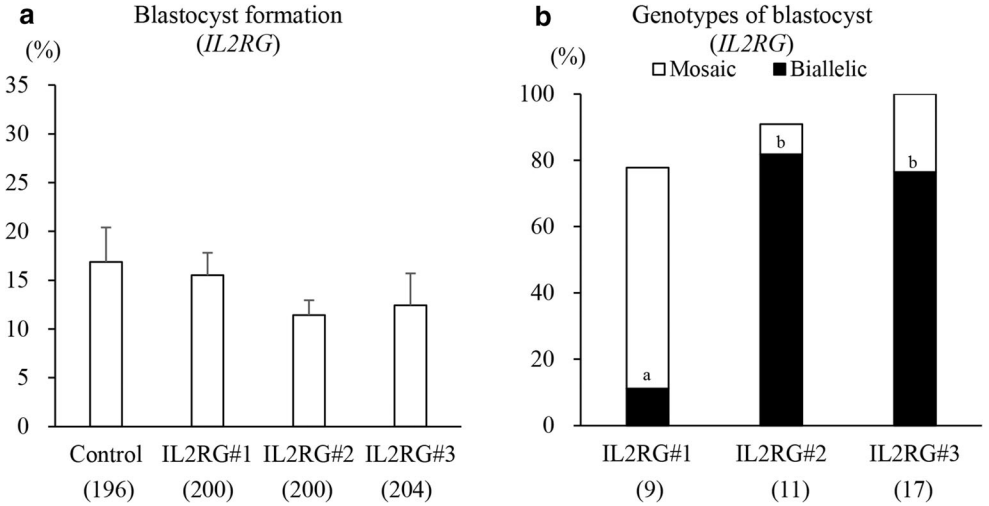

Figure 2. Development (*a*) and mutation (*b*) of porcine zygotes edited with gRNAs targeting *IL2RG*. The sequences of the gRNA target region and the genome editing efficiencies in the resulting blastocysts were determined using Sanger sequencing and TIDE analysis, respectively. The percentage of edited blastocysts was defined as the ratio of the number of edited blastocysts to the total number of sequenced blastocysts. Biallelic: biallelic mutant, Mosaic: mosaic mutant. The *numbers* within *parentheses* indicate the total number of oocytes and blastocysts examined for development and mutation, respectively. As a control for blastocyst formation, inseminated zygotes were cultured for 7 d without electroporation. [a–b]Values with different *superscripts* are significantly different ($p < 0.05$).

the gRNA can affect the efficiency of cleavage by the Cas9 nuclease (Doench *et al*. 2014; Ren *et al*. 2014; Sandoval *et al*. 2019); for example, a gRNA with GC content between 40 and 60% may be advantageous, as it allows a greater match to the target sequence (Liu *et al*. 2016; Sandoval *et al*. 2019). Although careful gRNA design considering target nucleotide sequences and the protospacer adjacent motif (PAM) sequence have been identified as significantly correlated with cleavage outcome, design strategies still vary between researchers and institutions (Liu *et al*. 2016, Sandoval *et al*. 2019). Therefore, in this study, we designed and tested three gRNA sequences for *IL2RG* with overall GC contents between 40 and 65%, which are considered highly effective for on-target cleavage. *IL2RG*-targeting gRNAs no. 2 and no. 3 resulted in a high proportion of biallelic mutations; thus, they were selected for the next experiment along with gRNAs no. 1 and no. 2 targeting *GHR*.

In Experiment 2, the porcine zygotes were electroporated with gRNAs targeting both *IL2RG* and *GHR*, and we analyzed the mutation efficiency and blastocyst formation. There were no significant differences in blastocyst formation rates with

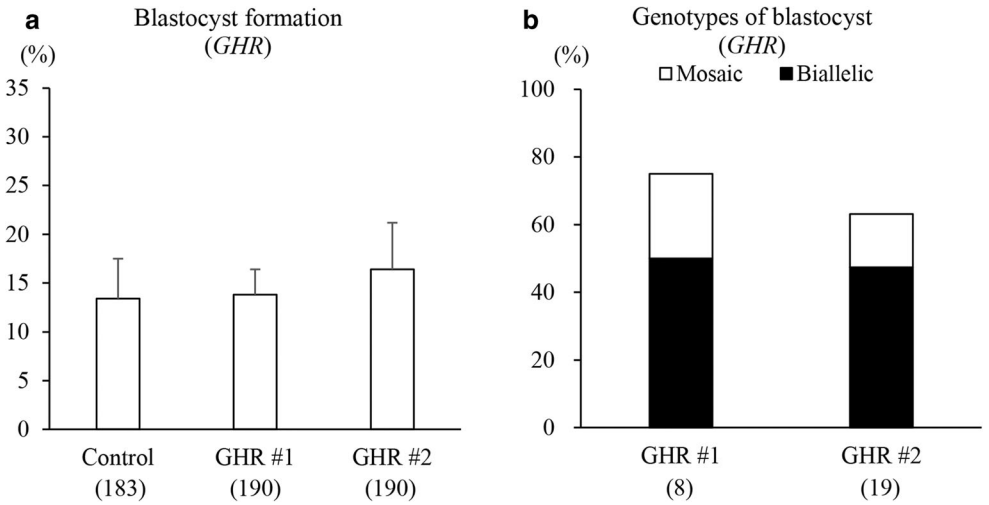

Figure 3. Development (*a*) and mutation (*b*) of porcine zygotes edited with gRNAs targeting *GHR*. The sequences of the gRNA target region and genome editing efficiencies in the resulting blastocysts were determined using Sanger sequencing and TIDE analysis, respectively. The percentage of edited blastocysts was defined as the ratio of the number of edited blastocysts to the total number of sequenced blastocysts. Biallelic: biallelic mutant, Mosaic: mosaic mutant. The *numbers* within *parentheses* indicate the total number of oocytes and blastocysts examined for development and mutation, respectively. As a control for blastocyst formation, inseminated zygotes were cultured for 7 d without electroporation.

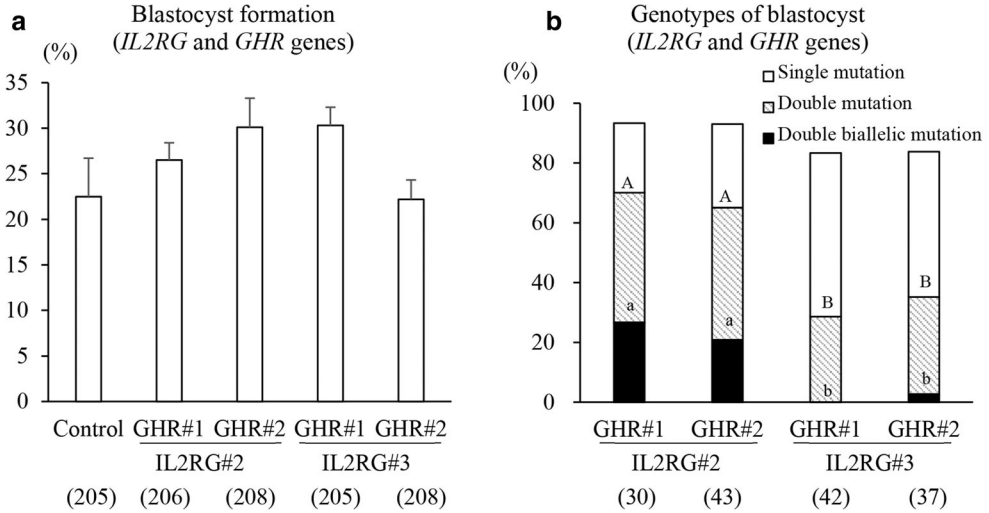

Figure 4. Development (*a*) and mutation (*b*) of porcine zygotes simultaneously edited in two target genes (*IL2RG* and *GHR*). The sequences of the gRNA target regions and genome editing efficiencies in the resulting blastocysts were determined using Sanger sequencing and TIDE analysis, respectively. The percentage of edited blastocysts was defined as the ratio of the number of edited blastocysts to the total number of sequenced blastocysts. Double biallelic mutation: biallelic mutation of both *IL2RG* and *GHR*. Double mutation: biallelic or mosaic mutations in both target genes (excluding double biallelic mutations). Single mutation: a biallelic or mosaic mutation in either *IL2RG* or *GHR*. The *numbers* within *parentheses* indicate the total number of oocytes and blastocysts examined for development and mutation, respectively. As a control for blastocyst formation, inseminated zygotes were cultured for 7 d without electroporation. $^{A–B,\ a–b}$Values with different *superscripts* within each mutation are significantly different ($p < 0.05$).

different gRNA pairs. In a previous study, we reported that the development of porcine zygotes electroporated with two different gRNAs targeting the same gene was affected by their sequence features, since one of the gRNAs used in the combination resulted in a low rate of blastocyst formation (Hirata *et al*. 2019b). In this study, the individual gRNAs did not affect the ability of electroporated zygotes to develop into blastocysts. Therefore, we concluded that electroporation of gRNAs targeting two different genes would not influence blastocyst formation.

When gRNA no. 3 targeting *IL2RG* was simultaneously electroporated with either gRNAs no. 1 or no. 2 targeting *GHR*, a lower double biallelic mutation was observed than when gRNA no. 2 targeting *IL2RG* was used. Our previous study demonstrated that biallelic mutation was found in only one target gene when the porcine zygotes were simultaneously

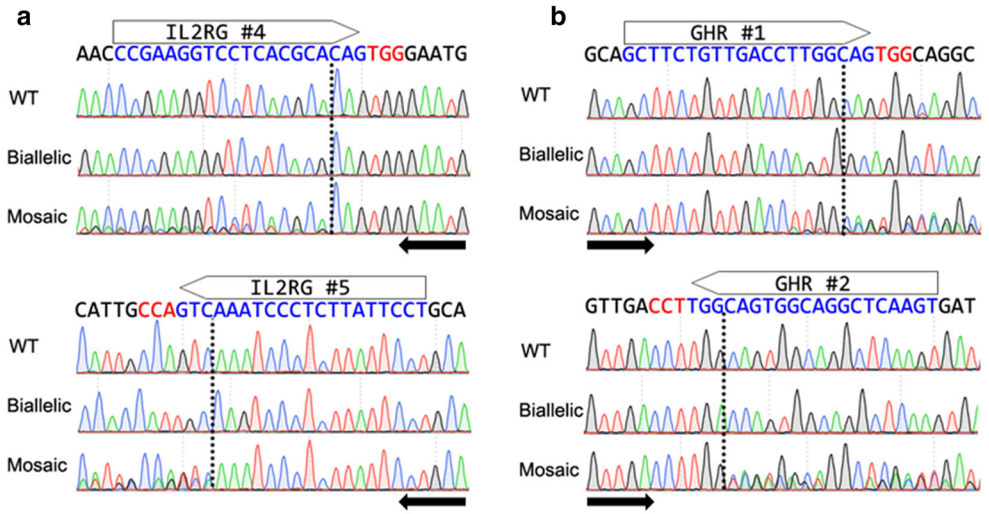

Figure 5. Representative results of Sanger sequencing of blastocysts formed after electroporation with Cas9 protein, *IL2RG* (*a*), and *GHR* (*b*) gRNAs. The TIDE bioinformatics package was used to determine the genotype of each blastocyst. *Arrows* at the bottom of each *panel* show the orientation of the primer used for sequencing. *Dotted lines* indicate the predicted Cas9 cleavage sites. Blue nucleotides indicate the target sequences, and red nucleotides indicate the protospacer adjacent motif (PAM) sequences. WT: wild-type, Biallelic: biallelic mutation, Mosaic: mosaic mutation.

electroporated with four gRNAs targeted different genes to introduce multiple biallelic mutations (Hirata *et al*. 2020). The different efficiencies between gRNA pairs could be explained by cross talk between the gRNAs, in which one gRNA could be affected by the presence of the other, lead to poor system efficiency (Qi *et al*. 2013; Tay *et al*. 2014). Although our study confirms the presence of cross talk activity that interferes with multiplex CRISPR/Cas9 genome editing efficiency, the specific mechanism through which this occurs remains to be determined.

On the other hand, there may be increasing concern about off-target events when performing genome editing using multiple gRNAs simultaneously. gRNAs should be carefully designed to minimize the off-target effects, but the possibility of off-target events cannot be completely excluded (Fu *et al*. 2013; Anderson *et al*. 2018). In addition, large deletions can be generated during double-strand break repair after Cas9 cleavage (Kosicki *et al*. 2018). In this study, we could not perform further analysis due to the limited amount of genomic DNA in each blastocyst, but off-target events and large deletions should be evaluated when producing founder animals with multiple genome edits using this technique.

In conclusion, using this one-step multiplex CRISPR/Cas9-based genome editing electroporation method, we successfully generated porcine embryos that developed to the blastocyst stage and harbored double biallelic mutations in *IL2RG* and *GHR*. However, the rates of double biallelic mutations in the blastocysts by one-step multiplex genome editing were still low. Therefore, extensive technical improvement is required to maximize the efficiency of multiplex-gene editing using electroporation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval The animal experiments were approved by the Institutional Animal Care and Use Committee of Tokushima University (approval number: T2019-11).

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