Animal Reproduction

Cloning, transgenesis and stem cells

Conventional CRISPR is unable to edit mtDNA in mouse zygotes

Alba López-Palacios, Ismael Lamas-Toranzo, Celia de Frutos, Esther Zurita, Pablo Bermejo-Álvarez

INIA, Spain.

Keywords: mitochondria, mtDNA, embryo, CRISPR.

Mitochondria contain their own genome (mtDNA) encoding for proteins essential for their function. Mutations in mtDNA can cause mitochondrial diseases and CRISPR may provide a way to correct these mutations or to eliminate mtDNA harbouring unwanted mutations. The objective of this study has been to analyse whether conventional CRISPR system, developed for nuclear DNA modification, is able to induce mtDNA mutation or mtDNA degradation. Mouse zygotes were injected with mRNA encoding for Cas9 alone (control group) or combined with sgRNA against one of two mitochondrial sequences: *Cytb* or *Nd4*. *In vitro* blastocysts produced from microinjected embryos were snap frozen. To determine whether mtDNA was edited by CRISPR, target sequences (*Cytb* or *Nd4*) were amplified by PCR and Sanger sequenced. Possible mtDNA degradation was determined by relative mtDNA content analysis by qPCR. Development to blastocyst was similar in all three groups (83±10 vs. 79±2 vs. 74±6 % for control, *Cytb* and *Nd4* groups, respectively, ANOVA $P<0.05$). In contrast to the >90 % edition rates we routinely obtain when targeting nuclear sequences, no mtDNA mutation was detected on embryos microinjected with CRISPR components against mitochondrial sequences (18 embryos analysed for *Cytb* and 20 embryos analysed for *Nd4*). Relative mtDNA content was also similar between groups (1.2±0.1 vs. 1±0.1 vs. 1±0.1 for control, *Cytb* and *Nd4* groups, respectively, ANOVA $P<0.05$). These results suggest that the CRISPR system conventionally used for nuclear genome modification, which includes a Cas9 protein tagged with a nuclear localization signal, is unable to access the mitochondrial matrix where mtDNA is located.

Animal Reproduction

Cloning, transgenesis and stem cells

Timing of mRNA CRISPR/Cas9 microinjection with respect to in vitro fertilization affects embryo development and mutation efficiency in porcine embryos

Sergio Navarro-Serna[1], Evelyne París-Oller[1], Analuce Canha-Gouveia[1], Alaa Hachem[2], César Flores-Flores[3], Pablo Bermejo-Álvarez[4], Raquel Romar[1], John Parrington[2], Joaquín Gadea[1]

[1]Department of Physiology, Faculty of Veterinary, University of Murcia, Campus Mare Nostrum and IMIB-Arrixaca, Murcia, Spain; [2]Department of Pharmacology, University of Oxford, Oxford, UK; [3]Molecular Biology Section, Scientific and Technical Research Area (ACTI), University of Murcia, Murcia, Spain; [4]Animal Reproduction Department, INIA, Madrid, Spain.

Keywords: CRISPR/Cas9, TPC2, pig.

Current advances on *in vitro* fertilization (IVF) techniques in pig, allow the generation of embryos with improved quality, enabling the use of IVF as a means to produce genetically modified pigs. In contrast to *in vivo* production, IVF allows precise control of microinjection timing of gene editing tools with respect to fertilization and the beginning of DNA replication. The objective of this study was to determine the effect of microinjection timing on embryo developmental and genome edition rates. For this aim, Cas9 mRNA and single guide RNA (sgRNA) targeting the two-pore channel 2 gene (TPC2) were microinjected at three different times: oocyte microinjection before IVF (BIVF), zygote microinjection 5-6h after IVF (5IVF) and zygote microinjection 10-12h after IVF (10IVF). A non-injected group was used as a control.

Porcine cumulus-oocyte complexes matured in NCSU37 medium for 40-44h (38.5ºC, 5% CO_2) were inseminated in TALP medium with ejaculated frozen-thawed boar spermatozoa selected with NaturARTs-PIG sperm swim-up medium (EmbryoCloud, Murcia, Spain). At 18h post insemination, putative zygotes were *in vitro* cultured in NCSU23 medium until day 6 post insemination. Blastocyst yield (blastocyst/oocyte) was recorded and mutation rates were analysed by fluorescent PCR-capillary gel electrophoresis.

A total of 869 oocytes were used for this experiment in four different replicates. Results showed that microinjection after IVF detrimentally affected the embryo development. Thus, blastocyst yield was significantly lower ($p<0.05$) in 5IVF (19.9±2.8 %) and 10IVF (17.9±2.7%) groups compared to the BIVF group (28.8±3.3%), but no group was significantly different to the control (21.6±2.8%). Mutation rates despite were numerically higher at the late microinjection times, being the highest edition rate obtained with 10IVF (14/33, 42.4%) followed by 5IVF (11/32, 34.4%) and BIVF (12/40, 30.0%), but these differences between groups were not statistically significant. Mosaicism incidence was low in all groups analysed (0, 9.1 and 21.4% for BIVF, 5IVF and 10IVF groups, respectively). Finally, the efficiency of the system, measured as the proportion of edited blastocysts without mosaicism from total oocytes injected, was 8.6% in BIVF, 6.2% in 5IVF and 6.0% in 10IVF. In conclusion, the efficiency of the system for obtaining pig embryos without mosaicism tended to be higher when oocytes were microinjected with Cas9 mRNA and sgRNA before IVF.

Supported by MINECO-FEDER (AGL 2015-66341-R), Fundación Séneca 20040/GERM/16, and a FPU fellowship (FPU16/04480) from the Spanish Ministry of Education, Culture and Sport.

Animal Reproduction

ABSTRACTS: 36TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

Cloning, transgenesis and stem cells

First generation of two-pore channel 2 (TPC2) mutant pigs by ribonucleoprotein CRISPR/Cas9 microinjection before in vitro fertilization

Sergio Navarro-Serna[1], Ali Hanbashi[2], Analuce Canha-Gouveia[1], Gabriela Garrappa[1], Jordana S. Lopes[1], Lucía Sarrías-Gil[1], Alaa Hachem[2], Ondrej Simonik[1,3], César Flores-Flores[4], Carmen Matás[1], Raquel Romar[1], John Parrington[2], Joaquín Gadea[1]

[1]Department of Physiology, Faculty of Veterinary, University of Murcia, Campus Mare Nostrum and IMIB-Arrixaca, Murcia, Spain; [2]Department of Pharmacology, University of Oxford, Oxford, UK; [3]Laboratory of Reproductive Biology, Institute of Biotechnology CAS, v.v.i., BIOCEV, Vestec, Czech Republic; [4]Molecular Biology Section, Scientific and Technical Research Area (ACTI), University of Murcia, Murcia, Spain.

Keywords: CRISPR/Cas9, TPC2, pig.

TPC2 is a lysosomal cation channel protein involved in NAADP-mediated Ca^{2+} signalling and endolysosomal trafficking in the cell. TPC2 has been shown to be involved in a variety of pathophysiological processes including smooth muscle and heart contraction, neo-angiogenesis and cancer, immune responses, autophagy, skeletal muscle development, diabetes, brown adipose thermogenesis (reviewed in Patel et al. 2018 Biochimica et Biophysica Acta. Molecular Cell Research), and SARS-CoV-2 (Ou et al., 2020 Nature Communications) and Ebola infection (Penny et al. 2019 Biochim Biophys Acta Mol Cell Res). Most insights into TPC2 function have come from studies of TPC2 knockout (KO) mice. The importance of TPC2 is such that further valuable insights might be gained by studying the consequences of loss of TPC2 expression in species physiologically closer to humans, and pig is a very suitable model in this respect (Perleberg et al., 2018 Disease Models & Mechanisms). Therefore, this study aimed to generate TPC2 KO pigs by using *in vitro* matured oocytes that were immediately microinjected with anti-TPC2 CRISPR/Cas9 ribonucleoprotein (RNP) before being subjected to *in vitro* fertilization (IVF). The goal of injecting before IVF was to see if this led to gene editing before the first DNA replication thus reducing mosaicism incidence.

Porcine cumulus-oocyte complexes matured in NCSU37 medium for 40-44h were inseminated in TALP medium with ejaculated frozen-thawed boar spermatozoa selected with NaturART-PIG sperm swim-up medium (EmbryoCloud, Murcia, Spain). At 18h post insemination (p.i.), putative zygotes were cultured *in vitro* in NCSU23 medium until day 2 p.i. Embryos at the 2-4 cell stage were surgically transferred (120±10 embryos/sow) to one oviduct of 6 sows 3-5 days after natural heat detection. After delivery, ear, tail, muscle and blood tissue were collected from born piglets and mutation rates were analysed by fluorescent PCR-capillary gel electrophoresis and Sanger DNA sequencing.

Embryo transfer resulted in 2 pregnancies (33.3%) with one litter of 5 piglets (4 males and 1 female) and another with 7 piglets (1 male and 6 females). The mean litter size was 6 piglets (41.7% males and 58.3% females). Regarding mutations, 2 females and 1 male were homozygous KO in all analysed tissues. One of the females and the male showed a double deletion of 11bp whereas the other female had a double insertion of 16 bp; both should result in a frameshift in the TPC2 open reading frame. From birth to day 28 of life, all mutant pigs had a phenotype and growth rate similar to that observed in wild-type animals. In conclusion, this is the first generation of KO TPC2 pigs generated by any means to our knowledge. The mutant animals obtained will be now used to generate a TPC2 KO pig colony to obtain more animals that will be available for different studies.

Supported by MINECO-FEDER (AGL 2015-66341-R), Fundación Séneca 20040/GERM/16, and a FPU fellowship (FPU16/04480) from the Spanish Ministry of Education, Culture and Sport.