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論 文 内 容 要 旨

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学位論文題目	**Studies on genome editing techniques using CRISPR/Cas9 in porcine embryos** **(ブタ胚におけるCRISPR / Cas9を用いたゲノム編集技術に関する研究)**		

内容要旨

Animal models has been used to study of human diseases and test clinical drugs, cosmetics, and vaccines before mass human application. Among these animals, the pig is an outstanding experimental animal model, and 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. Xenotransplantation is the use of organs, tissues or cells from other species for human transplantation. Particularly, the domestic pig is a potential organ donor resource because of its similarity to human in terms of anatomy, physiology, and organ size. However, the human immune system recognizes transplantated organs from wild type (genetically- unmodified) pigs as a foreign antigen, then raipidly rejects. Therefore, genetic enginerring is a feasible solution to solve these problems.The CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat/CRISPR-associated 9) system has become a research hotspot and is extensively used in genome editing. In this study, we employed porcine *in vitro* fertilized embryos and CRISPR/Cas9 component to generate genetically edited pigs.

In chapter 1, 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.

In chapter 2, 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.

In chapter 3, 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.

In conclusion, the issue of human immune rejection in organs xenotransplantation can be overcome using improved gene editing techniques. 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.