Animal Reproduction

THEMATIC SECTION: 39TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

CLONING, TRANSGENESIS, AND STEM CELLS

Effective generation of double knock-out TPC1 and TPC2 porcine embryos by CRISPR-Cas9 electroporation of oocytes before insemination

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Keywords: electroporation, knock-out, two pore channel

The production of gene edited porcine embryos has major implications for agriculture and biomedicine. Recently, the modification of oocytes prior to fertilization has gained importance due to the complexity of the cloning technique. One of the most promising methods is electroporation, which enables the delivery of the CRISPR/Cas9 system by applying an electric field that creates pores in the membrane. Two-pore channels (TPCs) are membrane proteins that form cation-permeable channels located on the surface of endolysosomal organelles such as lysosomes and endosomes. TPCs are responsible for regulating the release of Ca^{2+} ions in response to extracellular signals. These proteins are involved in various pathophysiological processes such as embryonic development, cell differentiation, cardiovascular function, autophagy regulation, the acrosome reaction in spermatozoa, and Ebola and SARS-CoV-2 infections, among others. We have previously generated embryos and pigs that are KO for TPC2 and embryos that are KO for TPC1. The double TPC1/2 KO model will provide a valuable experimental model for different biomedical areas, as has been shown previously in mice. The aim of this study is to evaluate the efficacy of electroporation to generate edited gene embryos in which two different genes (TPC1 and TPC2) are targeted in a single step. CRISPR sgRNAs were designed to target exon 9 of the TPC1 gene (Navarro-Serna, Int J Mol Sci 23, 2135, 2022) and exon 3 of the TPC2 gene (Navarro-Serna, CRISPR J 4, 132, 2021). *In vitro* matured oocytes were electroporated (128 oocytes) with Cas9 protein and simultaneously with sgRNAs targeting the TPC1 and TPC2 genes (25 µg/µl of each sgRNA and 100 µg/µl of Cas9 protein), with a negative control with no sgRNAs (control-C, 156 oocytes), followed by *in vitro* fertilization and embryo culture for up to 6 days. Cleavage and blastocyst (blastocyst/oocyte) rates were assessed, and mutation and mosaicism rates were analyzed by fluorescent PCR-capillary gel electrophoresis. The simultaneous application of two sgRNAs and the double concentration of Cas had no negative effect on *in vitro* embryo development, with a cleavage rate of 81.3% higher than control (64.7, p=0.002) and similar blastocyst rate (29.7 vs. 30.1%, p=0.94). When the mutation rates were evaluated separately for each gene, rates of 84.4% for TPC2 and 91.3% for TPC1 were observed. When both genes were evaluated simultaneously, 79% of the embryos had at least one allele of both genes mutated. Only 5% of the blastocysts had no mutations at all, while 79% had the double mutation and smaller percentages had mutations in one gene but not the other (TPC1 11% and TPC2 5%). Electroporation of oocytes prior to IVF is an efficient method for producing gene edited porcine embryos for a variety of models, including multiple modifications in one step. Transfer of these gene edited embryos will result in the generation of TPC1 and TPC2 KO pigs after embryo transfer. This model will be of great interest in the biomedical field.

Acknowledgements: Fundación Séneca 22065/PI/22; MICIN PID2020-113366RB-I00; funded by MCIN/AEI/10.13039/501100011033/ and FEDER Una manera de hacer Europa and Contrato predoctoral Universidad de Murcia R-496/2022.

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Creating porcine phospholipase C zeta knockout (PLCζ-KO) embryos by CRISPR-Cas9 electroporation of oocytes before insemination

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Keywords: electroporation, knock-out, male fertility

Phospholipase C zeta (PLCζ) is found in the cytosol of mammalian spermatozoa. It plays an important role in mammalian fertilization by entering the oocyte and contributing to oocyte activation through intracytoplasmic Ca^{2+} oscillations. Alterations in this protein have been associated with human infertility (Heytens, Human Reproduction 24, 10, 2417-2428). The availability of animal models with specific genetic alterations in this gene/protein will facilitate the study of infertility and the development of new treatments (Hachem, Development 144, 16, 2914-2924, 2017). The aim of this study was to evaluate the efficacy of electroporation of oocytes before *in vitro* fertilization for the generation of the PLCζ-KO edited porcine embryos and to optimize the efficiency of the system using different sgRNA-Cas9 concentrations. The sgRNA was designed to target exon 3 of the PLCζ gene using Braking-Cas software (BioinfoGP, CNB-CSIC, Madrid, Spain). *In vitro* matured oocytes were electroporated with sgRNA-Cas9 protein at two different concentrations, E-Low (6.25 µg/µl sgRNA and 12.5 µg/µl Cas9 protein) and E-High (12.5 and 25 µg/µl), with an unelectroporated control (control-C), immediately followed by *in vitro* fertilization and embryo culture for up to 6 days (Navarro-Serna, Theriogenology 186, 175, 2022; Piñeiro-Silva, Animals 13, 3, 342, 2023). Cleavage and blastocyst (blastocyst/oocyte) rates were assessed, and mutation and mosaicism rates in blastocysts were analyzed by fluorescent PCR-capillary gel electrophoresis. Oocyte electroporation tended to increase the cleavage rate (C: 63.5; E-Low: 77.4, E-High: 76%, p=0.052), although the blastocyst rate was similar for all groups (C: 23.1, E-Low: 18.3 and E-High 21%, p=0.712), indicating that the presence of mutations in PLCζ did not impair blastocyst formation in our model. The mutation rate was 61.2% and 80% for the low and high concentration groups (p=0.025), with an increase in the number of alleles detected per embryo from 2.49±0.53 in E-Low to 2.69±0.51 in E-High (p=0.038), which is related to the increase in mosaicism (E-Low: 47.8; E-High: 67.3%). This is the first reference to our knowledge about the production of porcine PLCζ KO embryos and this study confirms the effective use of electroporation for the transfer of gene editing material. The high level of mutation detected in blastocysts will facilitate the generation of KO pigs after embryo transfer, although the high rates of mosaicism will make it difficult to obtain a biallelic animal in a single step. PLCζ KO pigs will have the potential to generate data for further understanding of the role of PLCζ in mammalian fertilization and oocyte activation.

Acknowledgements: Fundación Séneca 22065/PI/22; MICIN PID2020-113366RB-I00; funded by MCIN/AEI/10.13039/501100011033/ and "FEDER Una manera de hacer Europa" and "Contrato predoctoral Plan de Fomento de la Investigación de la Universidad de Murcia para 2022" (R-496/2022).

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CLONING, TRANSGENESIS, AND STEM CELLS

OVGP1 KO female hamsters are infertile due to a failure in early preimplantation embryo development

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Keywords: oviduct, reproduction, CRISPR Cas9

Oviductal fluid (OF) is the result of blood plasma filtrate and the specific secretions of non-ciliated cells present in the oviductal epithelium. The most predominant protein secreted by these cells is oviductal glycoprotein 1, which is encoded by *OVGP1* gene. The relevance of *OVGP1* has been controversial since *OVGP1* is dispensable for mouse fertility (as evidenced by Knock-out -KO- experiments) (Araki *et al*. Biochemical Journal, 374:551-557, 2003), and it is absent in other mammals such as rats and some bats (Tian *et al*. Biology of Reproduction, 80:616621, 2009; MorosNicolás *et al*. Journal of Molecular Evolution, 86:655667, 2018). To determine the role of *OVGP1* in other mammals, we have generated *OVGP1* KO in the hamster model (*Mesocricetus auratus*) by CRISPRCas9 technology and compared their reproductive performance to that obtained from heterozygous (Hz) and wild type (WT) animals. Fertility assessment by natural mating revealed that KO and Hz males were fertile, being as prolific as their WT counterparts (litter sizes: KO 8.71±0.68; Hz 9.17±1.11; WT 8.25±0.85). Similarly, Hz and WT females were fertile (litter sizes: Hz 9.64±0.79; WT 8.42±0.62). However, female KO hamsters were infertile since no offspring was obtained after the observation of 40 matings of 6 females. Infertility was not caused by fertilization failure or polyspermy as all recovered zygotes showed two pronuclei (10/10 for embryos obtained from WT and KO females). However, embryos recovered from KO females at ~2.5 days *post coitum* (d.p.c.) and ~3 d.p.c. exhibited a significant reduction in blastomere number (~2.5 d.p.c.: KO 2.67± 0.50; WT 5.25 ± 0.30; ~3 d.p.c.: KO 3.69 ± 0.58; WT 7.08 ± 0.37), along with an obvious asymmetry in blastomere size. These embryos may be suffering cell death since microarray analysis revealed that the expression of several genes involved in autophagy and ubiquitin ligase pathway were altered (either, up-, or downregulated) between embryos obtained from WT and KO females. Further characterization of the phenotype by histochemistry and transmission electron microscopy (TEM) evidenced that oviducts from KO females were morphologically normal. On the other hand, transcriptomic analysis of hamster oviducts by RNA-seq showed that 7 genes were differentially expressed: *OVGP1, ENPEP, C1QTNF4, DERL3, AMY1*, and *WDR95* (downregulated in KO animals), and *GPR18* (upregulated in KO animals). Taken together our results evidence that oviductal glycoprotein 1 is essential for female reproductive function in hamsters and, specifically, for proper *in vivo* early embryo development.

This research is part of the projects PGC2018 094781 B I00 and PID2021-123091NB-C21 supported by MCIN/ AEI /10.13039/501100011033/ and by FEDER Una manera de hacer Europa.