様式８

論　文　内　容　要　旨

報告番号	甲　先　第　320　号	氏　名	西尾　克俊
学位論文題目	Studies of efficient conditions for generation of genetically modified pigs（遺伝子組換えブタの効率的作製条件に関する研究）		

内容要旨

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.