Studies of efficient conditions for generation of genetically modified pigs

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CHAPTER 1. GENERAL INTRODUCTION

The necessity of laboratory animals

New technologies such as genetic modification differ from breed improvement, which fixes mutations and trait changes such that one can also add useful attributes by manipulating genes directly. If it is advantageous to the industry, industrial animals can also be modified by this method. We aim to create animals with better traits for experimentation. Observation over many generations can be possible if we use animals with a short life cycle, for research on aging and inheritance. Additionally, in research on organ surgery methods and organ transplantation, medium-sized animals such as dogs and pigs are used. Dogs, rabbits, and cats can also be used for research on the mechanisms and treatments of diseases of the cardiovascular system. In particular, dogs are used for diagnostic techniques such as electrocardiograms, cardiac catheters, angiography, coronary blood flow measurement, and coronary arteries. This has greatly contributed to the development of surgical methods such as bypass surgery, pacemaker implantation, cardiac valvuloplasty, and heart transplantation. It is also well known that goats are used for the development of artificial heart, as the size of their tissue is close to that of humans. Mice, rats, rabbits, and monkeys are used for research in cancer and infectious diseases, and rats, cats, and monkeys are frequently used for nerve system research. Fish are used as model organisms for studying the life phenomena of vertebrates, as they also have spinal cords. One reason is that the fertilized eggs are relatively easy to manipulate but fish cannot generally survive without water. Drosophila has a number of excellent advantages as an experimental organism: it is easy to breed, because the time taken for one generation is as short as 2 weeks. In addition, there are only four pairs of chromosomes (one sex chromosome, three autosomes), which can be suitable for genetic research. Thomas Hunt Morgan was awarded the Nobel Prize in Physiology and Medicine in 1933, demonstrating the chromosome theory that chromosomes are carriers of genes in experiments with *Drosophila melanogaster*.

Superiority of pigs as laboratory animals

Various animals can be used in experiments as mentioned above. Although it is not possible to investigate what animals are being bred and used in research institutions in Japan, it is possible to estimate the usage of experimental animals through monitoring the number of sales (Table 1). A survey of the number of sales of laboratory animals in FY2008 was conducted, and responses have been obtained from almost all domestic companies. The sale of mice and rats also include genetically modified forms, but sale of those with artificially modified genes is as low as about 2% of the total. In addition, the sale of laboratory animals as a whole has decreased greatly. Among them, however, the number of pigs increased by 14% compared to that in the previous year, a remarkable increase compared to the others. Even though most of them are SPF, they were raised in a controlled environment.

Pigs have been well known for their traits from ancient times through selective breeding and are classified as industrial animals for consumption. To better serve research, the technology of management is improving, such as that through the advancement of SPF. The market is largely characterised by stability. Nowadays, modified species such as mini pigs are also fixed, species are specially created for experiments, and the environments for experimental animals are being set up.

Reasons why pigs have recently been used as laboratory animals

1. Cardiovascular research. As the size of the heart and the distribution of the coronary artery in a pig resembles that of a human being, the effect of medicine on myocardial infarction has been explored, cardiac output and heart failure have been monitored, and cardiac surgery and xenograft experiments have been performed. The similarity in heart size is key, and pigs originally meant for consumption are also used for endoscopic surgery training.
2. Gastrointestinal research. Diet and ulcers have been studied in pigs, as their ulcers, pig gastric cardia, occur due to a cause similar to that in humans, with a strong influence of diet. Since their digestive physiology/intestinal flora is similar to that in humans,

Table 1. Survey of total annual sales of laboratory animals (March 2016 – Aprial 2017)

（from Japan Experiment Animal Association）

Animal species	Conventional	Clean	SPF	Total (increase / decrease%)	
Mouse	0	11,504	3,189,298	3,200,802	-19.1
Rat	504	4,492	894,398	899,394	-26.3
guinea pig	18	6,897	62,231	69,146	-31.6
hamster	0	0	8,465	8,465	-35.1
Other rodents	0	0	1,266	1,266	-39.2
Rabbit	1,496	25,812	18,033	45,341	-24.2
Dog	4,754	0	0	4,754	-26.2
Cat	32	254	270	556	**+0.4**
Monky	3,131	130	0	3,261	**+9.9**
Pig	862	267	2,070	3,199	**+14.0**
Goat	6	0	0	6	-83.3
Sheep	2	0	0	2	-88.9
Birds	1,370	0	3,476	4,846	-49.5

pigs are also used to investigate diseases related to intestinal infection caused by viruses and *Escherichia coli*, which occur in neonates. Additionally, research on the secretion mechanism of gastric juice is progressing.

3. Nutritional research. Many studies exist on how to grow pigs normally and promptly for the purpose of breeding. As a model animal for the relationship between nutrition and growth in human children, using pigs has the advantage of being able to observe developments in a time-shortened form. When breeding several weeks immediately after weaning under low protein conditions, signs similar to protein deficiency seen in human children can be observed. Pigs are a model of the age and sex of a human child and how they affect body composition during the growth period.

4. Models of premature infancy. Pigs are bred in a comparatively immature state and are susceptible to the influence of the outside air temperature. If the temperature is not properly controlled, their body temperature drops, and hypoglycaemia can lead to

death. This relationship is common in human neonates. As such, pigs can act as an important model of premature babies, allowing for investigation of proliferation and preterm delivery associated with infertility treatment.

5. Skin models. It is well known that pig skin tissue is similar to that in humans. However, pigs do not sweat, unlike humans, so they cannot be used to completely imitate human skin. Research on how chemical substances contained in cosmetics and paints affect the skin (such as skin poisoning) is emphasized in medicine development. The relatively large surface area pigs offer is also regarded as an advantage.

6. Immunological research. SPF pigs and swine that were grown by artificial breastfeeding are used for immunological studies. We have achieved results in studies such as those on the defence mechanisms of living organisms, activity of immune cells, and immunological rejection reactions to organ transplants. On the other hand, there are many individuals who have hepatitis E, and it is still impossible to completely imitate the human system. This is regarded as a future research theme.

7. Xenograft donor. If a person's organs suffer a partial or complete loss of function, substitute functional organs are needed. One solution is an organ transplant, an artificial organ or a cross-species transplant. Bio-transplantation is one of the most promising methods, but the number of potential donors is insufficient owing to the brain-dead state or lack of a part of the living body to transplant. Additionally, some artificial organs have a high possibility of success such as blood vessels and bones, but artificial hearts are not sufficient. For organs with complicated functions such as the liver are unknown. Currently, heterogeneous transplantation holds promise. Monkeys are close to humans, but criticism and resistance to their use is also numerous, and their numbers are also small. Long-term breeding is an inevitable problem, as they need to grow slowly to be potential donors. Currently, minipigs are physiologically easy to obtain organs similar to humans, even in terms of size. It is expected that they could act as donors of alternative organs if the SPF pigs reach a state where they can be supplied sufficiently. Pigs already approved for transplantation are available for cardiac valves.

8. As a substitute animal for dogs. Dogs have been subjected to experiments since ancient times; there are homogeneous and improved dog breeds such as beagles, and highly accurate experiments are possible with dogs. However, the use of dogs for

anatomy experiments is strongly resisted on grounds of animal welfare, as they are companion animals and are usually captured for use in experiments (nearly 20,000 in 1998). On the other hand, pigs are readily consumed, less hurt by experiments, and show lesser resistance than dogs.

Pigs give birth more than twice a year, to around 20 piglets in total, they grow fast, and there is a possibility that they can be supplied relatively inexpensively as experimental animals. In addition, since the breed has been improved over many years, it has been systematically unified. In addition, as more individuals can be obtained from a single sow, the accuracy of the experiment also improves. Pigs as laboratory animals having these characteristics has led to the increase in their usage, and 10% of the total number of these pigs constitute a particular kind, called mini-pig.

Heat stress perturbs oocyte development in pigs

In gilts and sows, the summer-autumn period often is characterised by reduced fertility. Heat stress and long photoperiods during the warm season can cause a reduction in feed intake and an imbalance of the hypothalamic-hypophysial-ovarian axis. The increased variability in the interval between oestrus onset and ovulation results in an increased number of poorly timed inseminations. The altered endocrine activity compromises follicular and corpora luteal development, reduces oocyte quality, and increases embryo mortality. Heat stress affects the follicle and its enclosed oocyte, suggesting that perturbations in the follicular microenvironment, to which the oocytes are exposed for long periods of development, reduce their developmental competence. A better understanding of the underlying mechanisms by which heat stress impairs fertility may lead to the development of additional approaches to alleviate these effects. In vivo and in vitro studies support the view that porcine oocytes are susceptible to thermal stress at various stages of follicular development. Perturbation in the physiology of the follicle-enclosed oocyte during the lengthy period of follicular development could potentially lead to an oocyte with reduced competence for fertilization and subsequent development. Oocytes harvested from cows during the summer exhibit a reduced ability to develop to the blastocyst stage after in vitro

fertilization (Rocha et al., 1998; Al-Katanani and Hansen, 2002; Al-Katanani et al., 2002). It has been reported that cleavage to the two- and four-cell stages following chemical activation was delayed in oocytes collected during the hot season relative to oocytes collected during the cold season (Aroyo et al., 2007). The timing of first cleavage is considered to have major long-lasting effects on subsequent embryonic developmental potential (Fenwick et al., 2002), which may explain the inferior developmental competence of oocytes collected during the summer. Elevated air temperatures before oestrus have been associated with reduced fertility(Al-Katanani et al., 1999; Chebel et al., 2004). A study performed from late summer to early winter indicated that a period of two to three oestrous cycles is required for recovery from heat damage and appearance of competent oocytes (Roth et al., 2001). In support of this form of recovery, induction of maternal hyperthermia in mice carried over through three pregnancy cycles, as expressed by a lower pregnancy rate and smaller litter sizes in the first cycle and slight increases in these parameters through the second and the third cycles after heat exposure (Aroyo et al., 2007). It appears that not only the individual ovulated oocyte, but also the ovarian pool of oocytes can be damaged during heat exposure. Nevertheless, the exact follicular stage at which the enclosed oocyte is susceptible to thermal stress has not been defined.

Future of creating new experimental animals

It is a fact that the number of experimental animals used has decreased. Among them, we are continuing research to create new experimental animals. In this study, we aimed to optimize the introduction process of the CAS9 system into oocytes, which is necessary for genetic modification. The result could be of use to research institutions that genetically modify animals and aims to be used as a basic condition to support experiments. In many cases, it is impossible to grasp the real number of genetic engineering experiments that is conducted within research institutions in a country. However, some transgenic animals, which are the result of these studies, are on the market and the demand for genetically modified animals can be known from the number of their sales. The number of sales of genetically modified mice in Japan continued to increase from 13,721 in 2010 to 17,414 in 2013 and 18,969 in 2016 (Japan

Experiment Animal Association), even though sales of laboratory animals rapidly decreased. Regardless, the demand for animals that have recombined genes with certain characteristics continues to grow. Many experimental organisations are expected to increase demand in the future even though it is considered that voluntarily transformed animals are being created.

There is a limit to evaluating the number of genetic experiments based on the sales number of laboratory animals, but the trend can be confirmed with changes in the number of papers published. It is possible to compare the increase rate of the number of hits in the following year from the presentation year for ZFN (Zinc-finger nuclease), TALEN (Transcription activator effector-like nuclease), and CRISPR/Cas9. Notably, in the era in which the CRISPR(Clustered Regularly Interspaced Short Palindromic Repeat)/Cas9 technology was developed, peripheral technologies necessary for experiments, such as PCR and DNA synthesis, had already been developed. However, the rapid increase in the number of papers after the discovery of CRISPR/Cas9 shows the demand for genetic modification experiments and the number of themes to which this method can be applied to. In 2000, papers related to genome editing began to be reported, especially as the number of papers has increased remarkably after the appearance of CRISPR. The original paper on genome editing in our country is the first paper that used the 2010 ZFN method. Statistically, the number of papers changes to 4 in 2010, 0 in 2011, 5 in 2012, 22 in 2013, and 37 in 2014. Among them, 14 papers on the modification of cultured cells and 46 papers related to alteration of animals have been published. Since 2013 when CRISPR/Cas9 was developed, the number of overseas papers has increased greatly (44 reports).

Various gene introduction methods

In genetic recombination, it is necessary to incorporate exogenously generated genes into cells. Several methods have been studied to incorporate foreign genes into the chromosome.

1. Chemical gene transfer method

Cationic lipid-mediated transfection, calcium phosphate co-precipitation, and incorporation by DEAE-dextran method are examples of chemical gene transfer

methods. Cationic lipid-mediated transfection is one of the most common approaches for transferring foreign genetic material into cells. Conventional lipid-based transfection reagents utilize artificial liposomes that encapsulate nucleic acids, then lyse the cell membrane and accumulate it inside (Fraley et al., 1980). With a new cationic lipid-based reagent, spontaneous condensation occurs owing to electrostatic interaction between the negatively charged nucleic acid and the head group of the positively charged synthetic lipid reagent to form a nucleic acid-cationic lipid reagent complex. The complex is then taken up by cells via endocytosis and released into the cytoplasm. When the complex enters the cell, the incorporated DNA is transferred to the nucleus and expressed. RNA or antisense oligonucleotides skip the transfer step and remain in the cytoplasm.

The calcium phosphate coprecipitation method has been a commonly used transfection method since the early 1970's (Graham and van der Eb, 1973), because its components are readily available and inexpensive. It is effective for many types of cultured cells and is used for transient and stable transfection of various cultured cell types. Mixing DNA with calcium chloride solution in phosphate-buffered saline results in a calcium phosphate-DNA co-precipitate, which is taken up by placing a suspension of this precipitate on top of cultured cells. Calcium phosphate promotes the binding of concentrated DNA to the cell surface in the co-precipitate, and DNA invades the cell by endocytosis.

Diethylaminoethyl (DEAE)-dextran is a polycationic derivative of a carbohydrate polymer (dextran) and is one of the first chemical reagents used to introduce nucleic acids into cultured mammalian cells (Pagano and Vaheri, 1965). The cationic DEAE dextran molecule binds tightly to the negatively charged nucleic acid backbone. Since the resulting nucleic acid DEAE dextran complex has a positive charge, it adheres to the cell membrane and invades the cytoplasm by osmotic shock induced by endocytosis or DMSO or glycerol. It is characterised by high reproducibility and low cost. The disadvantage are cytotoxicity, low transfection efficiency in a wide range of cell types (typically less than 10% in primary cells), and the need to use a medium with low serum content during the transfection process. In addition, the use of this approach is limited to transient transfection and not suitable for the creation of stable cell lines.

2. Biological gene transfer method

Viruses can be used as a biological gene incorporation vehicles. Virus-mediated transfection, also known as transduction, is a method for protein overexpression and knockdown in difficult-to-transfer cell types and is most commonly used in clinical studies (Pfeifer and Verma, 2001; Glover et al., 2005). Adenovirus, onco-retrovirus, and lentiviral vectors are widely used for mammalian cell culture and *in vivo* gene transfer. Other well-known viral gene transfer examples are baculovirus and vaccinia virus-based vectors. Viruses are preferred as a system for gene transfer in clinical trials because the transfection efficiency *in vivo* is high and gene expression is maintained by the gene being integrated into the host genome. However, this approach has numerous shortcomings such as immunogenicity and cytotoxicity, technically difficult production of time- and labour-intensive vectors, high cost to meet the requirements of biosafety, and variability of virus infectivity (Glover et al., 2005) (Hunt and Vorburger, 2002; Glover et al., 2005; Kim and Eberwine, 2010).

In snake in viruses, viruses are a source of infection for cells. Therefore, one must use viruses with specific characteristics as vectors. Viral vectors are often made from pathogenic viruses; risks in handling are thereby incurred. It is possible to introduce the virus efficiently by infecting cells, and only those modified so as to prevent the production of new virus particles are available. Regarding the safety of using a viral vector, there is a concern that the ectopic chromosomal integration of viral DNA could activate an oncogene that hinders the expression of a tumour-suppressor gene or leads to malignant transformation of cells (Glover et al., 2005). The influence of viral vectors on the physiological functions of infected cells should also be minimized. This is particularly important in gene transfer *in vivo*. When a vector is regarded as a foreign invader, an immune response is induced *in vivo* (Nayak and Herzog, 2010).

Table 2. Types and characteristics of virus vectors

Virus system	Size	DNA insert size	Maximum titer (particles / mL)	Infection target	Expression	Disadvantage
Adenovirus	36 kb (dsDNA)	8 kb	1×10^{13}	Mitotic and non-dividing cells	Transient	Induce strong antiviral immune response. There is a limited dose
Retrovirus	7–11 kb (ssRNA)	8 kb	1×10^{9}	Dividing cell	Stable	Possibility of insertion mutation
Lentivirus	8 kb (ssRNA)	9 kb	1×10^{9}	Mitotic and non-dividing cells	Stable	Possibility of insertion mutation
Adeno associated virus	8.5 kb (ssDNA)	5 kb	1×10^{11}	Mitotic and non-dividing cells	Stable	Replication requires a helper virus: Difficult to produce highly pure virus stock
Baculovirus	80–180 kb (dsDNA)	upper limit is unknown	1×10^{8}	Mitotic and non-dividing cells	Transient or stable	Limited mammalian host range
Vaccinia virus	190 kb (dsDNA)	25 kb	1×10^{9}	Dividing cell	Transient	Possibility of cytopathic effect
Herpes simplex virus	150 kb (dsDNA)	30–40 kb	1×10^{9}	Mitotic and non-dividing cells	Transient	No gene is expressed during latent infection

(from “Gibco Cell Culture Basics Handbook” Thermo Fisher Scientific Inc.)

Besides this, few genomic rearrangements are likely to occur, the specificity of infected cell types is sometimes required, and the currently used viral vectors are limited (Table 2).

Physical gene transfer method

When using the physical gene incorporation method, it is necessary to note that two stages of assembling the gene and its target DNA and the task of bring in the package having the function into the cell are necessary. One of the major characteristics is that substances to be introduced into cells are not selected as compared with chemical or biological gene transfer methods in which these two operations are performed at the same time in many cases.

Electroporation is a method in which pores are temporarily formed in a cell membrane using electric pulses, and physical cargo such as nucleic acids are passed through the pores. This is a very efficient strategy for transferring foreign nucleic acids into many types of cells, including bacterial cells and mammalian cells. Electroporation is based on a simple process; the host cells are suspended with selected molecules in a conductive solution with a closed electrical circuit around the mixture. Electric pulses are applied to the cell suspension at optimum voltage for just a few microseconds to milliseconds. This destroys the phospholipid bilayer membrane of the cell membrane that temporarily forms pores. At the same time, the potential difference between the plasma membrane and the cytoplasm increases, and charged molecules like DNA pass through the cell membrane in a manner similar to that in electrophoresis (Shigekawa and Dower, 1988).

A major advantage of electroporation is its applicability to transient and stable transfection of all cell types. Since electroporation is simple and rapid, once optimal electroporation conditions are determined, a large number of transferring work can be performed in a short time. The major disadvantage of electroporation is that high voltage pulses cause a considerable number of cell deaths and cell membranes are only partially repaired so that a greater number of cells are needed compared to that in chemical transfection methods. Optimisation of pulse and electric field strength parameters to balance electroporation efficiency and cell viability is required, but it is also a disadvantage that all optimum conditions are different depending on the type of target cell and the state at that time. Our research aims to clarify one of these conditions.

Incorporation of microparticles by gene guns is also known as particle irradiation and can be performed by shooting heavy metal particles (often gold or tungsten) coated

with nucleic acid under a microscope into a host cell, using a ballistic device (i.e. "gene gun") It is a method to spray particles at high speed. Introduction of microparticles by gene gun can be used for transient transfection into dividing and non-dividing cells and *in vivo* cells in culture, which is frequently used for gene vaccination and agricultural applications (Klein et al., 1992; Sun et al., 1995). While this method is reliable and rapid, it requires expensive equipment, causes physical damage to the sample, and requires many cells because of the high cell death rate.

For direct microinjection, a fine needle is used to deliver the nucleic acid to the cytoplasm or nucleus one cell at a time. Thus, the use of this method is limited to *ex vivo* applications such as introducing genes into oocytes, creating transgenic animals, or delivering artificial chromosomes (Capecchi, 1980; 1989; deJong et al., 1999). The 100% direct incorporation efficiency of direct injection is the biggest advantage of this method, but success probability is extremely labour-dependent owing to the required degree of technical skill and frequent cell death. Thus, this method is not suitable for studies where transfection of a large number of cells is required. However, because it does not require any special equipment, it is also currently used frequently.

Laser-mediated transfection is also known as phototransfection, laser fusion, or opt-poration (Shirahata et al., 2001; Schneckenburger et al., 2002) and uses laser pulses to temporarily permeate the cell membrane. When holes are generated in the cell membrane by laser, penetration of nucleic acids and other desired substances (ions, low molecules, proteins, semiconductor nanocrystals, etc.) into the cells is promoted by the osmotic pressure difference between the medium and the cytosol. The advantage of laser-mediated transfection is its high transfection efficiency and the ability to form pores in every part of the cell. However, this method requires expensive laser microscope systems and cells to adhere to the substrate. It can be said that it is a technique similar to electro-ionization except that the drilling technique is different. However, since processing is performed for each cell, it cannot be advanced to many cells simultaneously.

DNA modification technology

Next, the target DNA must be modified by the substance introduced into the cell. The above retro/lentiviral vector or transposon vector method is a method aiming for accidental integration (random integration). Waiting for an accidental positive result cannot yield high efficiency. Therefore, there has been a growing demand for transferring genes for substance production and therapeutic genes in gene therapy to specific sites. This is made possible by a method using an artificial restriction enzyme that recognizes arbitrary gene sequences on the genome and triggers double strand breakage of DNA as a tool of genome modification, a new genome editing technique called target integration. The discovery of this new genomic editing technology has been the driving force for the increase in genetically engineered research. These discoveries have made it possible to efficiently perform experiments and allow for the rapid increase in the number of genetic recombination experiments in the past few years. These have been standardized as recombinant techniques and established as a highly reproducible technique.

The technique of cleaving DNA at a specific position rapidly developed following the discovery of the site-specific nuclease protein zinc-finger nuclease (ZFN). Transcription activator effector-like nuclease (TALEN) with improved DNA recognition ability was reported in 2010. At this stage, it was necessary to newly prepare a corresponding protein for each target DNA sequence, which took time, expertise, and labour. In 2013, Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/Cas9, which overcame these issues and triggered the explosive use of genome editing technology around the world, was developed. The following describes the features of these methods, especially points that are obstacles to the increase in further applied research.

Zinc-finger nuclease (ZFN) method

The ZFN method was developed around the Zinc Finger Consortium in the US. However, it is thought that the use of ZFN has been limited because it is complicated, expensive, and laborious in the laboratory. In October 2009, a cross license agreement was signed between Sigma Aldrich and Sangamo Bioscience. As a result, Sigma

Aldrich for research use, clinical research ~ production is monopolized by Sangamo Bioscience. In addition, it has the following disadvantages in practical use: owing to its high sensitivity to the chromatin structure, the recombination efficiency tends to be low and the incidence of off-target is large, and there is a possibility that unintended genome editing may occur at a site other than the targeted site. For cultured cells, knock-in efficiencies of SNP alteration and tag insertion are very poor. For animals, the knock-in technique of long-chain DNA sequence (2-3 kb) has not been established yet. It takes a lot of time to construct the vector, and it is technically difficult. A method for efficiently introducing ZFN into cells is necessary.

Transcription activator effector-like nuclease (TALEN) method

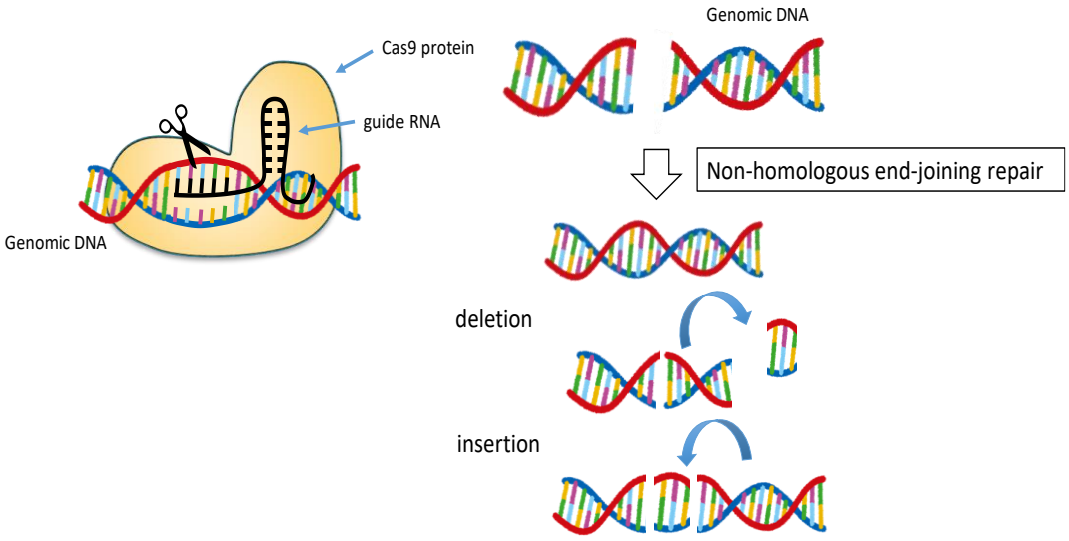
Development of the TALEN method was concluded in June 2014 with a cross license between Thermo Fisher SCIENTIFIC and Cellectis: Thermo Fisher SCIENTIFIC for research use and sublicense rights, and Cellectis for therapeutic applications. There are some problems in using this method, e.g. it is sensitive to chromatin structure. Compared to ZFN and CRISPR, the influence of off-target incorporation is small, but because of off-target incorporation by nonhomologous terminal recombination (NHEJ), it is still necessary to make improvements. For cultured cells, knock-in efficiencies of techniques such as SNP alteration and tag insertion are very poor. For animals, the knock-in technique of long-chain DNA sequence (2-3 kb) is not established. A method for efficiently bring in TALEN into cells is therefore required.

Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/Cas9 method

In 2014, Broad Institute's Feng Zhang, who acquired the basic patent (US 8697359) for the CRISPR/Cas9 method (Fig. 1) founded the medical education Editas Medicine company along with Jennifer Doudna and George Church, a leading researcher of genome analysis with the aim of applying genomic editing technology. The company licenses sales for therapeutic applications. For research use, Takara Bio entered into a license with the Broad Institute, developing it in the form of product and service provision. Horizon Discovery also entered into a license with Broad Institute,

Jennifer Doudna of the University of California at Berkeley, one of the discoverers of the mechanism of CRISPR/Cas9, is at the centre of Caribou Biosciences, and aims to treat human diseases. Her intellectual property is licensed to subsidiary Intellia Therapeutics. At CRISPR Therapeutics, Emmanuelle Charpentier, also responsible for the discovery of the mechanism, and Craig Mello and others who received the Nobel Prize in Physiology Medicine 2006 for the discovery of RNAi aim to investigate the medical applications of genomic editing technology.

Figure 1. Editing a gene using the CRISPR/CAS9 technique



A non-complementary chain for cleavage of the target double-stranded DNA, in addition to complementarity between the guide sequence and the target DNA

A nucleotide sequence (NGG) called PAM (protospacer adjacent motif) is required near DNA cleavage sites. Although it was said that its knockout efficiency is higher than that of ZFN or TALEN, the influence of off-target recombination is also great, but improvement measures such as target selection method, the double nicking method, and methods using short targets for sgRNA have been reported. Since the recognition motif that interacts when the guide RNA and Cas9 nuclease bind is the

same, it is difficult to ensure selectivity for the guide RNA. A method to efficiently introduce CRISPR/Cas9 into cells is necessary. It is also necessary to miniaturize Cas9 (1368 amino acid residues) derived from *S. pyogenes*, which is a huge protein. Problems exist in every technology, but what is commonly referred to in all the methods discussed is that a way to efficiently bring these systems into cells has not yet been found.

As mentioned thus far, experiments to modify genes have rapidly become common. Their aims are varied, ranging from research to elucidate the function of a gene to expressing the functional gene in an organism. Our aim is to produce swine models that eventually develops specific functions. As mentioned above, pigs as laboratory animals have advantages not found in other animal species.

Primarily, it is the experimental use of pig models of cardiovascular disease that is of interest. Similarity is seen in the circulatory systems of pigs and humans, and the size is also close to that of human beings, so pigs can be used for the development of medical equipment. Pigs can also potentially be used for cancer research as an animal that repeats more cell divisions in its lifetime than a mouse. With regard to the development of cancer therapeutic drugs for humans, it includes preferential treatment with existing drugs with clear indications for early and mid-term patients, and the use of new drugs for treatment of patients with end-stage cancer. In this situation, it is difficult to obtain more data and expect prompt results. The general lifespan of a mouse is two years, but that of a pig is less than 20 years. Additionally, pigs are characterised by multiple generations and it is possible to use animals with genetically characterised traits. By taking advantage of this feature, it will be possible to study and proceed with the development of more drastic treatments for early and mid-term cancers. Longer life expectancy is also an important characteristic for studying side effects and is expected to help develop effective medicines faster.

In addition, in order to produce edible pigs, SPF conversion progresses, and it is possible to utilize the breeding know-how for development of pigs as experimental animals. In the case of rearing a gene so as to produce a specific disease, it is easy to use animals in which breeding techniques are generalised. From the above, it is

assumed that the number of pigs used in the laboratory animal market will expand over a long period.

In the genetic modification technology, there are major problems when creating specific disease models. Depending on the targeted disease, there are things that cannot be created. In the case of diseases of the blood system, it is difficult to give birth safely in case of artificial pregnancy with a fertilized egg that has been genetically modified. Even when giving birth, it is often difficult to grow. Therefore, it is difficult to genetically tap the trait of interest, and gene transfer to fertilized eggs should be repeated as many times as necessary as. Therefore, a technique to reliably introduce genes into many fertilized eggs is required. In addition, in order to obtain many experimental bodies at the same time, it is necessary to complete the operation simultaneously on a large number of fertilized eggs.

We selected CRISPR/CAS9 and electroporation as techniques to introduce the same trait to more fertilized eggs. In this study, we aimed to search for and establish more stable gene incorporation conditions in porcine fertilized eggs by combining these two techniques. Specially, we clarified suitable conditions of electroporation for generation of modified pigs using the CRISPR/Cas9 system and investigated the meiotic stage of porcine oocytes that had the most sensitivity to hyperthermia.

CHAPTER 2.

EFFECTS OF VOLTAGE STRENGTH DURING ELECTROPORATION ON THE DEVELOPMENT AND QUALITY OF *IN VITRO*-PRODUCED PORCINE EMBRYOS

INTRODUCTION

The recently developed clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) 9 system has enabled high-efficiency genome modification in animal cells/embryos including site-specific modifications and gene knock-ins and knockouts (Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013). Using CRISPR/Cas9, efficient gene targeting has been achieved in mice, rats, and monkeys via the co-injection of zygotes with Cas9 mRNA and single-guide RNA (Ma et al., 2014; Niu et al., 2014; Yasue et al., 2014), and this strategy has been applied to gene targeting in pigs (Wang et al., 2015). However, the microinjection of CRISPR/Cas9 system into zygotes requires a high level of skill, is time-consuming and may cause damage to embryos. Thus, the widespread production of gene-modified pigs may remain limited due to the use of micromanipulator systems for the microinjection of endonucleases into the cytoplasm of zygotes (Fan and Lai, 2013). Recently, we established the GEEP (gene editing by electroporation of Cas9 protein) method (Tanihara et al., 2016), a method in which the CRISPR/Cas9 system is introduced into porcine zygotes by electroporation, which leads to high-efficiency disruption of the targeted gene. Previous studies have also reported the generation of knockout animals (mice and rats) by introducing the CRISPR/Cas9 system into intact zygotes using a similar electroporation method (Kaneko et al., 2014; Hashimoto and Takemoto, 2015; Kaneko and Mashimo, 2015). Kaneko and Mashimo (Kaneko and Mashimo, 2015) have suggested that the pulse polarity affects the success rate of transferring mRNA into intact zygotes. In a previous study, we demonstrated that when the presumptive zygotes were electroporated with Cas9 mRNA and single-guide RNA (sgRNA) targeting the *FGF10* gene, the frequency of base insertions or deletions (indels) in the targeted gene and blastocyst formation rates were influenced by electroporation conditions such as duration and number of pulses (Tanihara et al., 2016). However, information on the conditions suitable for the introduction of the CRISPR/Cas9 system into intact embryos of pigs and other species by electroporation is limited.

To clarify suitable conditions for electroporation, we investigated the effects of

pulse polarity and voltage on the development and quality of *in vitro*-produced porcine embryos. We then confirmed whether the selected conditions could be used to edit the *FGF10* gene in porcine embryos.

MATERIALS AND METHODS

There were no live animals used in this study, so no ethical approval was required.

Oocyte collection, *in vitro* maturation and fertilization

Pig ovaries were obtained from prepubertal crossbred gilts (Landrace × Large White × Duroc breeds) at a local slaughterhouse. Cumulus-oocyte complexes (COCs) with a uniform ooplasm and compact cumulus cell mass were collected from follicles 2–6 mm in diameter; the COCs were cultured in maturation medium at 39 °C in a humidified incubator containing 5% CO_2 as described previously, with minor modifications (Do et al., 2015). The maturation medium consisted of 25 mM HEPES tissue culture medium 199 with Earle's salts (TCM 199; 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 sodium pyruvate (Sigma-Aldrich), 2 mg/ml D-sorbitol (Wako Pure Chemical Industries Ltd., Osaka, Japan), 1 μg/ml 17 β-estradiol (Sigma-Aldrich), 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). After maturation for 20–22 h, the COCs were cultured for 24 h in maturation medium without hormones.

The matured oocytes were subjected to *in vitro* fertilization (IVF), as described previously (Do et al., 2015). Briefly, frozen-thawed spermatozoa were transferred into 6 ml of porcine fertilization medium (PFM; Research Institute for the Functional Peptides Co., Yamagata, Japan) and washed by centrifuging at 500 × *g* for 5 min. The pelleted spermatozoa were resuspended in PFM and adjusted to 5×10^6 cells/ml. Next, COCs were transferred to the sperm-containing PFM and co-incubated for 12 h at 39 °C under 5% CO_2 and 5% O_2. After co-incubation, the putative zygotes were denuded from the cumulus cells and the attached spermatozoa by mechanical pipetting.

Preparation of sgRNA targeting *FGF10*, and *Cas9* mRNA

We introduced Cas9 mRNA and sgRNA targeting *Fgf10*, which was previously transferred into eggs by the electropolation method (Tanihara et al., 2016) and elicited the limbless phenotype (Hashimoto and Takemoto, 2015). pDR274 plasmids carrying target sequences were constructed by inserting annealed oligos into the BsaI site. The oligos (Fwd: 5′-TAGGAAAAGGAGCTCCCAGGAG-3′; and Rev: 5′-AAACCTCCTGGGAGCTCCTTTT -3′) were purchased from Sigma-Aldrich. After DraI digestion, sgRNAs were synthesized using the MEGAshortscript T7 Transcription Kit (Ambion, Austin, TX, USA) and then purified by phenol-chloroform-isoamylalcohol extraction and isopropanol precipitation. The precipitated RNA was dissolved in Opti-MEM I (Life Technologies, Gaithersburg, MD, USA). The RNAs were quantified by absorption spectroscopy and agarose gel electrophoresis and were stored at -30˚C until use. *Cas9* mRNA was prepared as described previously (Hashimoto and Takemoto, 2015).

Electroporation and embryo culture

Electroporation was performed 13 h after the initiation of IVF as described previously (Tanihara et al., 2016). Briefly, an electrode (LF501PT1-20; BEX, Tokyo, Japan) was connected to a CUY21EDIT II electroporator (BEX) and placed under a stereoscopic microscope. The putative zygotes (approximately 30 – 40 zygotes) were washed with Opti-MEM I solution and placed in a line in the electrode gap, in a chamber slide filled with 10 μl of Opti-MEM I solution with or without sgRNA and Cas9 mRNA. After electroporation, the zygotes were washed with pig zygote medium (PZM-5; Research Institute for the Functional Peptides Co.) and cultured for 3 days. Embryos cultured for 3 days were subsequently incubated in porcine blastocyst medium (PBM; Research Institute for the Functional Peptides Co.) for 4 days. As a control, some zygotes were cultured with PZM-5 and PBM for 7 days without performing electroporation.

Assessment of blastocyst quality

To evaluate the total cell number and existence of apoptosis in the blastocysts, the blastocysts were fixed on day 7 (day 0; insemination) and were analysed using a

combined technique for simultaneous nuclear staining and terminal deoxynucleotidyl transferase nick-end labelling (TUNEL) modified from previously described procedures (Otoi et al., 1999). Briefly, blastocysts were fixed overnight at 4°C in 3.7% (w/v) paraformaldehyde diluted in PBS. After fixation, the blastocysts were permeabilized in PBS containing 0.1% (v/v) Triton-X100 for 40 min. The blastocysts were subsequently incubated overnight at 4°C in PBS containing 10 mg/ml bovine serum albumin (blocking solution) and then incubated in fluorescein-conjugated 2-deoxyuridine 5-triphosphate and terminal deoxynucleotidyl transferase (TUNEL reagent; Roche Diagnostics Co., Tokyo, Japan) for 1 h at 38.5°C. After TUNEL staining, the embryos were counterstained with 1 μg/ml DAPI (Invitrogen Co., Carlsbad, CA, USA) for 10 min and then treated with an anti-bleaching solution (Slow-Fade; Molecular Probes Inc., Eugene, OR, USA), mounted on glass slides and sealed with clear nail polish. Labelled blastocysts were examined using an epifluorescence microscope (Eclipse 80i, Nikon, Tokyo, Japan). Apoptotic nuclei exhibited condensed and fragmented morphology (Brison and Schultz, 1997). The apoptotic index was calculated by dividing the number of cells containing apoptotic nuclei (labelled by TUNEL) by the total number of cells.

Analysis of targeted genes after electroporation

Genomic DNA was isolated by boiling individual blastocysts in 50 mM NaOH solution. After neutralization, the genomic regions flanking the sgRNA target sequences were PCR-amplified using specific primers (Fwd: 5′-CCATCCCATTTGATCTGCTT-3′; and Rev: 5′-CTTCAACTGGCAGCACAATG-3′). The PCR products were extracted using agarose-gel electrophoresis and the targeted genomic regions were sequenced. Sequencing was performed using a BigDye Terminator Cycle Sequencing Kit ver. 3.1 (Thermo Fisher Scientific, Waltham, MA, USA) and an ABI 3500 Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

Experimental design

In the first experiment, we examined the effect of pulse polarity with unipolar and bipolar pulses on the development of porcine embryos. Putative zygotes were placed

in Opti-MEM I solution without sgRNA and Cas9 mRNA and were electroporated by either unipolar or bipolar pulses, keeping the voltage, pulse duration and pulse number fixed at 30 V/mm, 1 msec and five repeats, respectively.

In the second experiment, we tested the effect of electroporation voltages on the development and quality of porcine embryos. In the first experiment, the unipolar pulse was better than the bipolar pulse for the development of embryos. Thus, putative zygotes were electroporated in Opti-MEM I solution without sgRNA and Cas9 mRNA by electroporation voltages ranging from 20 V/mm – 40 V/mm with five 1-msec unipolar pulses.

The electroporation voltage found to be most suitable for the development and quality of embryos in the second experiment was 25 V/mm, but the frequency of base insertions or deletions (indels) in the target gene after introducing the CRISPR-Cas9 system into zygotes remained unclear. In the third experiment, we used two electroporation voltages (25 V/mm and 30 V/mm) to compare the efficiency of genome editing in porcine zygotes. The putative zygotes were electroporated with 400 ng/μl of *Cas9* mRNA and 200 ng/μl of sgRNA targeting the *FGF10* gene (Sekine et al., 1999) by electroporation at 25 V/mm and 30 V/mm with five 1-msec unipolar pulses. The electroporated zygotes were cultured for 7 days until blastocyst formation. The frequencies of base insertions or deletions (indels) in the *FGF10* gene of individual blastocysts derived from zygotes electroporated at 25 V/mm (26 embryos) and 30 V/mm (28 embryos) were analysed.

Statistical analysis

Statistical significance was inferred from analysis of variance (ANOVA) tests followed by Fisher's protected least significant difference (PLSD) tests using STATVIEW (Abacus Concepts, Inc., Berkeley, CA, USA). All percentage data were subjected to arcsin transformation before statistical analysis. Differences with a probability value (p) of 0.05 or less were regarded as significant.

RESULTS

As shown in Fig. 2, when putative zygotes were electroporated by either unipolar or bipolar pulses, cleavage rates did not differ among the groups. However, the rate of blastocyst formation from zygotes electroporated by bipolar pulses was significantly lower ($p < 0.05$) than from zygotes electroporated by unipolar pulses. The rates of blastocyst formation from electroporated zygotes decreased compared with control zygotes cultured without electroporation, irrespective of pulse polarity.

As shown in Table 3, when putative zygotes were electroporated by electroporation voltages ranging from 20 V/mm – 40 V/mm, the rate of cleavage and blastocyst formation of zygotes electroporated at 40 V/mm was significantly lower ($p < 0.05$) than that of zygotes electroporated at less than 30 V/mm. Moreover, the apoptotic nuclei indices of embryos derived from zygotes electroporated at voltages greater than 30 V/mm significantly increased compared with those from zygotes electroporated at voltages less than 25 V/mm ($p < 0.05$). The apoptotic nuclei indices of embryos from electroporated zygotes increased compared with embryos from control zygotes cultured without electroporation, irrespective of the voltage used.

When putative zygotes were electroporated with Cas9 mRNA and sgRNA targeting site in *FGF10* exon 3, the proportions of blastocysts with targeted genomic sequences were 7.7% (2/26) and 3.6% (1/28) in embryos derived from zygotes electroporated at 25 V/mm and 30 V/mm, respectively. All mutated blastocysts (3 embryos) carried wild-type sequences at variable ratios (Fig. 3).

DISCUSSION

Electroporation, which permeabilizes the plasma membrane with an electric pulse, can deliver exogenous molecules into cells (Mir, 2001). However, the method has been limited by low gene transfer efficiency compared with viruses and their transient gene expression (Nishikawa and Huang, 2001). As a function of the field strength and duration, the permeabilization of the cell membrane can be reversible or irreversible. Irreversible electroporation occurs if the cell cannot recover from the membrane disruption (Davalos et al., 2005). Therefore, the high levels of cell damage incurred by

electroporation must be considered to obtain successful transfection efficiency. Changing pulse polarity may increase permeabilized membrane area and, consequently, increase gene expression (Faurie et al., 2004). Tekle et al. (Tekle et al., 1991) reported that the efficiency of DNA transfection *in vitro* was significantly higher when using bipolar pulses than when using unipolar pulses. A bipolar pulse is a sequence of two

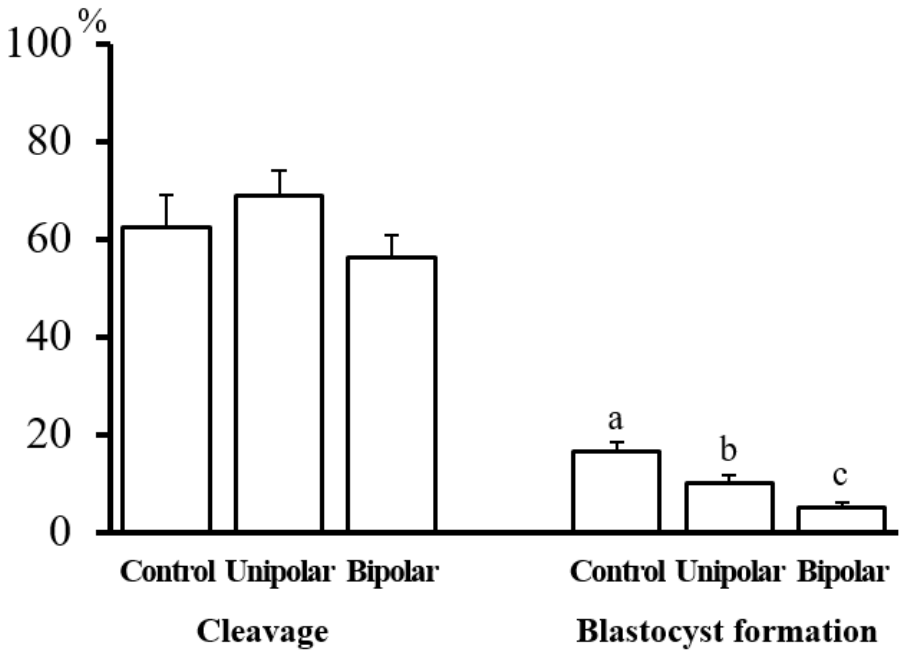


Figure 2. Efficiency of unipolar and bipolar rectangular electroporation pulses on the development of porcine embryos. Zygotes were electroporated using unipolar (549 zygotes) and bipolar (520 zygotes) pulses, using five 1-msec pulses at 30 V/mm. As a control, a set of zygotes (531 zygotes) was cultured without electroporation. Eleven replicates were analysed per treatment group. [a-c]Bars with different letters differ significantly ($p < 0.05$).

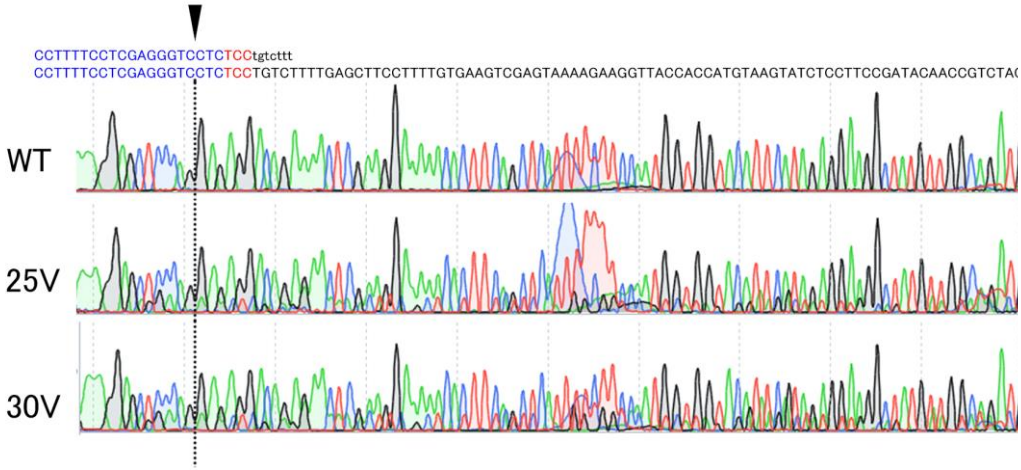


Figure 3. Representative genomic sequences of porcine blastocysts formed after zygote electroporation with Cas9 mRNA and *FGF10* sgRNA at 25 V/mm and 30 V/mm showing wild type (WT) and mutated type (25V and 30V). There were more than two peaks in the mutated embryos, indicating that *FGF10* mutation (deletion or/and insertion) had occurred. The arrowhead indicates the Cas9 cleavage site.

Table 3. Effects of voltage strength on the development and quality of embryos electroporated after in vitro fertilization*

Voltage strength (V/mm) **	No. of oocytes examined	No. (%) of embryos		Total cell number in blastocyst	Apoptotic nucleus index***
		cleaved	developed to blastocysts		
Control	125	108 (86.4 ± 1.6)^{a,b}	33 (26.4 ± 2.8)^{a}	51.1 ± 2.7^{a}	3.0 ± 0.4^{a}
20	115	105 (91.4 ± 2.1)^{a}	24 (21.0 ± 5.4)^{a,b}	47.5 ± 4.6^{a}	6.6 ± 0.6^{b}
25	121	108 (89.4 ± 2.3)^{a}	31 (25.8 ± 6.3)^{a,b}	49.4 ± 4.5^{a}	6.4 ± 0.6^{b}
				46.7 ± 3.7^{a}	10.6 ± 1.1^{c}
30	127	103 (82.4 ± 7.2)^{a,b}	21 (17.0 ± 3.8)^{a,b}	33.4 ± 2.5^{b}	16.1 ± 1.8^{d}
35	118	83 (70.2 ± 6.0)^{b,c}	17 (14.3 ± 2.2)^{b,c}	36.4 ± 3.4^{a,b}	16.9 ± 1.3^{d}
40	121	74 (61.1 ± 9.3)^{c}	5 (4.1 ± 1.6)^{c}		

*Four replicate trials were carried out. Data are expressed as mean ± SEM.

**Electroporation was performed by five 1-ms pulses at various voltages. As control, the zygotes were cultured without performing electroporation.

***The apoptotic index was defined as the ratio of the number of cells containing apoptotic nucleus and the total number of cells in a blastocyst.

$^{a-d}$ Values with different superscripts in the same column are significantly different ($P < 0.01$).

consecutive, oppositely polarized unipolar pulses. If the survival rate of embryos electroporated by bipolar pulses is similar to the survival rate of embryos electroporated by unipolar pulses, permeabilization could be expected to achieve increased molecular uptake. In the present study, however, we found that the development of zygotes electroporated by bipolar pulses decreased compared with those electroporated by unipolar pulses. Kotnik et al. (Kotnik et al., 2001) reported that pulse strengths were lower when using bipolar pulses. Under our conditions, the same voltage, pulse duration and pulse number were used for electroporation. Therefore, the decreased development of zygotes electroporated by bipolar pulses might result from damage induced by higher pulse strengths.

While high levels of gene expression are required following transfection by electroporation, it is also desirable to minimize damage to embryos. Therefore, optimizing electroporation conditions have become a key factor affecting the development and quality of embryos. In a previous study, we reported that the frequency of indels increased with increasing pulse duration and number, whereas blastocyst formation rates markedly decreased (Tanihara et al., 2016). Moreover, the optimal duration and number of pulses were 1-ms and five, respectively, for introducing *Cas9* mRNA and sgRNA into pig IVF zygotes. However, the optimal voltage to use for electroporation has remained unclear. In the present study, zygotes were electroporated using electroporation voltages ranging from 20 V/mm – 40 V/mm with five 1-msec unipolar pulses. We found that 25 V/mm was most suitable for embryo development and quality, although the apoptotic nuclei indices of electroporated embryos were higher than those of control embryos. Moreover, when zygotes were electroporated with Cas9 mRNA and sgRNA targeting site in *FGF10* exon 3 at 25 V/mm and 30 V/mm, 25 V/mm resulted in a high genome editing efficiency in the resulting blastocysts. Although we succeeded in introducing indels into the porcine embryos, the mutation frequency was lower than that observed in mice (Hashimoto and Takemoto, 2015). Our results showed that all mutated blastocysts carried wild-type sequences. These results indicate that electroporation at 25 V/mm may be an acceptable condition for introducing *Cas9* mRNA and sgRNA into pig IVF zygotes, but further studies are necessary to achieve higher genome editing in porcine embryos using the electroporation.

In conclusion, our results demonstrate that bipolar pulses have a detrimental effect on the development of zygotes electroporated under our study conditions. Moreover, when using five 1-msec unipolar pulses, electroporation at 25 V/mm is suitable for introducing the CRISPR/Cas9 system into pig IVF zygotes.

CHAPTER 3.

SENSITIVITY OF THE MEIOTIC STAGE TO HYPERTHERMIA DURING *IN VITRO* MATURATION OF PORCINE OOCYTES

INTRODUCTION

Heat stress (HS) can compromise reproductive events by decreasing the expression of oestrous behaviour, altering follicular development, compromising oocyte competence, and inhibiting embryonic development (Wolfenson et al., 2000; Hansen et al., 2001). HS disrupts the synthesis of the steroid hormone involved in the regulating mechanism of oocyte maturation. Moreover, HS during maturation has been suggested to alter both nuclear and cytoskeletal configurations in oocytes, reduce developmental competence, and increase oocyte apoptosis (Ju and Tseng, 2004; Roth and Hansen, 2004). In a previous study, we demonstrated that the exposure of porcine oocytes at the germinal vesicle stage to an elevated temperature (41 °C) causes a reduction in their maturation rate and increases the proportion of oocytes with DNA-fragmented nuclei. Hypothermia-mediated DNA damage to the cumulus cells surrounding the oocyte during maturation reduces the porcine oocyte quality, resulting in failure of meiotic maturation (Yuan et al., 2008). The deleterious effects of hyperthermia on porcine oocytes are potentially irreversible, even if the oocytes are returned to normal culture conditions (Ju and Tseng, 2004). However, the meiotic stage of oocytes, during maturation, with the most sensitivity to hyperthermia remains unclear.

The objective of this study was to clarify the meiotic stage of porcine oocytes that has the most sensitivity to hyperthermia by assessing the meiotic maturation and DNA damage of oocytes exposed to an elevated temperature (41 °C) for 12 h at various intervals during maturation culture.

MATERIALS AND METHODS

Exposure to an elevated temperature and in vitro maturation (IVM) of oocytes

Porcine ovaries were obtained from prepubertal cross-bred gilts (Landrace, Large White and Duroc breeds) at a slaughterhouse for an April-June 2014 and transported to the laboratory within 3 h in physiological saline (0.9% (w/v) NaCl) at 30 °C. The ovaries were washed three times with modified phosphate-buffered saline (m-PBS; Nihonzenyaku, Fukushima, Japan) that was supplemented with 100 IU/ml penicillin

G potassium (Meiji, Tokyo, Japan) and 0.1 mg/ml streptomycin sulfate (Meiji). The cumulus–oocyte complexes (COCs) were collected from 3-6-mm follicles using a surgical blade. Only COCs with a uniform, dark-pigmented ooplasm and an intact cumulus cell mass were collected. Approximately 50 COCs were then cultured in 500 μl of maturation medium consisting of 25 mM HEPES tissue culture medium 199 with Earle's salts (TCM 199; Invitrogen Co., Carlsbad, CA, USA) that was supplemented with 10% (v/v) porcine follicular fluid, 0.6 mM cysteine (Sigma-Aldrich, St. Louis, MO, USA), 50 μM sodium pyruvate (Sigma-Aldrich), 2 mg/ml D-sorbitol (Wako Pure Chemical Industries Ltd., Osaka, Japan), 1 μg/ml 17 β-estradiol (Sigma-Aldrich), 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) for 24 h in 4-well dishes (Nunc A/S, Roskilde, Denmark). Subsequently, the COCs were transferred to maturation medium without hormone supplementation and cultured for an additional 24 h according to the method previously described by Namula et al. (Namula et al., 2013).

Analysis of the meiotic stage and DNA damage of oocytes

After maturation culture, the meiotic stage and DNA damage of oocytes were analysed with a combined technique for simultaneous nuclear staining and terminal deoxynucleotidyl transferase (TdT) nick-end labelling (TUNEL) by a modification of the procedures previously described by Otoi et al. (Otoi et al., 1999). Briefly, oocytes were mechanically denuded from cumulus cells in Dulbecco's PBS (DPBS; Invitrogen Co) that was supplemented with 1 mg/mL hyaluronidase (Sigma). Denuded oocytes were fixed overnight at 4°C in 3.7% (w/v) paraformaldehyde diluted in DPBS. After fixation, the oocytes were permeabilized in DPBS containing 0.1% (v/v) Triton-X100 for 40 min. They were subsequently incubated overnight at 4°C in DPBS containing 10 mg/ml bovine serum albumin (A9647, Sigma-Aldrich). The oocytes were then incubated in fluorescein-conjugated 2′-deoxyuridine-5′-triphosphate and terminal deoxynucleotidyl transferase (TUNEL reagent; Roche Diagnostics, Tokyo, Japan) for 1 h at 38.5°C. After TUNEL staining, the oocytes were counterstained with 1 μg/ml DAPI (Invitrogen Co.) for 10 min. Then, they were treated with an anti-

bleaching solution (Slow-Fade; Molecular Probes Inc., Eugene, OR, USA), mounted on a glass slide, and sealed with clear nail polish. Labelled oocytes were examined using a microscope (Eclipse 80i, Nikon, Tokyo, Japan) with epifluorescence illumination. They were classified according to chromatin configuration as being in the germinal vesicle (GV), condensed chromatin (CC), metaphase I (MI), anaphase I to telophase I (AT), or metaphase II (MII) stage. Those with diffusely stained cytoplasm characteristics of nonviable cells and those in which chromatin were unidentifiable or not visible were excluded from DNA damage analysis.

To assess the meiotic stage of oocytes cultured at 38.5 °C for each period, oocytes were fixed and permeabilized in DPBS containing 3.7% (w/v) paraformaldehyde and 1% (v/v) Triton X-100 (Sigma-Aldrich) at room temperature for 15 min. They were then incubated in DPBS containing 0.3% (w/v) polyvinylpyrrolidone at room temperature for another 15 min. The oocytes were placed in a drop of mounting medium consisting of 90% (v/v) glycerol with 1.9 μM Hoechst 33342 (Sigma-Aldrich) on a slide, covered with a cover slip supported by four droplets of Vaseline/paraffin, incubated overnight at 4 °C and examined under a fluorescence microscope. The meiotic stage of oocytes was classified as described above.

Experiment 1

To assess the sensitivity of the porcine oocyte meiotic stage to hyperthermia, the COCs were randomly assigned to five treatment groups and then cultured in maturation medium at 41 °C for 12 h in each period during maturation culture. The COC incubations were performed in a 38.5 °C humidified incubator containing 5% CO_2 with an exposure period of 41 °C. After 48 h of IVM culture, the oocytes were fixed and stained to examine the nuclear status and DNA damage of oocytes exposed to 41 °C.

Experiment 2

In Experiment 1, the sensitivity of porcine oocytes exposed to hyperthermia from 12 h to 24 h after the start of maturation culture was higher than the other exposed groups. Therefore, the meiotic stages of oocytes cultured in a 38.5 °C humidified

incubator containing 5% CO_2 for each period during 48 h of maturation culture and between 12 and 24 h were examined.

Statistical analysis

The data are expressed as the means ± SEMs. The proportions of oocytes reaching each stage and oocytes with DNA-fragmented nuclei were subjected to arc sin transformation before performing an analysis of variance (ANOVA). The transformed data were tested by ANOVA, which was followed by the post hoc Fisher's protected least significant difference test (PLSD test) using the Statview program (Abacus Concepts, Inc., Berkeley, CA, USA). Differences at a probability value (P) of 0.05 or less were considered significant.

RESULTS

Experiment 1

As shown in Table 4, when the oocytes were exposed to 41 °C from 12 to 24 h, the proportions of oocytes that remained at MI increased and of oocytes reaching MII decreased compared with control oocytes cultured at 38.5 °C ($P < 0.05$). Moreover, the proportions of DNA fragmentation in the total oocytes exposed to 41 °C at each culture period after 12 h from the start of maturation culture were significantly higher ($P < 0.05$) than those of control oocytes (Fig. 4). The proportions of MII-stage oocytes with DNA-fragmented nuclei tended to be higher in oocytes exposed to 41 °C after 12 h of maturation culture than the control oocytes ($P < 0.1$).

Experiment 2

As shown in Fig. 5A, when the meiotic stages of the oocytes were examined at various intervals during maturation culture, the proportions of oocytes remaining at the GV stage dramatically decreased from 74.8% to 22.5% between 12 and 24 h after the start of maturation culture. The proportions of oocytes at the CC and MI stages increased at 24 h of maturation culture. The proportion (47.5%) of CC-stage oocytes reached a maximum at 20 h, and approximately half (44.9%) of the oocytes reached MI at 24 h (Fig. 5B).

Table 4. Meiotic maturation of porcine oocytes exposed to 41 °C during in vitro maturation*

Exposure period	No. of oocytes examined	No. (%) of oocytes with**					No. (%) of unidentifiable oocytes
		GV	CC	MI	AT	MII	
Control	133	3 (2.0 ± 2.0)	8 (6.0 ± 3.2)	20 (15.1 ± 4.1)[a]	2 (1.5 ± 1.0)	95 (72.2 ± 3.6)[a]	5 (3.3 ± 1.9)[a, b]
0 h – 12 h	129	2 (1.8 ± 1.8)	4 (3.2 ± 1.5)	41 (30.8 ± 7.8)[b]	4 (3.4 ± 2.2)	76 (59.3 ± 7.1)[a, b]	2 (1.5 ± 1.0)[a]
12 h – 24 h	128	5 (4.3 ± 2.0)	5 (3.6 ± 1.3)	38 (28.8 ± 2.7)[b]	5 (4.1 ± 2.1)	66 (51.7 ± 4.3)[b]	9 (7.6 ± 2.9)[b]
24 h – 36 h	132	2 (1.3 ± 1.3)	9 (6.2 ± 2.2)	24 (19.1 ± 3.1)[a, b]	2 (2.0 ± 2.0)	86 (64.8 ± 2.2)[a, b]	9 (6.7 ± 2.2)[a, b]
36 h – 48 h	134	4 (3.2 ± 1.6)	3 (2.0 ± 1.4)	28 (20.9 ± 2.9)[a, b]	3 (2.5 ± 1.7)	92 (68.4 ± 4.2)[a]	4 (3.0 ± 1.6)[a, b]

*All experiments were repeated 6 times. Data are expressed as the mean ± SEM.

**GV, germinal vesicle; CC, condensed chromatin; MI, metaphase I; AT, anaphase I to telophase I; and MII, metaphase II.

[a-b] The values with different superscript letters in the same column are significantly different ($P < 0.05$).

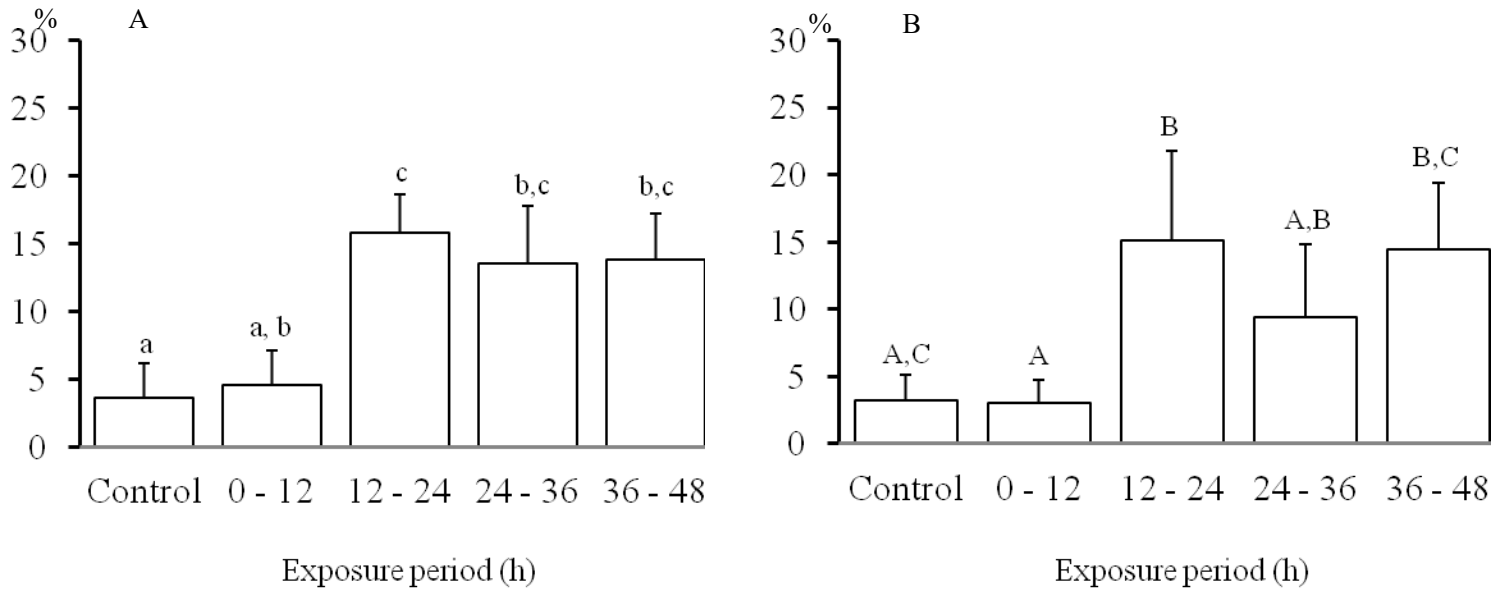


Figure 4. Effects of porcine oocyte exposure to 41 °C for 12 h during maturation culture on the proportions of total (A) and metaphase II (B) oocytes with DNA-fragmented nuclei. Control oocytes were cultured for 48 h without exposure to 41 °C. Proportions were calculated by dividing the number of oocytes with DNA-fragmented nuclei by the total number of oocytes examined and metaphase II oocytes. Each bar represents the mean ± SEM. Bars with different letters differ significantly (a-c; $P < 0.05$, A-C; $P < 0.1$).

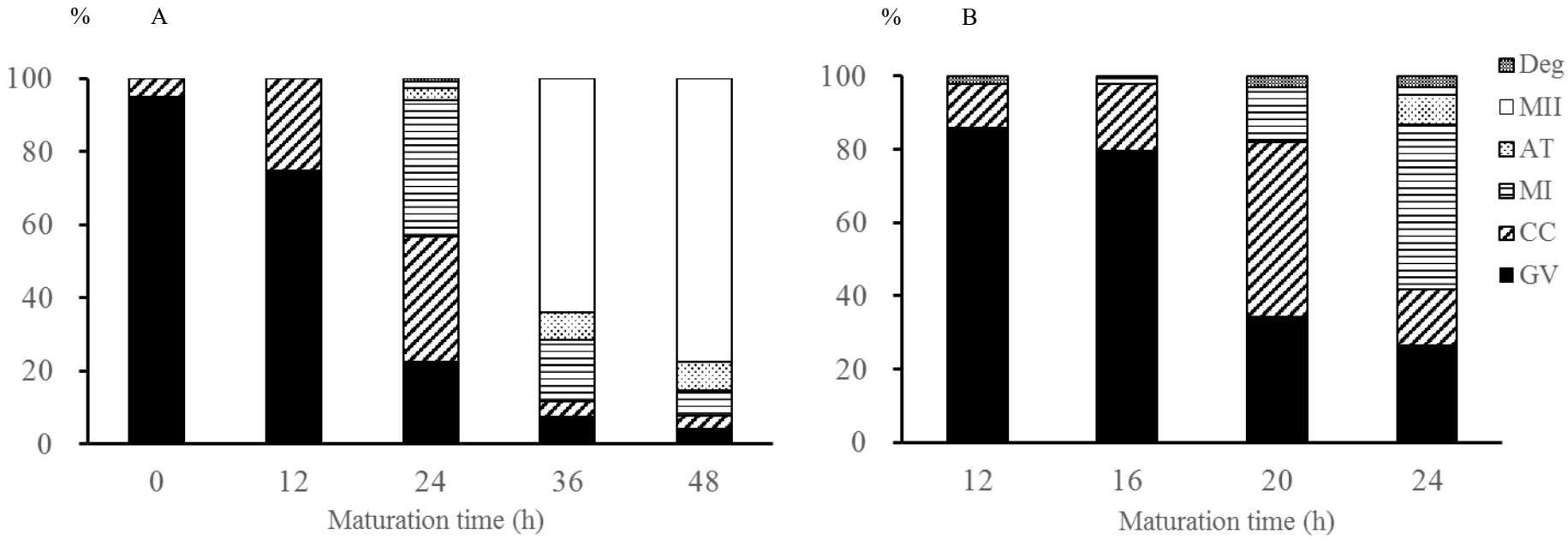


Figure 5. Meiotic stage of porcine oocytes cultured for each time period during 48 h (A) and between 12 and 24 h (B) of maturation culture. All oocytes cultured at 38.5 °C for each time period during 48 h (116 -120 oocytes) and between 12 and 24 h (98 - 99 oocytes) were used to estimate the meiotic stage.

DISCUSSION

Our previous study demonstrated that when porcine oocytes were exposed to 41.0 °C for the entire period of maturation culture, their meiotic competence decreased, but the oocytes could mature and develop to the blastocyst stage after fertilization (Do et al., 2015). In the present study, porcine oocytes were exposed to 41.0 °C for 12 h at each period of maturation culture to clarify the meiotic stage of porcine oocytes that had the most sensitivity to hyperthermia. We confirmed that the exposure of porcine oocytes to 41 °C for 12 h decreased the meiotic competence of oocytes and increased the DNA damage of total and MII-stage oocytes. Moreover, porcine oocytes cultured from 12 to 24 h after the start of maturation culture had a higher sensitivity to the elevated temperature.

The cooling of mammalian oocytes to sub-physiological temperatures is well known to affect their viability through inducing various abnormalities at all stages of meiosis (Moor and Crosby, 1985; Heyman et al., 1986; Pickering et al., 1990; Aman and Parks, 1994). In particular, porcine oocytes at the GV stage have been demonstrated to have a high sensitivity to chilling (Didion et al., 1990). Similarly, heat stress during porcine oocyte maturation has been shown to retard the nuclear maturation of oocytes, resulting in the poor oocyte quality and low potency of their development (Tseng et al., 2006; Yuan et al., 2008). Our previous study demonstrated that exposure of porcine oocytes at the GV stage to 41 °C for 1 h reduced their maturation rate and increased the proportion of oocytes with DNA-fragmented nuclei (Barati et al., 2008). Yuan et al. (Yuan et al., 2008) also reported that the maturation rates of oocytes at the germinal vesicle breakdown (GVBD) stage decreased with exposure to 42 °C for 1 h. It has been suggested that abnormalities in the chromosomes, spindle microtubules, and pericytoplasmic microtubules of porcine oocytes occurred when the oocytes were exposed to an elevated temperature for even a short time (Ju and Tseng, 2004). Moreover, heat shock during oocyte maturation has been shown to promote an apoptotic response that is mediated by group II caspases, which are responsible for destruction of structural and regulatory proteins that leads to DNA damage and cell demise (Chang and Yang, 2000; Roth and Hansen, 2004). Activation of the apoptotic processes mediated by the group II caspases is a critical mechanism

that is responsible for disrupting the oocyte capacity to cleave and further develop (Roth and Hansen, 2004). Although the detrimental effects of heat shock on the meiotic competence and quality of oocytes has been demonstrated, the meiotic stage of oocytes with high sensitivity against hyperthermia has remained unclear. In the current paper, we clearly showed that the detrimental effects of hyperthermia become more apparent for the maturation rates and DNA damage of oocytes that were exposed to 41.0 °C between 12 and 24 h after the start of maturation culture. At that time, oocytes resumed meiosis from the GV stage, and the majority of the oocytes reached the CC- or MI-stage after 24 h in culture. In the detailed analysis of the meiotic stage of oocytes between 12 and 24 h in culture, GVBD started in the majority of oocytes after 20 h in culture. These results were similar to the experiment by Nobata et al. (Nobata et al., 2013), who reported that porcine oocytes remained at the GV stage after 12 h of maturation culture and GVBD started after 18 h. Moreover, we observed that the proportion of oocytes at the GVBD stage reached maximum at 20 h, and approximately half of oocytes reached the MI stage at 24 h. Therefore, our results indicate that the transition period to the MI stage from the GV stage has higher sensitivity to the elevated temperature.

In summary, the results of the present study demonstrate that porcine oocytes cultured from 12 to 24 h after the start of maturation culture had a higher sensitivity to hyperthermia, and their meiotic stages were from the GV to MI stage.

CHAPTER 4. SUMMARY

Recently, the value of pigs as laboratory animals has become widely recognized. Genetically modified pigs are generated by preparation of genetically modified embryos and subsequent implantation of modified embryos to surrogate pigs. Electroporation is the technique of choice to introduce an exogenous gene into embryos for transgenic animal production. On the other hand, heat stress has been demonstrated to significantly reduce conception rates in gilts exposed to higher ambient temperature during the early period of implantation. The present studies were conducted to clarify suitable conditions of electroporation for the generation of modified pigs using the CRISPR/Cas9 system and to investigate the meiotic stage of porcine oocytes that has the most sensitivity to hyperthermia.

In the first experiment, we aimed to determine suitable conditions for an experimental method in which the CRISPR/Cas9 system is introduced into *in vitro*-produced porcine zygotes by electroporation. When putative zygotes derived from *in vitro* fertilization (IVF) were electroporated by either unipolar or bipolar pulses (keeping the voltage, pulse duration, and pulse number fixed at 30 V/mm, 1 msec, and five repeats, respectively), the rate of blastocyst formation from zygotes electroporated by bipolar pulses decreased compared to that for zygotes electroporated by unipolar pulses. When the putative zygotes were electroporated by electroporation voltages ranging from 20 V/mm – 40 V/mm with five 1-msec unipolar pulses, the rates of cleavage and blastocyst formation of zygotes electroporated at 40 V/mm were significantly lower ($p < 0.05$) than those of zygotes electroporated at less than 30 V/mm. Moreover, the apoptotic nuclei indices of blastocysts derived from zygotes electroporated by voltages greater than 30 V/mm were significantly increased compared to those for zygotes electroporated by voltages less than 25 V/mm ($p < 0.05$). When zygotes were electroporated with Cas9 mRNA and a single-guide RNA (sgRNA) targeting a site in the *FGF10* exon 3, the proportions of blastocysts with targeted genomic sequences were 7.7% (2/26) and 3.6% (1/28) in the embryos derived from zygotes electroporated at 25 V/mm and 30 V/mm, respectively. Our results indicate that electroporation at 25 V/mm may be an acceptable condition for introducing *Cas9* mRNA and sgRNA into pig IVF zygotes where the viability of the

embryos is not significantly affected.

In the second experiment, we aimed to clarify the meiotic stage of porcine oocytes having the highest sensitivity to hyperthermia during *in vitro* maturation by evaluating the meiotic competence and DNA damage. Oocytes were exposed to 41 °C for 12 h at various intervals during 48 h of culture maturation. When the oocytes were exposed to 41 °C from 12 to 24 h of the culture maturation, the proportion of oocytes reaching metaphase II (MII) decreased as compared to that of the control oocytes cultured at 38.5 °C ($P < 0.05$). Moreover, the proportions of DNA fragmentation in all oocytes exposed to 41 °C in each culture period after 12 h from the start of maturation culture were significantly higher ($P < 0.05$) than that for the control oocytes. When the meiotic stage of oocytes cultured at 38.5 °C between 12 and 24 h was examined, the majority of oocytes remained at the germinal vesicle (GV) stage at 12 h and approximately half of the oocytes reached metaphase I (MI) at 24 h. These results indicate that the meiotic stage of porcine oocytes having the highest sensitivity to hyperthermia during *in vitro* maturation is a transition period from the GV stage to the MI stage.

In conclusion, our studies demonstrate that bipolar pulses have a detrimental effect on the development of zygotes electroporated under our study conditions. Moreover, electroporation at 25 V/mm may be an acceptable condition for introducing *Cas9* mRNA and sgRNA into pig IVF zygotes. On the other hand, we confirmed that the exposure of porcine oocytes to 41 °C for 12 h decreased the meiotic competence of oocytes and increased the DNA damage of total and MII-stage oocytes. Moreover, porcine oocytes cultured from 12 to 24 h after the start of maturation culture had a higher sensitivity to the elevated temperature. We have established the GEEP (gene editing by electroporation of Cas9 protein) method, in which the CRISPR/Cas9 system is introduced into porcine zygotes by electroporation, which leads to high-efficiency disruption of the targeted gene. Our studies will contribute to increasing the efficiency of the GEEP method adapted to genome editing using *in vitro*-produced porcine zygotes.

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CHAPTER 7. APPENDIX

1) Effects of voltage strength during electropolation on the development and quality of *in vitro*-produced porcine embryos.

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