Graduation thesis

Studies on genome editing techniques using CRISPR/Cas9 in porcine embryos

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CONTENTS

ABBREVIATION

ANOVA	Analysis of variance
COCs	Cumulus-oocyte complexes
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
DMEM	Dulbecco's modified eagle's medium
EGF	Epidermal growth hormone
EP	Electroporation
GGTA1	Glycoprotein galactosyltransferase alpha-1,3
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IVP	*In vitro* production
MI	Cytoplasmic Microinjection
PBM	Porcine blastocyst medium
PCR	Polymerase chain reaction
PFM	Porcine fertilization medium
PZM5	Porcine zygote medium-5
SCNT	Somatic cell nuclear transfer
SEM	Standard error of the mean

I. INTRODUCTION

1. Experimental animal models

1.1. Pig is an excellent animal model in research

Animal models has been used to study of human diseases and test clinical drugs, cosmetics, and vaccines before mass human application. They have been utilized since the ancient Greek period to understand human anatomy, physiology and pathology (Ericsson et al. 2013). As selection criteria, the anatomy, physiology, and body structure of animals should be similar to humans. In additional, characteristics, such as longevity, body weight, reproductive behavior, litter size, should also be considered. Mammals, such as rats, dogs, monkeys, have been widely employed in biotechnology and biomedical research. Among these, the pig is an outstanding experimental animal model (Meurens et al. 2012), and their advantages include similar anatomy, genome, and chromosomal structures to human. Pig genome has been mapped and completely characterized, and cells and organs for xenotransplantation can be generated (Schook et al. 2005). Furthermore, pigs have a relatively long lifespan (10 to 20 years), early sexual maturity (5-8 months). Their average litter size varies from 4 to 16 piglets per time, and a sow usually delivers two to three times yearly (Meurens et al. 2012; Rothschild et al. 1996). Therefore, the pig has gained acceptance from the biomedical community and has been widely

used as a biomedical animal model for studies on human health and genetic diseases (Walters et al. 2013).

1.2. *In vitro* porcine oocytes maturation and fertilization

Recently, the *in vitro* production (IVP) of pig embryos has dramatically improved through developments in the techniques of *in vitro* maturation (IVM) and *in vitro* fertilization (IVF).

Generally, the main source of pig ovaries is local slaughterhouses. These are transported to the laboratory and used as source of oocytes for experiments. The use of *in vitro* matured oocytes is more suitable because obtaining a large quantity is less expensive and less time-consuming than the use of *in vivo* mature oocytes. IVF techniques have been used to produce large quantities of pig embryos, thus allowing the conduct of important experiments, such as pronuclear microinjection, cytoplasmic microinjection, electroporation, and other genome editing studies.

2. Objectives of the thesis

Nowadays, organ, tissue and cell transplantation is used to treat heart, liver, lung, pancreas diseases and diabetes. However, the aging of the global population leads to the shortage of human donor organs (Tonelli et al. 2014). In some countries, such as Japan, the use of deceased human organs is associated with cultural and

ethical issues (Cooper et al. 2015). Therefore, xenotransplantation, an alternative source of donor organs, addresses this shortage.

Xenotransplantation is the use of organs, tissues or cells from other species for human transplantation. It can address the growing demand for suitable human donor organs. Particularly, the domestic pig is a potential organ donor resource because of its similarity to human in terms of anatomy, physiology, and organ size. (Fan et al. 2013; Niemann et al. 2012).

However, the human immune system recognizes transplantated organs from wild type (genetically- unmodified) pigs as a foreign antigen, thus resulting in rapid antibody-dependent complement-mediated rejection. The survival of transplantated organs in the recipients is extremely short lasting in minutes rather than hours or days. Therefore, genetic enginerring is a feasible solution to solve these problems (Byrne et al. 2015; Cooper et al. 2015; Shimizu et al. 2000).

Particularly, the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat/CRISPR-associated 9) system, a genome engineering method based on the bacterial CRISPR immune system, has become a research hotspot and is extensively used in genome editing (Barrangou et al. 2016; Brinkman et al. 2018; Niu et al. 2014). The development of other genetic techniques, such as somatic cell nuclear transfer (SCNT), sperm-mediated gene transfer (SMGT), cytoplasmic

microinjection (CI), gene editing by electroporation of Cas9 protein (GEEP), have also promoted the production of genetically modified animals. Therefore, in this study, we employed porcine *in vitro* fertilized embryos to generate genetically edited pigs.

II. LITERATURE REVIEW

1. CRISPR/Cas9 system

CRISPR is a family of DNA sequences that was found in the genome of bacteria and archaea. The three major components of a CRISPR locus are *cas* genes, a leader sequence, and a series of repeat-spacer array (Figure 1). *Cas* genes are often located next to CRISPR repeat-spacer arrays. Base on the similarity of the encoded proteins, 93 *cas* genes are divided into 35 families, of which, 11 form the *cas* core. A complete CRISPR-Cas locus has at least one gene belonging to the *cas* core (Makarova et al. 2015). The leader region, an AT-rich sequence, is located in the upstream of CRISPR locus and does not contain an open reading frame. Short repeated sequences by unique spacers are derived from the DNA fragments of bacteriophages that had previously infected the prokaryote. These fragments are used to detect and destroy DNA from similar bacteriophages during subsequent infections.

The CRISPR method is based on a natural occurring genome editing system in bacteria that confers immunity form infection caused by viruses. When the bacterium detects the presence of viral DNA, it produces two types of short RNA, one of which contains a sequence that matches the invading virus. These two RNAs form a complex with a protein Cas9. Cas9 is a nuclease, a type of enzyme that can cut DNA. When the matching sequence, known as a guide RNA, find its target

within the viral genome, Cas9 cuts the target DNA, thus disabling the virus. Recently, the CRIPSR/Cas9 system has been engineered to cut not only viral DNA but also any DNA sequence at a precisely location by changing the guide RNA to match the target. This also can be done within the nucleus of a living cell. Once inside the nucleus, the resulting complex locks onto a short sequence known as the PAM (protospacer adjacent motif), which is a DNA sequence containing 2-6 base pair DNA immediately following the DNA sequence targeted by the Cas9 nuclease in the CRISPR bacterial adaptive immune system (Hsu et al. 2014). Cas9 unzips the DNA and matches it to its target RNA. When the match is completed, Cas9 uses two tiny molecular scissors to cut the DNA. The viral infected cell subsequently attempts to repair the cut, but this repair process is error prone, leading to mutations that can disable the gene, which allows researchers to understand the gene's function. Although these mutations are random, replacing the mutant gene with a healthy copy by adding another DNA piece carrying the desired sequence.

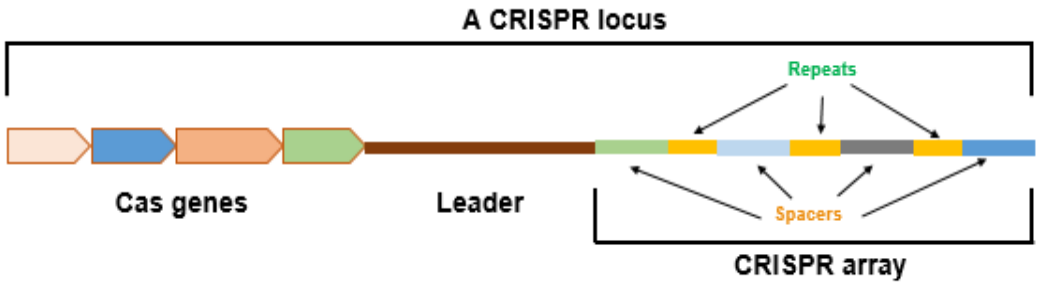

Figure 1. Typical structure of a CRISPR locus

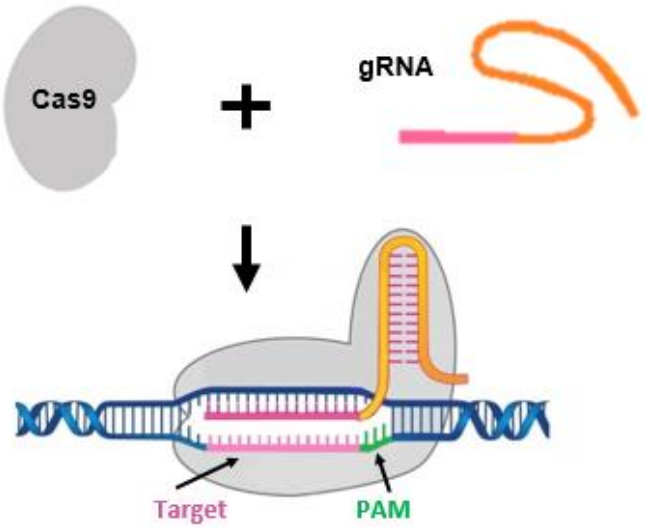

Figure 2. The CRISPR/Cas9 system. The CRISPR-associated endonuclease Cas9 can target specific DNA loci and produce double-strand breaks under the guidance of the gRNA. gRNA: guide RNA, PAM: proto-spacer-adjacent motif.

Once the CRISPR system has made a cut, this DNA template can pair up with the cut ends and recombine and replacing the original sequence with the new version. This process can be performed not only in cultured cell, but also in fertilized oocytes, resulting in the generation of transgenic animals with targeted mutations. Because the CRISPR/Cas9 system can be used to target many genes at once, it is advantageous in studying complex human diseases caused by multiple genes acting together (Barrangou et al. 2016; Guan et al. 2016; Wang et al. 2014) (Figure 2).

The CRISPR/Cas9 system is becoming a powerful tool in genome editing to generate diseased models and treat genetic and infectious diseases (Hsu et al. 2014; Yang et al. 2015).

2. Genome editing

Genome editing, or genome engineering, is a type of genetic engineering in which DNA is inserted, deleted, modified or replaced in the genome of a living organism. Unlike previous genetic engineering techniques that randomly inserts genetic material into a host genome, genome editing targets the insertions at specific locations. Various developments in genetic engineering have contributed to the foundation of gene editing tools, the most recent one being the CRISPR/Cas9 system.

Three mechanisms of CRISPR have been discovered, in which the type II (CRISPR- associated Cas9 protein or CRISPR/Cas9) is the most interesting.

CRISPR/Cas9 system is increasingly being developed and has been extensively applied for gene editing in biomedical and biological studies (Barrangou et al. 2016; Yu et al. 2014).

In this study, using a pig model and the CRISPR/Cas9 system, we aimed to generate genetically edited pigs from porcine *in vitro* fertilized embryos for xenotransplantation. This thesis is divided into three chapters and is outlined as follows:

Chapter 1: Effects of electroporation treatment using different concentrations of Cas9 protein with gRNA targeting Myostatin (*MSTN*) genes on the development and gene editing of porcine zygotes.

Chapter 2: Comparison of the effects of introducing the CRISPR/Cas9 system by microinjection and electroporation into porcine embryos at different stages.

Chapter 3: Comparison of the genome editing efficiency through a combination of microinjection and electroporation method to generate *GGTA1/CMAH/β4GalNT2* triple gene knockout in porcine embryos.

III. RESEARCH CONTENTS

Chapter 1

Effects of electroporation treatment using different concentrations of Cas9 protein with gRNA targeting Myostatin (*MSTN*) genes on the development and gene editing of porcine zygotes

1. Abstract

Using gene editing by electroporation of Cas9 protein (GEEP) system and gRNAs targeting myostatin (*MSTN*) genes, we investigated the effect of different concentrations of Cas9 (0, 25, 50, 100, 200, 500 and 1000 ng/μl) on the development and gene editing of porcine embryos. This study included the target editing and off-target effect of embryos developed from zygotes edited via the electroporation of the Cas9 protein with guide RNA targeting *MSTN* genes. We found that the development up to the blastocyst stage was not affected by the concentration of Cas9 protein. Although the editing rate, defined as the ratio of edited blastocysts to total examined blastocysts, did not differ with Cas9 protein concentration, the editing efficiency, defined as the frequency of indel mutations in each edited blastocyst, was significantly decreased in the edited blastocysts from zygotes electroporated with 25 ng/μl of Cas9 protein compared with blastocysts from zygotes electroporated with higher Cas9 protein concentrations. Moreover, the frequency of indel events at the

two possible off-target sites was not significantly different among different concentrations of Cas9 protein. These results indicate that the concentration of Cas9 protein affects gene editing efficiency in embryos but not the embryonic development, gene editing rate, and non-specific cleavage of off-target sites.

2. Introduction

The myostatin (*MSTN*) gene codes for a protein myostatin, a member of the transforming growth factor-β superfamily, that controls the growth and development of skeletal muscle in animals. Myostatin restrains muscle growth (Mcpherron et al. 1997), whereas the lack of myostatin leads to the overgrowth of skeletal muscle. Therefore, blocking the *MSTN* gene has been suggested as a potential treatment for muscle-wasting disease.

Myostatin is found in various animals, such as mice, cows, sheep, pigs, and others, and it has a similar function in humans (Bellinge et al. 2005; Mcpherron et al. 1997). Among the genetically modified animal models used to elucidate the pathogenesis and develop therapeutic strategies for human diseases, pigs are considered as one of the best because they are similar to humans in terms of anatomy, physiology, and genome (Fan et al. 2013; Niemann et al. 2012). Therefore, the generation of pigs with blocked *MSTN* provides a deeper understanding of muscle development, meat performance, and treatment of muscle-wasting disorders.

The CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat/CRISPR-associated 9) technique is a genome engineering method based on the bacterial CRISPR immune system. It has been recently developed and is widely used for genome editing to produce genetically modified animals (Brinkman et al. 2018; Niu et al. 2014a; Wang et al. 2015). Other modern gene modification techniques, such as somatic cell nuclear transfer (SCNT), sperm-mediated gene transfer (SMGT), and microinjection, also promote the production of genetically modified animals.

The GEEP (gene editing by electroporation of Cas9 protein) method was previously established to generate high-efficiency disruption of the targeted gene (Tanihara et al. 2016). This is a simple method for CRISPR/Cas9 gene editing and delivers the Cas9 protein and single-guide RNA (sgRNA) into *in vitro* fertilized zygotes via electroporation. Thus, the GEEP method does not require complexity techniques associated with micromanipulation for microinjection of CRISPR/Cas9 system into zygotes.

The efficiency of electroporation using CRISPR/Cas9 system is dependent on the ability to reduce embryonic damage. In addition, a previous study revealed that the concentration of Cas9 protein introduced into mouse zygotes via electroporation influences gene editing efficiency (Hashimoto et al. 2015). Therefore, to increase

the efficiency of gene editing in the CRISPR/Cas9 system by electroporation, the optimized concentrations of Cas9 protein and gRNA should be identified.

In this study, we investigated the effect of different concentrations of Cas9 (0, 25, 50, 100, 200, 500 and 1000 ng/μl) on the efficiency of target mutation and the degree of mosaicism in blastocysts obtained from electroporation using the GEEP method and gRNAs targeting *MSTN* gene. However, the CRISPR/Cas9 system also causes unexpected mutations at off-target sites (Fu et al. 2013). These off-target effects can cause confusion for experiment results. To avoid this, the genomic regions flanking the sgRNA target sites or potential off-target sites were amplified using two-steps PCRs, and the genome sequences of possible off-target sites were analyzed.

3. Materials and methods

Because live animals were not used in this study, ethical approval was not required. The study protocol has been approved by the Animal Research Committee of the Tokushima University.

3.1. Oocyte collection and in vitro maturation

Pig ovaries were obtained from prepubertal, crossbreed gilts (Landrace × Large White × Duroc breeds) at a local slaughterhouse and were transported to the laboratory in physiological saline at 30 °C. The o varies were washed three times

with prewarmed physiological saline solution supplemented with 100 IU/ml penicillin G potassium (Meiji, Tokyo, Japan) and 0.1 mg/ml streptomycin sulfate (Meiji). Follicles (3–6 mm diameter) on the ovarian surface were sliced on a sterilized dish using a surgical blade, and cumulus-oocyte complexes (COCs) were visualized using a stereomicroscope. Approximately 50 COCs were cultured in 500 µl of maturation medium consisting of tissue culture medium 199 (TCM 199) with Earle's salts (Gibco/Invitrogen Co., Carlsbad, CA, USA) supplemented with 10% (v/v) porcine follicular fluid, 0.6 mM cysteine (Sigma-Aldrich, St. Louis, MO, USA), 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 (Kyoritu Seiyaku, Tokyo, Japan), 10 IU/ml human chorionic gonadotropin (Kyoritu Seiyaku), and 50 µg/ml gentamicin (Sigma-Aldrich). The COCs were covered with mineral oil (Sigma-Aldrich) in 4-well dishes (Nunc A/S, Roskilde, Denmark), and after 22 h, they were transferred to a maturation medium without hormones and then cultured for another 22 h. Incubation was conducted at 39 °C in a humidified incubator containing 5% CO_2.

3.2. *In vitro* fertilization

The matured oocytes were subjected to IVF as described previously (Nguyen et al. 2017). Briefly, frozen-thawed ejaculated spermatozoa were transferred into 5 ml of 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 fertilization medium at a final concentration of 1 × 10^6 cells/ml. Approximately 50 oocytes were transferred to 500 µl of sperm-containing fertilization medium, 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 using mechanical pipetting, transferred to porcine zygote medium (PZM-5; Research Institute for the Functional Peptides Co.) and cultured for 7 h until electroporation.

3.3. 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. Approximately 50 putative zygotes were washed with Opti-MEM I solution (Gibco/Invitrogen Co.) and placed in a line between the electrodes on the

chamber slide filled with 10 μl of Nuclease-Free Duplex Buffer (Integrated DNA Technologies (IDT), Coralville, IA, USA) with 100 ng/μl of gRNA (Alt-R CRISPR crRNAs and tracrRNA, chemically-modified and length-optimized variants of the native guide RNA purchased from IDT) targeting porcine *MSTN* genes (target sequence: 5′- AGGAAAATGTGGAAAAAGAG-3′) (Figure 3), and various concentrations (0, 25, 50, 100, 200, 500 and 1000 ng/μl) of Cas9 protein. gRNA was designed in the first exon of *MSTN* to knockout *MSTN* by a frameshift caused by an indel mutation introduced by the non-homologous end joining (NHEJ)-mediated repair of double-strand break (DSB) generated by Cas9. After electroporation with 5 1-ms pulses at 25 V, the zygotes were washed with PZM-5 and cultured for 3 days. Then, the embryos were cultured in the porcine blastocyst medium (PBM; Research Institute for the Functional Peptides Co.) for 4 days to evaluate their development to the blastocyst stage and to examine the genotype of the resulting blastocysts. Incubation was conducted at 39 °C in a humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2.

3.4. Analysis of targeted gene sequence after electroporation

Genomic DNA was isolated from individual blastocysts by boiling them in a 50 mM NaOH solution at 98 °C for 10 min, followed by neutralizing with 100mM Tris HCl. The genomic regions flanking the *MSTN* gRNA target sequences were amplified by polymerase chain reaction (PCR) with the following primers: 5′-ATGCAAAAACTGCAAATCTATG-3′ (forward) and 5′-TGTAGGCATGGTAATGATCG-3′ (reverse). The PCR products were extracted using agarose gel electrophoresis with a Fast Gene Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan). The targeted genomic regions of the PCR products were directly sequenced using Sanger sequencing with a BigDye Terminator Cycle Sequencing Kit version 3.1 (Thermo Fisher Scientific K.K., Tokyo, Japan) and an ABI 3500 genetic analyzer (Applied Biosystems, CA, USA).

The TIDE (tracking of indels by decomposition) bioinformatics package was used to determine the genotype of each blastocyst (https://www.tide.deskgen.com)(Brinkman et al., 2018). Blastocysts that carried no WT sequences were classified as having biallelic editing, whereas those carrying more than one type of editing (in addition to the WT sequence) were classified as a mosaic. Those having only the WT sequence were classified as WT. Editing rate was defined as the ratio of the number of gene edited blastocysts to the total number

of sequenced blastocysts. Editing efficiency was defined as the proportion of indel mutation events in the blastocyst that carried the mosaic or biallelic editing.

3.6 Off-target analysis using next generation sequencing

The COSMID webtool (Cradick et al. 2014), which ranks the potential off-target sites based on the number and location of mismatches, was used to select the candidate off-target sites. The two top-ranked sites were analyzed using deep-sequencing. The genomic DNA of three blastocysts from the control zygotes or four blastocysts from edited zygotes at each concentration of the Cas9 protein was individually used as templates for PCR. The genomic regions flanking the potential off-target sites were amplified using two-step PCRs with specific primers and Index PCR primers following the manufacturer's instructions (Illumina, CA, USA). After gel purification, the barcoded amplicons were pooled and sequenced using the MiSeq System (Illumina, Paired-End, 250-bp runs). Indel quantitation was performed using CRISPResso (Pinello et al. 2016) and indels were measured within a 5–bp window surrounding the predicted cleavage site to minimize false positives (Gaj et al. 2017).

3.7. Statistical analysis

Data of embryonic development and off-target frequency were evaluated using the analysis of variance (ANOVA) test followed by Fisher's protected least

significant difference (PLSD) test using STATVIEW version 5.0 (Abacus Concepts, Inc., Berkeley, CA, USA,). All percentage data

were subjected to an arcsin transformation before ANOVA. The percentages of gene edited blastocysts within all sequenced blastocysts were analyzed using a chi-squared analysis with Yates' correction. $P < 0.05$ was considered statistically significant.

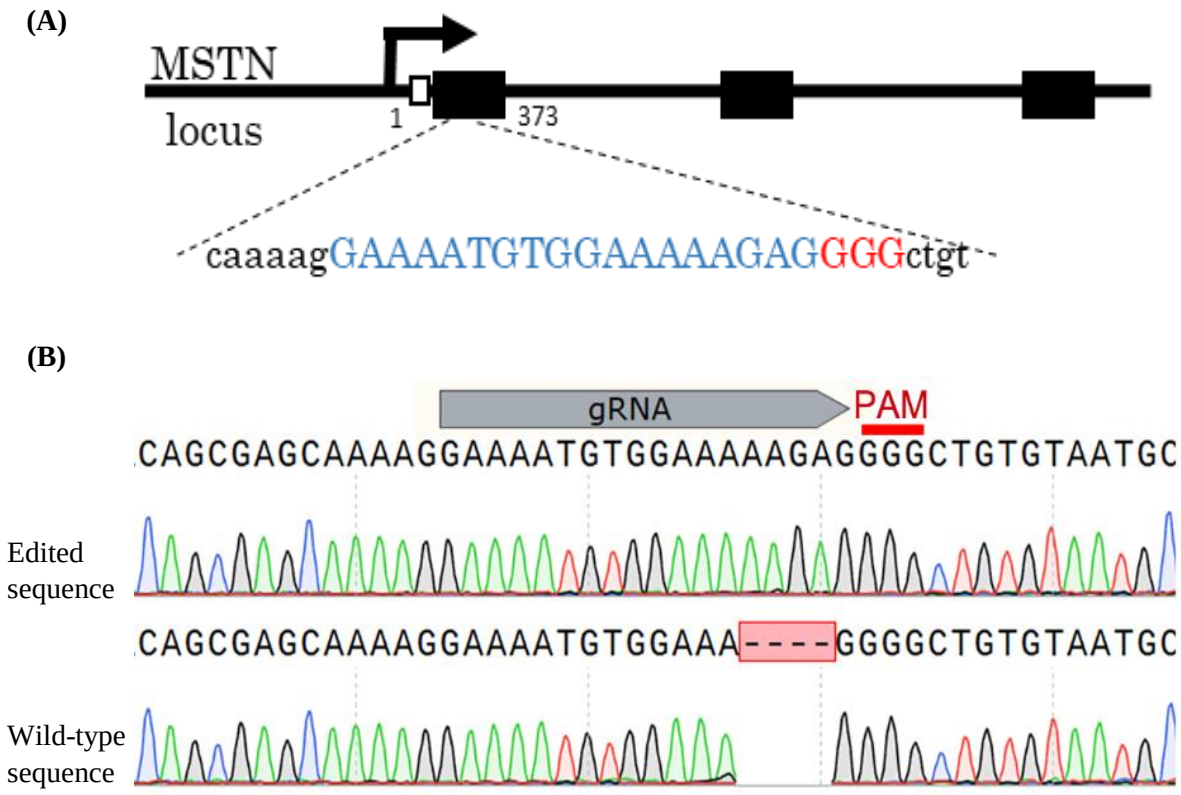

Figure 3. Genomic structure of the MSTN locus and sequence of gRNA targeting the first exon. (A) The nucleotides in blue and red text represent the target sequences and the PAM sequences of gRNA, respectively. (B) Representative sequencing data of the wild-type sequence and edited sequence. MSTN is expected to be knocked out because of frameshift due to insertion or deletion (indel) mutations generated by gRNA.

4. Results

The percentage of cleavage and blastocyst formed from electroporated porcine zygotes was not affected by the concentration of Cas9 protein (Table 1), and found that biallelic mutations in the blastocysts did not differ with varying Cas9 concentrations (Figure 4). However, biallelic editing rate in the blastocysts from zygotes electroporated with 500 ng/μl of Cas9 protein was significantly higher ($P < 0.05$) than that with less than 100 ng/μl. The biallelic editing was not observed in blastocysts from zygotes electroporated with 25 ng/μl Cas9 protein. Moreover, mutation efficiency was lower in the blastocysts from zygotes electroporated with 25 ng/μl of Cas9 than in those with different concentrations. (Figure 5).

To evaluate determine whether there exists a correlation between the concentration of Cas9 protein and non-specific cleavage of off-target sequence, two possible off-target sites were analyzed using deep-sequencing. No significant differences were detected (Table 2).

Table 1. Effects of electroporation treatment using different concentrations of Cas9 protein on the development of porcine zygotes*

Cas9 concentration (ng/μl)	Number of oocytes	Number of embryos (%)	
		Cleaved	Developed to blastocysts
Control	165	130 (76.9 ± 8.0)	37 (22.9 ± 5.4)
0	226	168 (74.3 ± 8.2)	51 (23.1 ± 5.7)
25	227	151 (65.6 ± 10.3)	37 (16.5 ± 2.6)
50	226	173 (75.3 ± 6.7)	39 (17.1 ± 1.6)
100	231	184 (78.6 ± 6.5)	39 (16.9 ± 1.2)
200	235	186 (78.8 ± 4.4)	45 (18.6 ± 5.0)
500	250	197 (78.9 ± 6.5)	53 (21.1 ± 3.1)
1000	248	186 (75.0 ± 4.5)	33 (13.3 ± 2.0)

*Experiments were repeated 4 or 5 times. Data are expressed as the mean ± SEM.

Table 2. Frequencies of sequences with substitutions, deletions, or insertions at candidate off-target (OT) sites measured by deep sequencing*

Cas9 concentration (ng/μl)	% of sequences with substitutions, deletions, or insertions	
	OT1	OT2
Control	0.664 ± 0.053	0.642 ± 0.04
25	0.891 ± 0.033	0.693 ± 0.062
50	0.788 ± 0.041	1.276 ± 0.668
100	0.803 ± 0.094	0.624 ± 0.042
200	0.724 ± 0.045	5.246 ± 4.464
500	0.893 ± 0.342	0.696 ± 0.013
1000	1.783 ± 0.094	0.690 ± 0.016

* Experiments were repeated 4 or 5 times. Data are expressed as the mean ± SEM.

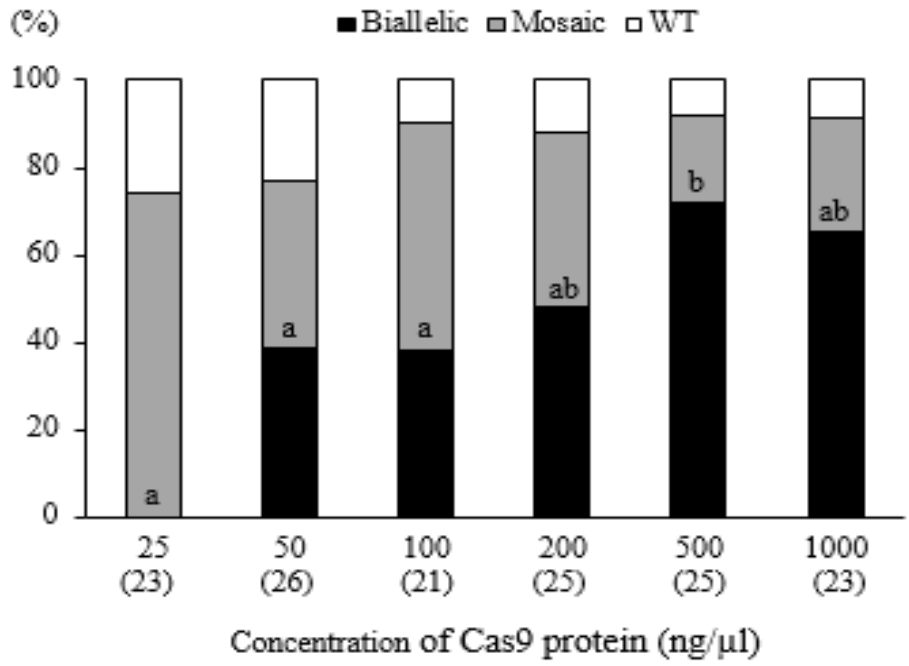

Figure 4. Editing frequency in the blastocysts derived from porcine zygotes electroporated with different concentrations of Cas9 protein. Numbers inside parentheses indicate the total number of examined blasto l cysts. Different superscripts in the proportion of biallelic editing are significantly different (*P*< 0.05). Biallelic: biallelic editing; Mosaic: mosaic editing; WT: wild type.

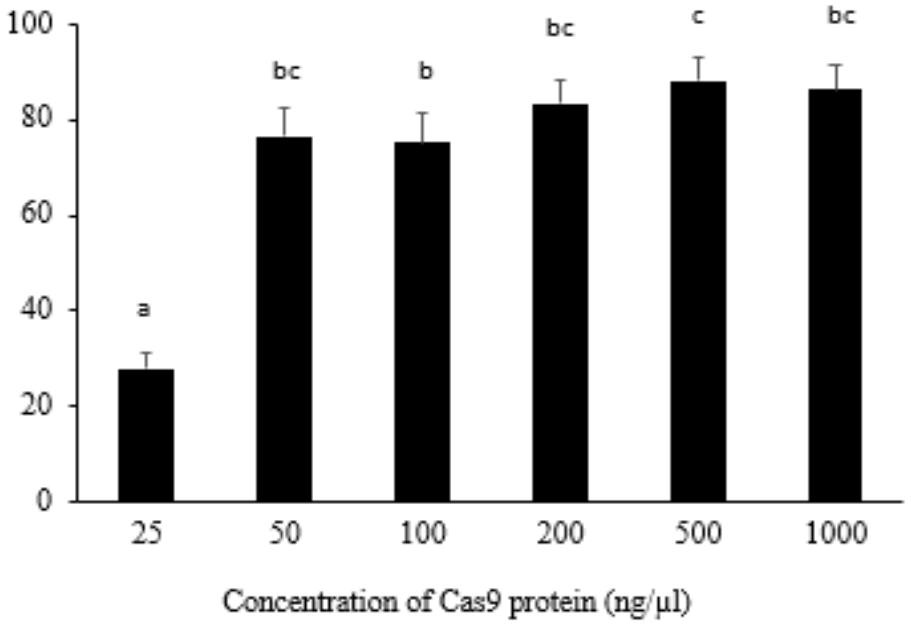

Figure 5. Gene editing efficiency in the blastocysts derived from zygotes electroporated with different concentrations of Cas9 protein. Data are presented as the mean ± SEM. Different superscript letters indicate significantly different ($P < 0.05$).

5. Discussion

Using a well- designed sgRNA, the specificity and efficiency of the CRISPR/Cas9 system can be enhanced, thus achieving minimal off-target (Cradick et al. 2013). Direct microinjection of zygotes to deliver the CRISPR/Cas9 system has been reported to yield highly efficient biallelic mutation when it was performed prior to DNA replication; nevertheless, when a large number of embryos were treated, the operability of Cas9 protein was reduced, causing mosaicism in embryos (Vilarino et al. 2017). Electroporation has been used to introduce DNA or RNA into embryos and is less time-consuming that the microinjection method.

Previous studies in pigs have used various concentrations of Cas9 mRNA, Cas9 protein, and gRNa for microinjection. The following concentrations have been employed: 20 ng/µl Cas9 mRNA and 10 ng/µl gRNA (Yu et al. 2016), 250 ng/µl Cas9 mRNA and 250 ng/µl gRNA (Inui et al., 2014), 100 ng/µl Cas9 mRNA and 50 ng/µl gRNA (Vilarino et al. 2017), 50 ng/µl Cas9 protein and 200 ng/µl gRNAs (Hashimoto et al. 2016), 250 ng/µl Cas9 protein and 300 ng/µl gRNA (Wang et al. 2016). However, only few studies compared Cas9 concentrations. Genome editing efficiency has been reported to be influenced by Cas9 protein concentration, even after its introduction in the early stage of zygotes without delay (Hashimoto et al. 2016).

In this study, we varied Cas9 protein concentrations and maintained the number of pulses, duration, and voltage constant at five repeats, 1 ms, and 25 V, respectively. We found that no difference in the development rate of electroporated porcine zygotes to the blastocyst stage at varying Cas9 protein concentrations. These findings indicate that Cas9 protein concentration does not influence embryonic development. However, it affected both the editing rate and editing efficiency of the blastocysts from electroporated zygotes. Particularly, more blastocysts having biallelic editing were observed in the zygotes electroporated with 500 ng/μl of Cas9 protein than in those with less than 100 ng/μl of Cas9 protein. Zygotes electroporated with 25 ng/μl of Cas9 protein exhibited no biallelic editing in the blastocysts and lower gene editing efficiency in the edited blastocysts.

These results are consistent with those of a previous study(Hashimoto et al. 2015), which demonstrated that electroporation with a lower concentration of Cas9 protein generated heterozygous mutant mice carrying an intact WT sequence. In our previous study, we showed that cytoplasmic microinjection using higher concentration of Cas9 protein and gRNA led to increased gene editing efficiency and biallelic editing rate (Tanihara et al. 2019). Therefore, the gene editing efficiency may dependent on the concentration of Cas9 protein induced into zygotes in electroporation. However, the optimal concentration of Cas9 protein might be dependent on both the targeting genes and gRNA sequences because it has been

revealed that the gene editing efficiency varied along with targeting genes (Tanihara et al. 2019; Tanihara et al. 2016). Because our result might only apply to *MSTN*, further investigation targeting other genes is required to confirm the effect of Cas9 protein concentration on gene editing by electroporation.

Although the CRISPR/Cas9 system is a highly efficient on genome editing method, it does not exhibit high specificity, similar to its predecessors Zinc finger nucleases (ZFNs) and Transcription activator like effector nucleases (TALENs) (Cradick et al. 2013; Kleinstiver et al. 2016; Lee et al. 2016; Wu et al. 2014; Zhang et al. 2015). The CRISPR/Cas9 system is associated with off-target cleavage and subsequent off-target mutation. The Cas9 protein is directed by a gRNA to a target site in the genome containing matching the first 17–20 nucleotides of gRNA, followed by a protospacer adjacent motif (PAM) sequence. After the Cas9 /gRNA complex binds to the target site, Cas9 induces a DSB three bases upstream of the PAM (Wu et al. 2014). Therefore, the occurrence of an off-target mutation may increase as the concentrations of Cas9 protein and gRNA increase. In this study, however, we found no significant differences at the off-target sites among different Cas9 protein concentrations. The mechanism responsible for Cas9-induced, off-target effects has been proposed to be associated with the tolerance of gRNA mismatches, especially the seed sequence of gRNA (Shin et al. 2017; Zhang et al. 2015). Therefore, the use of well-designed gRNA may result in increased specificity

and efficiency and minimal off-target effects in genome editing via the CRISPR/Cas9 system, irrespective of Cas9 concentration.

In conclusion, we demonstrated that Cas9 concentration affected mutation efficiency and that of biallelic editing in the embryos but had no effect on the development of the electroporated zygotes to the blastocyst stage. Furthermore, higher Cas9 protein concentration was associated with increased biallelic editing rate. Our findings indicated that off-target effects may not be related to Cas9 protein concentration in editing *MSTN* genes of porcine embryos via electroporation. Therefore, off-target effects may be reduced by choosing unique target sequences, instead of changing the concentration of Cas9. However, the optimal concentration of the Cas9 protein to achieve efficient gene editing and minimal off-target effects may change due to use of different targeting genes. To generate valuable genetically modified founder pigs with high efficiency, gene editing conditions in the CRISPR/Cas9 system using electroporation should be evaluated for each target gene.

Chapter 2

Effects of microinjection and electroporation in the CRISPR/Cas9 system in porcine embryos at different stages

1. Abstract

The cytoplasmic microinjection and electroporation of the CRISPR/Cas9 system into zygotes are used to generate genetically modified pigs. However, these methods generate mosaic mutations in embryos. In this study, we evaluated whether the method and the embryonic stage for gene editing affect the gene editing efficiency in porcine embryos.

First, we designed five guide RNAs (gRNAs) targeting the *B4GALNT2* gene and evaluated mutation efficiency through the introduction of each gRNA and Cas9 protein into zygotes via electroporation. Next, the optimized gRNA with Cas9 protein was introduced into 1-cell and 2-cell stage embryos via microinjection and electroporation. We found that the gRNA sequence affected the biallelic mutation rate and mutation efficiency in blastocysts derived from electroporated embryos. Microinjection significantly decreased the cleavage rates ($p < 0.05$) but not the blastocyst formation rates compared with electroporation. Furthermore, the biallelic mutation rate and mutation efficiency in blastocysts from the 1-cell stage embryos edited using microinjection were significantly higher ($p < 0.05$) than those from the

2-cell stage embryos edited by both methods. These results indicate that the method and embryonic stage for gene editing may affect the genotype and mutation efficiency of the resulting embryos.

2. Introduction

The pig is an ideal experimental animal model because of its close similarity to humans, particularly in anatomy and physiology. Genetically modified pigs have been used as disease models (Fan et al. 2013) and organ donors for human xenotransplantation (Klymiuk et al. 2010; Zeyland et al. 2015). To generate gene-modified animals, the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated gene (CRISPR/Cas9) system has been recently used. This method delivers the Cas9/gRNA complex into the embryos mostly via microinjection (Niu et al. 2014; Wang et al. 2015), which induces site-specific mutations in zygotes and embryos (Tan et al. 2016). Particularly, mutant embryos often exhibit a mosaic pattern, i.e. the presence of more than two alleles in an individual (Burkard et al. 2017; Sato et al. 2015; Sato et al. 2018; Whitworth et al. 2014).

In our previous study, we have developed the GEEP (gene editing by electroporation of Cas9 protein) method exhibiting high-efficiency disruption of the targeted genes in pigs (Tanihara et al. 2016). GEEP is a simple gene editing method

wherein the CRISPR/Cas9 system is introduced into porcine zygotes via electroporation and does not cause the damage associated with micromanipulation for injection of the CRISPR/Cas9 system into zygotes. However, the GEEP method also induces the mosaic pattern in the embryos (Tanihara et al. 2020). The occurrence of genetic mosaics in the resulting pigs is a serious problem because mosaicism, including wild-type (WT) cells, complicates the phenotype analysis. Although the production of a non-mosaic F1 generation from the mosaic mutants is possible, the production of the next generation is time-consuming and labor-intensive, particularly in large animals because of longer gestation than that in mice.

Mosaic mutants are suggested to arise owing to Cas9/gRNA complexes that remain active throughout several cell divisions or to delayed mRNA expression possibly triggered by cell division (Burkard et al. 2017). The continuous function of Cas9 on the targeting site during embryonic development induces the mosaic. Hashimoto et al. (Hashimoto et al. 2016) have demonstrated that the generation of mosaic mutants via introduction of Cas9 protein/gRNA complexes into 1-cell stage embryos depends on the time window between fertilization and the first DNA replication. Moreover, Gu et al. (Gu et al. 2018) have suggested that major zygotic genome activation associated with an open chromatin state occurs during the extended G2 phase in the 2-cell stage embryos, resulting in decreased mosaic after gene editing via the CRISPR/Cas9 system.

β-1,4-N-acetyl-galactosaminyltransferase 1 (*B4GALNT2*) synthesizes carbohydrate xenoantigens and accounts for the majority of human anti-pig antibody reactivity (Byrne et al. 2018). Thus, the generation of *B4GALNT2*-deficient animals is necessary step to achieve successful pig-to-human xenotransplantation. To reduce mosaic mutation in the early embryos, we compared the developmental competence and gene editing efficiency of porcine embryos at the 1-cell and 2-cell stage after introduction the complexes Cas9/gRNA (targeting the *B4GALNT2* gene) via microinjection and electroporation methods.

3. Materials and methods

3.1. Oocyte collection and in vitro maturation

Pig ovaries were obtained from prepubertal crossed gilts (Landrace × Large White × Duroc breeds) at a local slaughterhouse and were transported to the laboratory in physiological saline at 30°C. The ovaries were washed thrice with prewarmed physiological saline solution supplemented with 100 IU/ml penicillin G potassium (Meiji) and 0.1 mg/ml streptomycin sulfate (Meiji). Follicles (3–6 mm diameter) on the ovarian surface were sliced on a sterilized dish using a surgical blade, and COCs were visualized and collected using a stereomicroscope. Approximately 50 COCs were cultured in 500 µl of maturation medium consisting of the TCM 199 with Earle's salts (Thermo Fisher Scientific) and supplemented

with 10% (v/v) porcine follicular fluid, 0.6 mM cysteine (Sigma-Aldrich), 50 μM β-mercaptoethanol (Wako Pure Chemical Industries Ltd), 50 μM sodium pyruvate (Sigma-Aldrich), 2 mg/ml D-sorbitol (Wako Pure Chemical Industries Ltd.), 10 IU/ml equine chorionic gonadotropin (Asuka Pharmaceutical), 10 IU/ml human chorionic gonadotropin (Nippon Zenyaku Kogyo), and 50 μg/ml gentamicin (Sigma-Aldrich). The COCs were covered with mineral oil (Sigma-Aldrich) in 4-well dishes (Nunc A/S), and after 22h, they were transferred to a maturation medium without hormones and cultured for another 22 h. Incubation was conducted at 39°C in a humidified incubator containing 5% CO_2.

3.2. *In vitro* fertilization

The matured oocytes were subjected to IVF as described previously (Nguyen et al. 2017). Briefly, frozen-thawed ejaculated spermatozoa were transferred to 5 ml PFM medium (Research Institute for the Functional Peptides Co.) and washed using centrifugation at 500 × *g* for 5 min. The pelleted spermatozoa were resuspended in the fertilization medium at a final concentration of 1×10^6 cells/ml. Approximately 50 oocytes were transferred to 500 μl sperm-containing fertilization medium, 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 using mechanical pipetting, transferred to PZM-5 (Research Institute for the Functional Peptides Co.), and cultured until microinjection and electroporation treatments.

3.3. Electroporation

Electroporation was performed as described previously(Tanihara et al. 2016). Briefly, the electrode (LF501PT1-20; BEX Co. Ltd.) was connected to a CUY21EDIT II electroporator (BEX Co. Ltd.) and placed under a stereoscopic microscope. Approximately 50 embryos were washed with Opti-MEM I solution (Gibco/Invitrogen Co.) and placed in a line between electrodes on the chamber slide filled with 10 μL of Nuclease-Free Duplex Buffer (IDT), along with 100 ng/μl of gRNA (Alt-R^{TM} CRISPR crRNAs and tracrRNA, chemically-modified and length optimized variants of the native guide RNA purchased from IDT) and 100 ng/μl Cas9 protein (Takara Bio). After electroporation using 5 1-ms pulses at 25 V, the embryos were washed and cultured in PZM-5 for 3 days. At Day 3 after fertilization (Day 0), all of the cleaved embryos were subsequently cultured in PBM (Research Institute for the Functional Peptides Co.) for 4 days at 39°C in a humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2.

3.4. Cytoplasmic microinjection

The CRISPR/Cas9 components were injected into 1-cell and 2-cell stage embryos in a 20 μl drop of PZM-5 (Research Institute for the Functional Peptides Co.) covered by mineral oil. The duplex buffer containing 100 ng/μl of gRNA and 100 ng/μl of Cas9 protein was loaded into an injection pipette (Femtotips II, Eppendorf, Hamburg, Germany), the tip of which was gently inserted into the cytoplasm of embryos immobilized by a holding pipette. Subsequently, the duplex buffer was injected into the cytoplasm by air pressure using a microinjector (FemtoJet 4i; Eppendorf) with the following parameters: injection pressure, 20–30 hectopascals (hPa); compensation pressure, 10–12 hPa; and injection time, 0.15 s). A slight swelling of the embryo cytoplasm was identified by visual inspection indicated successful microinjection. Then, the embryos were washed and cultured in PZM-5. Three days after fertilization, all of the cleaved embryos were subsequently cultured in PBM for 4 days at 39 °C as described above.

3.5. Analysis of targeted gene sequence after microinjection and electroporation

Genomic DNA was isolated from individual blastocysts by boiling them in a 50 mM NaOH solution at 98°C for 10 min, followed by neutralization 100 mM Tris HCl. The genomic regions flanking the *B4GALNT* gRNA target sequences were amplified using PCR with the following specific primers: for gRNA #1 and #2, 5'-GACCAGACATCGTTCCCAGT-3' (forward) and 5'-GGGAACTGGCTGTAAAGTGG-3' (reverse); for gRNA #3, #4 and #5, 5'-TAGGGGGAAAAACACACTGG-3' (forward) 5'-CACCCTCGGGAATGAGTAGA-3' (reverse). The PCR products were extracted using agarose gel electrophoresis. The targeted genomic regions of the PCR products were directly sequenced using Sanger sequencing with a BigDye Terminator Cycle Sequencing Kit ver. 3.1 (Thermo Fisher Scientific) and the ABI 3500 genetic analyzer (Applied Biosystems). The TIDE bioinformatics package (Brinkman et al. 2014) was used to determine the genotypes of the blastocysts. Blastocysts were classified as having bi-allelic mutations (without WT sequences), mosaics (with more than one type of mutation and the WT sequence), or WT (with only the WT sequence).

3.6. Experimental design

3.6.1. *Experiment 1: Comparison of gRNA gene-targeting efficiency*

To confirm the optimal gRNA for efficient gene editing, we designed five gRNAs (#1–#5) targeting different sites of the *B4GALNT2* gene (Table 3). Each gRNA with Cas9 protein was introduced into porcine embryos via electroporation at 12 h after the start of IVF. The blastocyst formation rate from the embryos post-introduction of each gRNA and the mutation efficiency in the resulting blastocysts were evaluated as described above. To serve as the control, some embryos were cultured with PZM-5 and PBM for 7 days without electroporation treatment.

3.6.2. *Experiment 2: Comparison of the development stage and gene editing methods*

Embryos at the 1-cell and 2- cell stages were collected at 12 h and 24 h after the start of IVF, respectively. gRNA #1, which has the highest targeting efficiency as confirmed by Experiment 1, was used here. Cas9 protein with gRNA #1 was introduced into the embryos at each stage via microinjection and electroporation. For the 2-cell stage embryos, we injected Cas9 protein with gRNA into both blastomeres separately. After the *in vitro* culture, the resulting blastocysts were collected to genotype analysis as described above.

Table 3. The information of gRNAs targeting *B4GALNT* gene

gRNA	Sequence	PAM	Strand	Position
#1	TTGAGGATCGACAGACATCT	AGG	Antisense	Exon 2
#2	ACATAAAGAGTCCAACGCTC	AGG	Antisense	Exon 2
#3	GATGCCCGAAGGCGTCACAT	TGG	Antisense	Exon 3
#4	GTCTCCTCAGGTTCACTGCG	GGG	Antisense	Exon 3
#5	ATGTGACGCCTTCGGGCATC	AGG	Sense	Exon 3

3.7. Statistical analysis

Percentage data were subjected to arcsine transformation and ANOVA, followed by Fisher's protected least significant difference test using StatView software (Abacus Concepts). In the experiment 2, the statistical model included embryonic stage, gene editing method, and two-way interactions. When the interactions were not significant, they were excluded from the model but retained to determine the effects of treatment. The percentages of mosaic and bi-allelic blastocysts in the total number of blastocysts were analyzed using chi-squared analysis with Fisher's exact test. $P < 0.05$ was considered statistically significant.

4. Results

4.1. Experiment 1

As shown in Fig. 6A, there were no significant differences in the blastocyst formation rates of embryos edited via electroporation among the different gRNA groups. The total mutation efficiency in blastocysts derived from the embryos electroporated with gRNA #4 significantly higer ($p < 0.05$) than those with gRNAs #2 and #5 (Fig. 6B), whereas the biallelic mutant rate using gRNA #1 was significantly higher ($p < 0.05$) than those using gRNAs #2 and #5. Moreover, the mutation efficiency using gRNA #1 was significantly higher ($p < 0.05$) than those using gRNAs #2 and #3 (Fig. 6C).

4.2. Experiment 2

No significant differences in the rates of cleavage, blastocyst formation, and mutation efficiency were observed in porcine embryos with varying embryonic stage× gene editing method.

The cleavage rates of embryos edited via microinjection were significantly lower than those via electroporation ($p < 0.05$), irrespective of the embryonic stage (Table 4). The blastocyst formation rate in 1-cell stage embryos edited via the microinjection was significantly lower ($p < 0.05$) . However, blastocyst formation rates did not differ between the two gene editing methods in each embryonic stage.

From Figure 7A, the total mutation efficiency was significantly lower ($p < 0.05$) in blastocysts derived from the 2-cell stage embryos edited via the microinjection than those form other treatment groups. Both the biallelic mutation rate and mutation efficiency were significantly higher ($p < 0.05$) in blastocysts from the 1- cell stage embryos edited via microinjection than in those form the 2-cell stage embryos edited using both methods (Figure 7A and 7B).

Table 4. Delivery of the CRISPR/Cas9 system into embryos at different stages using cytoplasmic microinjection and electroporation

Embryonic stage	Gene editing method	Total number of embryos	No. (%) of embryos	
			Cleavage stage*	Blastocyst stage
1-cell	MI	248	155 (62.4 ± 2.9)[a]	20 (8.1 ± 1.1)[a]
	EP	250	230 (92.0 ± 0.9)[b]	40 (16.0 ± 3.1)[ab]
2-cells	MI	119	73 (61.5 ± 6.2)[a]	15 (13.4 ± 6.4)[ab]
	EP	128	114 (89.7 ± 4.3)[b]	33 (26.3 ± 7.7)[b]

Experiments were performed five times. Data are expressed as the mean ± SEM.

*At 2-cell stage, embros were identified as on the cleavage stage when more than 3 blastomeres on day 7.

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

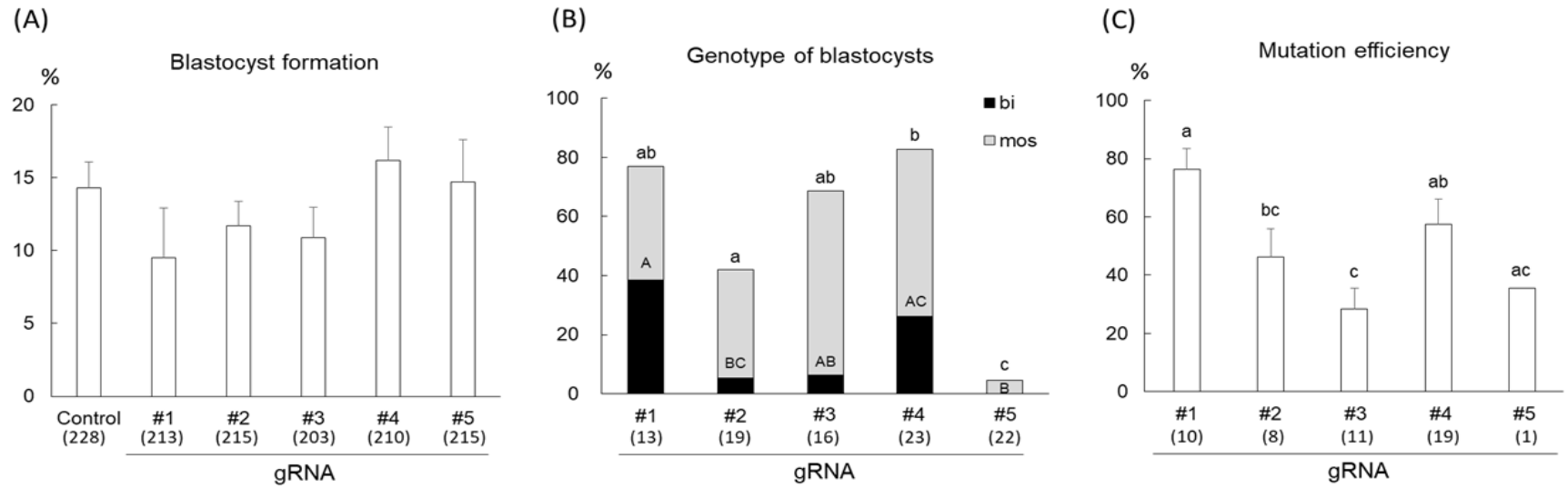

Figure 6. Gene targeting efficiency of five designed gRNAs. (A) Effects of electroporation treatment using different gRNAs on the development of porcine oocytes. (B) Editing frequency of blastocysts derived from porcine zygotes electroporated with different gRNAs. (C) The gene editing efficiency in the blastocysts derived from porcine zygotes electroporated with different gRNAs. Data represents the mean ± SEM. A-C and a-c: values with different superscripts in the proportion of biallelic editing are significantly different ($p < 0.05$). Bi: biallelic editing, mos: mosaic editing.

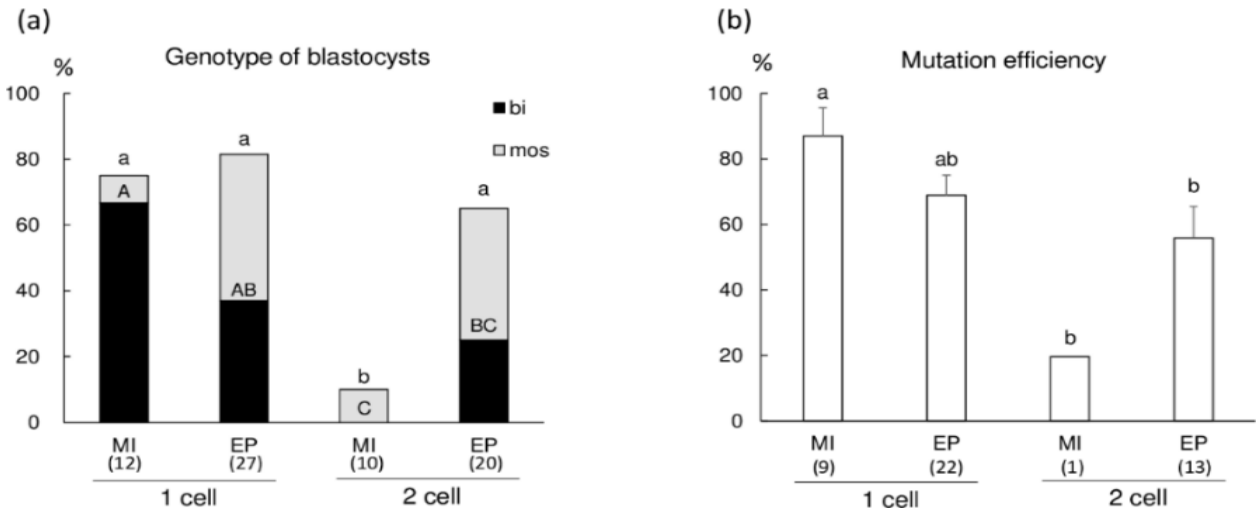

Figure 7. Effect of electroporation and cytoplasmic microinjection in the (A) editing frequency and (B) the gene editing efficiency in blastocysts derived from porcine zygotes and 2- cell stage embryos. Data represents the mean ± SEM. A-C and a-c: values with different superscripts in the proportion of biallelic editing are significantly different. ($p < 0.05$).

5. Discussion

Xenotransplantation can address the growing demand for organs suitable for transplantation. The *B4GALNT2* synthesizes carbohydrate xenoantigen, one of the major xenoantigen expressed at high levels in porcine tissue but is absent in human tissue (Byrne et al. 2018). Therefore, generating *B4GALNT2*-deficient pigs is the first step to achieve successful pig-to-human xenotransplantation. To date, highly efficient gene modification of embryos using the CRISPR/Cas9 system delivered by microinjection and electroporation has been reported in porcine and bovine embryos (Niu et al. 2014; Sato et al. 2016; Tanihara et al. 2016). However, mosaicism including WT cells, need to be overcome when using the CRISPR/Cas9 system (Tu et al. 2015). The one-step generation of F0 pigs with the completely desired gene modification saves cost and time; therefore, gene editing efficiency should be enhanced.

We previously demonstrated that the development and mutation efficiency of porcine embryos edited by the CRISPR/Cas9 system were affected by the sequence of the gRNA (Hirata et al. 2019; Tanihara et al. 2019). Therefore, in this study, we first identified the gRNA yielding the most efficient *B4GALNT2* targeting using the electroporation. We found that sequence used in this study did not affect the embryonic development following electroporation treatment; however, the mutation

efficiency in the resulting blastocysts was affected. These results are consistent with those of previous studies demonstrating that gRNA design is a key factor in gene targeting and mutation efficiency in the CRISPR/Cas9 system (Doench et al. 2014; Ren et al. 2014).

In addition, we evaluated the effects of the gene editing method (i.e., cytoplasmic microinjection and electroporation) and embryonic stage on the development and mutation efficiency of porcine embryos. We showed that the gene editing method affected the cleavage rates but not those of blastocyst formation. However, the mean rates of blastocyst formation in the microinjection method was lower by approximately half of that obtained by electroporation, irrespective of the embryonic stage. We attribute this decrease in blastocyst formation rates in the occurrence of mechanical invasion during microinjection, which may reduce the developmental competence of embryos (Brinster et al. 1985; Menchaca et al. 2020). In addition, the amount of expressed protein and toxicity that depends on the Cas9 concentration injected may have affected embryonic development. However, one study revealed that injecting 200 ng/μl Cas9 mRNA is nontoxic to embryos (Wang et al. 2013). In this study, we injected only 100 ng/μl Cas9 protein (160 kDa), which has a lower concentration than the Cas9 mRNA (~1,500 kDa), suggesting low toxicity.

In this study, we demonstrated that using microinjection, the embryonic stage affected the mutation of blastocysts, i.e., blastocysts from 1-cell stage embryos had higher rates of total mutation, biallelic mutation, and mutation efficiency than those from 2-cell stage embryos. However, our results were not consistent with those of Gu et al. (Gu et al. 2018). They found that the knock-in efficiency in mice was higher in the 2-cell microinjection of CRISPR reagents than zygote microinjection. They proposed that major zygotic genome activation, which occurs during the extended G2 phase of the 2-cell stage, is associated with an open chromatin state, resulting in increased accessibility of the chromatin to the editing enzymes and repair templates. In contrast, the delivery by electroporation into early 1-cell stage embryos prior to our soon after the first cleavage divisions has been reported to generate non-mosaic mutants in mouse embryos (Hashimoto et al. 2016). The authors (Hashimoto et al. 2016) speculated that gene editing occurred before the first genome replication in the 1-cell stage embryos, thus resulting in the generation of non-mosaic mutants. However, because the degree of mosaicism greatly varies from embryo to embryo and from gene to gene (Gu et al. 2018), the discrepancy in the effects on the embryonic stage need to be investigated. We hypothesize that this might be partly due to the differences in the animal species or target genes.

Mosaicism remains a challenge in genetic engineering, particularly in large animal models, whose long breeding periods necessitate considerable cost and time

for production and rearing. Here, we have demonstrated that the microinjection of the CRISPR/Cas9 system into 1-cell stage embryos may be suitable in pigs to obtain highly efficient gen edited embryos for one-step generation. However, the success of CRISPR/Cas9 method depends on the ability to minimize embryo damage and to conserve high levels of gene expression following transfection. Therefore, gene delivery via electroporation is a more suitable method when embryonic viability is a priority.

Chapter 3

Comparison of the genome editing efficiency through a combination of microinjection and electroporation method to generate GGTA1/CMAH/β4GalNT2 triple gene knockout in porcine embryos.

1. Abstracts

The cytoplasmic microinjection (CI) and electroporation (EP) of the CRISPR/Cas9 system into zygotes are used for generating genetically modified pigs. However, these methods create mosaic mutations in embryos. To the best of our knowledge, the combination of these delivery methods of the CRISPR/Cas9 system have not yet been studied. Thus, in this study, we compared the gene editing efficiency in procine zygotes at 1 cell-stage using the single EP method and the combination of EP and CI.

The combination of the two methods had significantly lower cleavage rates and blastocyst formation rates ($p< 0.05$) than the single EP method. However, the biallelic mutation rates and genome editing efficiencies in blastocysts from the combination method were significantly higher than those from single EP. These results indicated that the combination of two delivery methods highly is associated with high biallelic mutation in the porcine embryos, thus resulting in high genome editing efficiency.

2. Introduction

Organs transplantation has been receiving increasing research attention and has been used to treat genetic diseases. However, organ shortage, including hearts, livers, kidneys, lungs, is becoming an issue worldwide (Iwase et al. 2015; Patel et al. 2017; Laird et al. 2016; McGregor et al. 2017; Wilczek et al. 2015). Xenotransplantation is an attractive method to address this, particularly the use of pig organs (Tonelli et al. 2014). However, the human immune system may reject pig grafts as they are sometimes detected as foreign antigens (Byrne et al. 2015; Cooper et al. 2015; Shimizu et al. 2000).

The α1,3-galactosyltransferase *(GGTA1*) gene encodes αGal antigen; cytidine monophospho-N-acetylneuraminic acid hydroxylase (*CMAH*) gene encodes N-glycolylneuraminic acid (*Neu5Gc*), and β-1,4-N-acetylgalactosaminyl transferase 2 *(β4GalNT2*) encodes DBA-reactive glycans also known as the Sd(a) antigen. These antigens have been recognized as causative xenoantigens associated with hyperacute rejection of a xenograft (Byrne et al. 2014; Chen et al. 2005; Dai et al. 2002; Lai et al. 2002). Estrada et al. (Estrada et al. 2015) and Zhang et al. (Zhang et al. 2018) have previously generated *GGTA1/CMAH/β4GalNT2* triple gene-knockout (TKO) pigs through somatic cell nuclear transfer technique using the cells deficient in *GGTA1*, *CMAH* and *β4GalNT2*. They found that the binding levels of human

IgG/IgM, peripheral blood mononuclear cells (PBMCs) and red blood cells from TKO pigs were significantly reduced compared with those from wild-type pigs (Estrada et al. 2015; Wang et al. 2017; Zhang et al. 2018).

The pancreatic duodenal homeobox 1 (*PDX1*, also known as *IPF-1*, *IDX-1*, and *STF-1*) gene plays an important role in pancreas development in fetuses. PDX1 mutation in mice and pigs revealed that this biallelic mutation caused infant deaths (Bonal et al. 2008; Jonsson et al. 1994; McKinnon et al. 2001). In humans, a monoallelic 1-bp deletion in *PDX1* (Pro63fsdelC) have been shown to be a nonsense mutation that causes diabetes (Clocquet et al. 2000; Fajans et al. 2010; Stoffers et al. 1998). *PDX1*-modified pigs are attractive animal models to study the pathogenesis of diabetes in human; however, there are only a few phenotypic studies of *PDX1*-modified pigs, and their evaluation as diabetes models is insufficient.

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated gene (CRISPR/Cas) system has been recently used to establish genetically modified animals. This method delivers the Cas9/gRNA complex into embryos mostly via microinjection (Niu et al. 2014; Yong Wang et al. 2015). However, the genotype of resulting mutant embryos often exhibits a mosaic pattern, i.e. the presence of more than two alleles in an individual (Burkard et al. 2017; Sato et al. 2018; Whitworth et al. 2014).

In our previous study, we have developed the GEEP (gene editing by electroporation of Cas9 protein) method that exhibited high-efficiency disruption of the targeted genes in pigs (Tanihara et al. 2016). GEEP is a simple gene editing method wherein the CRISPR/Cas9 system is introduced into porcine zygotes via EP and does not cause the damage associated with micromanipulation for injection of the CRISPR/Cas9 system into zygotes. However, the GEEP method also induces mosaic pattern in the embryos (Tanihara et al. 2020). Hashimoto et al. (Hashimoto et al. 2016) have previously revealed that the generation of mosaic mutants via introduction of Cas9 protein/gRNA complexes into 1-cell stage embryos depends on the time window between fertilization and the first DNA replication. Therefore, in this study, we evaluated the developmental competence and gene editing efficiency of porcine zygotes at 1-cell stage mediated by CRISPR/Cas9 and gRNAs targeting *GGTA1*, *CMAH* and *PDX1* and delivered by EP or the combination of EP and MI.

3. Materials and Methods

Because live animals were not used in this study, ethical approval was not required.

3.1. Oocyte collection, in vitro maturation

Pig ovaries were obtained from prepubertal crossed gilts (Landrace × Large White × Duroc breeds) at a local slaughterhouse and were transported to the laboratory in physiological saline at 30°C. The ovaries were washed thrice with prewarmed physiological saline solution supplemented with 100 IU/ml penicillin G potassium (Meiji) and 0.1 mg/ml streptomycin sulfate (Meiji). Follicles (3–6 mm diameter) on the ovarian surface were sliced on a sterilized dish using a surgical blade, and COCs were visualized and collected using a stereomicroscope. Approximately 50 COCs were cultured in 500 µl of maturation medium consisting of the TCM 199 with Earle's salts (Thermo Fisher Scientific) and supplemented with 10% (v/v) porcine follicular fluid, 0.6 mM cysteine (Sigma-Aldrich), 50 µM β-mercaptoethanol (Wako Pure Chemical Industries Ltd), 50 µM sodium pyruvate (Sigma-Aldrich), 2 mg/ml D-sorbitol (Wako Pure Chemical Industries Ltd.), 10 IU/ml equine chorionic gonadotropin (Asuka Pharmaceutical), 10 IU/ml human chorionic gonadotropin (Nippon Zenyaku Kogyo), and 50 µg/ml gentamicin (Sigma-Aldrich). The COCs were covered with mineral oil (Sigma-Aldrich) in 4-

well dishes (Nunc A/S), and after 22h, they were transferred to a maturation medium without hormones and cultured for another 22 h. Incubation was conducted at 39°C in a humidified incubator containing 5% CO_2.

3.2. In vitro fertilization

The matured oocytes were subjected to IVF as described previously (Nguyen et al. 2017). Briefly, frozen-thawed ejaculated spermatozoa were transferred to 5 ml PFM medium (Research Institute for the Functional Peptides Co.) and washed using centrifugation at 500 × *g* for 5 min. The pelleted spermatozoa were resuspended in the fertilization medium at a final concentration of 1×10^6 cells/ml. Approximately 50 oocytes were transferred to 500 µl sperm-containing fertilization medium, 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 using mechanical pipetting, transferred to PZM-5 (Research Institute for the Functional Peptides Co.), and cultured.

3.3. Electroporation

EP was performed as described previously(Tanihara et al. 2016). Briefly, the electrode (LF501PT1-20; BEX Co. Ltd.) was connected to a CUY21EDIT II electroporator (BEX Co. Ltd.) and placed under a stereoscopic microscope. The

inseminated zygotes were washed with Opti-MEM I solution (Gibco/Invitrogen Co.) and placed in a line between the electrodes on the chamber slide filled with 10 μL of Nuclease-Free Duplex Buffer (IDT), gRNA (Alt-R^{TM} CRISPR crRNAs and tracrRNA, chemically-modified and length optimized variants of the native guide RNA purchased from IDT) and Cas9 protein (Takara Bio, Inc.). After EP using 5 1-ms pulses at 25 V, the zygotes were washed with PZM-5 and cultured. At Day 3 after fertilization (Day 0), all of the cleaved embryos were subsequently cultured in PBM (Research Institute for the Functional Peptides Co.) for 4 days at 39°C in a humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2.

3.4. Cytoplasmic mircoinjection

Microinjection was performed using a 20 μl drop of PZM-5 (Research Institute for the Functional Peptides Co.) covered by mineral oil. The duplex buffer containing 100 ng/μl of 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 of Cas9 protein (Guide-it™ Recombinant Cas9; Takara Bio) was loaded into an injection pipette (Femtotips II). The zygotes were immobilized with a holding pipette, and the tip of the injection pipette was inserted into the cytoplasm through the zona pellucida and cell membrane at 3 o'clock position. Subsequently, the duplex buffer containing Cas9/gRNA complex was

injected into the cytoplasm by air pressure using a microinjector (FemtoJet 4i; Eppendorf) with the following parameters: injection pressure, 20–30 hectopascals (hPa); compensation pressure, 10–12 hPa; and injection time, 0.15 s).

3.5. Design of gRNAs

gRNA was designed by using the CRISPR direct webtool (https://crispr.dbcls.jp/) (Naito et al. 2015). We confirmed that 12 bases at the 3' end of designed gRNAs have no identical sequence in the pig genome, excepting for the targeting region of the INS gene to minimize the possibility of the off-target effect by using COSMID webtool (https://crispr.bme.gatech.edu/) (Cradick et al. 2014). We designed three kinds of gRNA targeting *PDX1*, *GalT*, and *CMAH*. The target sequences of gRNAs are listed in Table 5.

Table 5. The information of gRNAs targeting *CMA*, *GGTA1* and *PDX1* genes

gRNA	Sequence	PAM	Strand
CMAH	GAAGCTGCCAATCTCAAGGA	AGG	Sense
GGTA1	AGACGCTATAGGCAACGAAA	AGG	Sense
PDX1	TGGCGAGGAGCAGTACTACG	CGG	Sense

3.6. In vitro culture

Approximately 50 zygotes were cultured continuously in 500 µl PZM-5 covered with mineral oil for 3 days in 4-well dishes. Then, the zygotes were cultured in 500 µl PBM (Research Institute for the Functional Peptides Co.) covered with mineral oil for another 4 days in 4-well dishes. Incubation was conducted at 39 °C in a humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2. To evaluate gene editing efficiency, blastocysts were collected on day 7 (day 0 = insemination) and subjected to gene analysis.

3.7. Analysis of targeted genes

Genomic DNA was isolated from individual blastocysts by boiling them in a 50 mM NaOH solution at 98 °C for 10 minutes, followed by neutralization with 100mM Tris HCl. The genomic regions flanking the gRNA target *B4GalNT2* sequences were amplified with the following primers: 5'-TAGGGGGAAAAACACACTGG -3' (forward) and 5'-CACCCTCGGGAATGAGTAGA -3' (reverse). The PCR products were extracted using agarose gel electrophoresis with a Fast Gene Gel/PCR Extraction Kit (Nippon Genetics) according to the manufacturer's instructions. The targeted genomic regions of the PCR products were directly sequenced using Sanger sequencing with

a BigDye Terminator Cycle Sequencing Kit v3.1 (Thermo Fisher Scientific K.K.) and an ABI 3500 genetic analyzer (Applied Biosystems).

The TIDE bioinformatics package was used to determine the genotype of each blastocyst (Brinkman et al. 2018). Blastocysts that carrying no WT sequences were classified having biallelic editing, whereas those carrying more than one type of editing (in addition to the WT sequence) were classified as mosaic. Those having only the WT sequence were classified as WT.

3.8. Experimental design

To compare the effects of the single or combination genome delivery methods on editing efficiency, we performed single electroporation and double treatments of EP and CI in porcine zygotes after IVF.

3.8.1. *Electroporation with three kinds of gRNA/Cas9 complexes*

The inseminated zygotes were electroporated with 10 μl of Nuclease-Free Duplex Buffer containing 100 ng/μl of gRNAs, targeting *PDX1, GalT, CMAH* gene and 100 ng/μl of the Cas9 protein (EP group) 10 h after IVF.

3.8.2. *Electroporation with two kinds of gRNA/Cas9 complexes before and after microinjection with gRNA targeting PDX1 gene*

Electroporation was conducted to the inseminated zygotes 10 h after IVF using 10 μl of Nuclease-Free Duplex Buffer containing 100 ng/μl of gRNA targeting *GalT*, 100 ng/μl of gRNA targeting *CMAH*, and 100 ng/μl of the Cas9 protein. One hour before and after EP treatment (MI-EP and EP-MI, respectively), MI was performed on these zygotes using gRNA/Cas9 complexes targeting *PDX1*. After treatments, the zygotes were cultured for 7 days as described above. (Figure 8). To serve as the control, untreated zygotes were similarly cultured. On day 7, blastocysts from each group were separately collected and analyzed.

3.9. Statistical analysis

All percentage data were subjected to arcsine transformation and ANOVA, followed Fisher's protected least significant difference test using StatView software (Abacus Concepts). In experiment 2, the statistical model included embryonic stage, gene editing method, and two-way interactions. When the interactions were not significant, they were excluded from the model but retained to determine the effects of treatment. The percentages of mosaic and biallelic blastocysts in the total number of blastocysts were analyzed using chi-squared analysis with Fisher's exact test. $P < 0.05$ was considered statistically significant.

4. Results

As shown in Table 6, the cleavage rates of embryos edited by single EP were not significantly different with those of control group. Those from the EP-MI and MI-EP treatment groups were significantly lower than those from the EP and control groups. In addition, the blastocyst formation rates in control group was significantly higher than those from EP, MI-EP and EP-MI group.

From Figure 9, the total mutation efficiency in blastocysts derived from the MI-EP and EP-MI group were significantly higher than those from the EP group. However, the number of blastocysts carrying 3 biallelic mutations of EP with 3 genes tended to be higher than that from the MI-EP and EP-MI groups ($p<0.1$). Interestingly, all blastocysts from the EP-MI treatment group had at least one biallelic mutation. However, the genome editing efficiency in the blastocyst was not significantly different among the treatment groups (Figure 10).

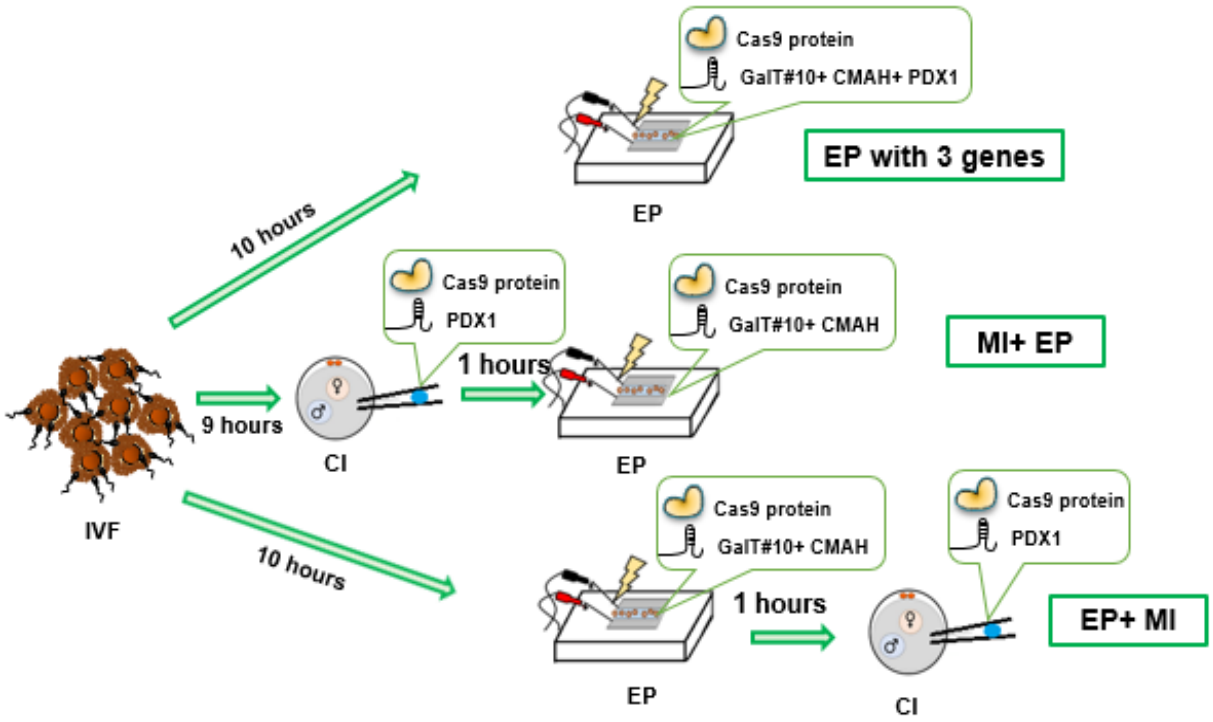

Figure 8. Schematic diagram of experimental design. IVF: *in vitro* fertilization, MI: cytoplasmic microinjection, EP: electroporation.

Table 6. Effects of electroporation and/or microinjection on the development of porcine zygogtes

Treatment groups*	Total number of embryos	No. of embryos (%)	
		Cleavage stage	Blastocyst stage
Control	251	238 (94.8 ± 1.3)[a]	72 (28.7 ± 2.6)[a]
EP	257	235 (91.5 ± 1.1)[a]	52 (20.3 ± 2.7)[b]
MI-EP	254	162 (63.7 ± 5.1)[b]	26 (10.2 ± 1.9)[c]
EP-MI	260	107 (40.9 ± 3.6)[c]	10 (3.8 ± 1.3)[c]

Experiment were performed using five replications. Data are expressed as the mean ± SEM.

* EP: Zygotes treated using EP 10 hours after IVF. MI-EP: Zygotes injected into cytoplasmic 9 hours after IVF and treated using EP at 10 hours after IVF. EP-MI: Zygotes treated using EP 10 hours after IVF and injected into cytoplasmic 11 hours after IVF.

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

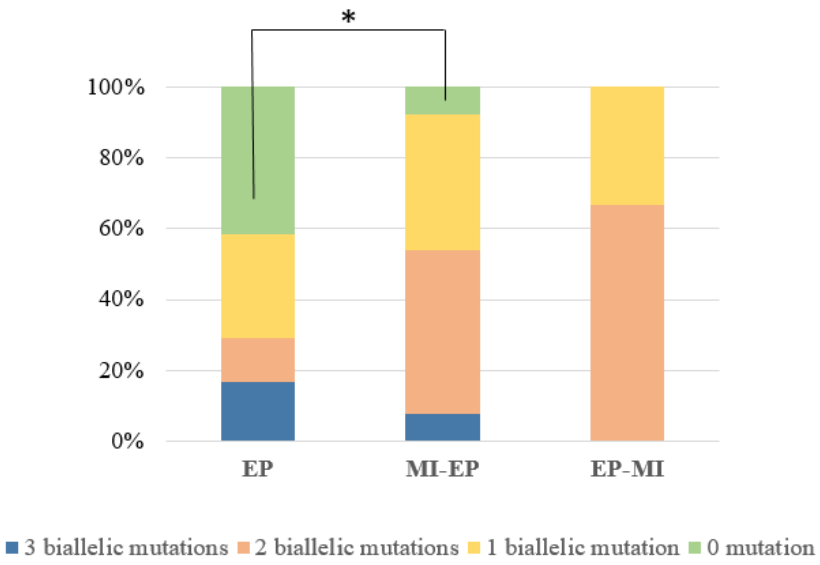

Figure 9. Biallelic mutation efficiency in blastocyst derived from porcine zygotes treated with EP or combination of EP and MI. EP: electroporation, MI: cytoplasmic microinjection

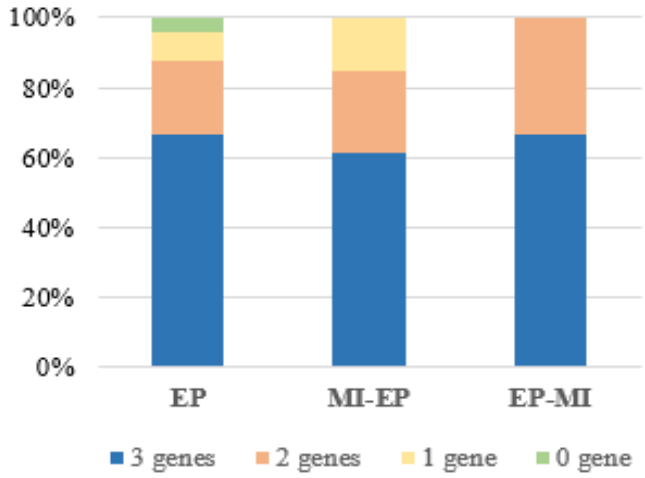

Figure 10. Genome editing efficiency in blastocyst derived from porcine zygotes treated with EP or combination of EP and MI. EP: electroporation, MI: cytoplasmic microinjection.

5. Discussion

Xenotransplantation can address the growing demand for organs suitable for transplantation. *GGTA1* and *CMAH* are the main xenoantigens associated with hyperacute rejection of a xenograft (Byrne et al. 2014; Dai et al. 2002; Lai et al. 2002) whereas *PDX1* is important pancreatic development in fetus (Bonal et al. 2008; Jonsson et al. 1994; McKinnon & Docherty. 2001). Therefore, generating *GGTA1/CMAH/ PDX1* triple gene knockout pig advances pig-to-human pancreas xenotransplantation. To establish experimental animal models, the CRISPR/Cas9 system is a highly efficient gene editing method delivered by microinjection and electroporation (Niu et al. 2014; Sato et al. 2016; Tanihara et al. 2016). To reduce mosaic, gene editing efficiency should be improved.

In this study, we compared the effects of gene delivery methods, i.e., EP and MI, on the development and mutation efficiency of 1 cell-stage porcine oocytes. Our results showed that both of cleavage rates and blastocyst formation rates were influenced the delivery. Particularly, these were significantly lower in the EP-MI and MI-EP treatment groups than in the EP and control groups. We attribute the decrease in blastocyst formation rates in the mechanical invasion occurring during microinjection, which may reduce the developmental competence of embryos (Brinster et al. 1985; Menchaca et al. 2020). In additional, the cleavage rate of the

embryos from the MI-EP group was higher than that from EP-MI group, indicating that MI conducted at the earlier stage is beneficial to their development.

Furthermore, more blastocysts having 3 biallelic mutations were obtained higher in the EP group than combination treatment groups. A single treatment of EP may be more gentle to the development of porcine zygotes than double treatments. The developmental ability in electroporated zygotes from EP was also high, almost equal to those in control group (Tanihara et al. 2019). On the other hand, the rate of blastocysts carrying at least 1 biallelic mutation obtained from double treatment methods were high (92.3%, MI-EP; 100%, EP-MI). The genome editing efficiency of both groups also reached 100%. These result suggested that the combination of these methods can lead to a higher number of blastocysts carrying biallelic mutation while reducing mosaicism in porcine zygotes (Tanihara et al. 2019).

In conclusion, although the combination EP and MI treatments resulted in lower cleavage rate and lower blastocyst formation than the single EP treatment, combination treatment achieved greater number of blastocysts having biallelic mutations and higher genome editing efficiency.

IV. SUMMARY

Biotechnology in livestock involves reproductive procedures and molecular genetic methods. Although there are several ethical issues involve in the use of animals in biomedical, the importance of animal experimentation cannot be denied. Animal models play an important role in biomedical and behavioral studies as they are essential in the prevention, cure, and treatment of various diseases. Small mammals, such as the rabbit, guinea pig, rat and mouse, are frequently used in wound healing studies as they are inexpensive and easy to handle; however, their anatomy and physiology are vastly different from humans. In contrast, pigs, which exhibit similar anatomy, genome, and chromosomal structures to humans, have been used to generate cells and organs for xenotransplantation. In addition, pig genome has also been mapped and completely characterized. Therefore, they are promising experimental animal models and have been widely used in studies involving human health and genetic diseases.

The CRISPR/Cas9 method is a powerful tool to generate gene edited pig organs for xenotransplantation. This method is increasing research attention and has been widely used for genome editing.

In chapter 1, we employed EP to deliver the CRISPR/Cas9 system, comprising gRNA targeting myostatin (*MSTN*) and various concentrations of Cas9 protein (i.e.,

0, 25, 50, 100, 500 to 1000 ng/µl), into porcine zygotes to determine the optimal Cas9 concentration for high-efficiency gene editing. We revealed that the Cas9 protein concentration did not affect the development of electroporated zygotes, editing rate, and the frequency of indel events at two possible off-target sites. However, gene editing efficiency was significantly lower in the edited blastocysts derived from zygotes electroporated with 25 ng/µl Cas9 protein than those with higher Cas9 protein concentrations. These results indicated that Cas9 protein concentration influences gene editing efficiency but not development, editing rate, and non-specific cleavage at off-target sites.

In chapter 2, we aimed to evaluate the influence of the delivery method, i.e., electroporation and cytoplasmic microinjection, and the embryonic stage, i.e., 1-cell and 2 cells, on CRISPR/Cas9-mediated gene editing in porcine embryos. First, we determined the efficiency of 5 designed gRNAs targeting *B4GALNT*. Then, using the most highly efficient gRNA, we compared the gen editing efficiencies using MI and EP, and 1-cell and 2-cells embryos. We found that the gRNA sequence affected the biallelic mutation rate and mutation efficiency in blastocysts derived from electroporated embryos. The MI method yielded significantly lower ($p < 0.05$) cleavage rate, but achieved the highest biallelic mutation rate and mutation efficiency in blastocysts form the 1- cells stage embryos. These results suggested

that gen editing rate and efficiency in blastocyst are influenced by the delivery method and embryonic stage.

Lastly, in chapter 3, we compared the gene editing efficiency using single electroporation and electroporation-cytoplasmic microinjection combination treatment in CRIPR/Cas9-mediated gene editing of *GGTA1/CMAH/ PDX1* in porcine zygotes. We revealed that the cleavage rate and blastocyst formation rate were significantly lower in embryos derived from the combination treatment than the single electroporation. Interestingly, the biallelic mutation rate and genome editing efficiency of blastocysts from the combination method were significantly higher than those from the single treatment ($p<0.05$). These results indicated that the use of 2 gene delivery methods may generate more blastocysts with biallelic mutations and results in higher gene editing efficiency in porcine embryos.

In conclusion, the issue of human immune rejection in organs xenotransplantation can be overcome using improved gene editing techniques, thus addressing the global issue of organ shortage. The CRISPR/Cas9 method is an emerging tool to generate genetically modified animal that can be used to study the mechanisms underlying immune rejection in animal-to-human xenotransplantation. In this study, we focused on the effect of the CRISPR/Cas9 component and gRNAs targeting genes involved in the human immune system aiming to generate

genetically modified pig organs containing genes suitable for xenotransplantation. Our findings serve as foundational guide for future studies on xenotransplantation research.

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