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Original Research Article

Oocyte electroporation prior to *in vitro* fertilization is an efficient method to generate single, double, and multiple knockout porcine embryos of interest in biomedicine and animal production

Sergio Navarro-Serna [a,1], Celia Piñeiro-Silva [a,1], Irene Fernández-Martín [a], Martxel Dehesa-Etxebeste [b], Adolfo López de Munain [b,c,d,e], Joaquín Gadea [a,*]

[a] Department Physiology, University of Murcia, International Excellence Campus for Higher Education and Research "Campus Mare Nostrum" and Institute for Biomedical Research of Murcia (IMIB-Arrixaca), 30100, Murcia, Spain
[b] IIS Biodonostia, Neuroscience, San Sebastián, Spain
[c] Department of Neurology. Hospital Universitario Donostia-OSAKIDETZA, San Sebastián, Spain
[d] Department of Neurosciences. University of the Basque Country (UPV-EHU), San Sebastián, Spain
[e] CIBERNED (CIBER), Institute Carlos III, Madrid, Spain

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Disease resistance

A B S T R A C T

Genetically modified pigs play a critical role in mimicking human diseases, xenotransplantation, and the development of pigs resistant to viral diseases. The use of programmable endonucleases, including the CRISPR/Cas9 system, has revolutionized the generation of genetically modified pigs. This study evaluates the efficiency of electroporation of oocytes prior to fertilization in generating edited gene embryos for different models. For single gene editing, phospholipase C zeta (PLC ζ) and fused in sarcoma (FUS) genes were used, and the concentration of sgRNA and Cas9 complexes was optimized. The results showed that increasing the concentration resulted in higher mutation rates without affecting the blastocyst rate. Electroporation produced double knockouts for the TPC1/TPC2 genes with high efficiency (79 %). In addition, resistance to viral diseases such as PRRS and swine influenza was achieved by electroporation, allowing the generation of double knockout embryo pigs (63 %). The study also demonstrated the potential for multiple gene editing in a single step using electroporation, which is relevant for xenotransplantation. The technique resulted in the simultaneous mutation of 5 genes (GGTA1, B4GALNT2, pseudo B4GALNT2, CMAH and GHR). Overall, electroporation proved to be an efficient and versatile method to generate genetically modified embryonic pigs, offering significant advances in biomedical and agricultural research, xenotransplantation, and disease resistance. Electroporation led to the processing of numerous oocytes in a single session using less expensive equipment. We confirmed the generation of gene-edited porcine embryos for single, double, or quintuple genes simultaneously without altering embryo development to the blastocyst stage. The results provide valuable insights into the optimization of gene editing protocols for different models, opening new avenues for research and applications in this field.

1. Introduction

The pig (*Sus scrofa*) is an excellent model for the study of human diseases because of its many similarities to humans. The physiological and anatomical characteristics of the pig make it particularly well suited for research in the clinical areas of human medicine [1,2]. Therefore, genetically modified pigs are used worldwide to mimic human diseases [2] or for xenotransplantation purposes [3–5].

Since 1985, when the creation of the first genetically modified pigs by random insertion of DNA by microinjection was reported [6,7], the use of this technology has increased exponentially. The development of the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated gene 9 (Cas9) system has had a major impact on the production of genetically modified animals. These tools have made this process more efficient, accurate and rapid (revised by Refs. [8,9]. To date, there are two main methods for generating genetically modified

* Corresponding author.
E-mail address: jgadea@um.es (J. Gadea).
[1] These two authors contributed equally to this work.

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pigs using the CRISPR/Cas9 system: somatic cell nuclear transfer (SCNT) of modified cells [10] and direct embryo/oocyte editing [11]. With SCNT, gene editing changes are made in the donor cell before it is transferred to the enucleated egg, so the resulting piglets will have the desired mutations in all their cells, including gametes. However, SCNT is associated with significant technical challenges, such as low efficiency and potential abnormalities in newborns due to epigenetic alterations [12,13]. The alternative is to introduce the CRISPR/Cas9 system directly into the oocyte or an early-stage embryo so that the editing occurs before the embryo has started dividing. This can be achieved by a variety of techniques, including microinjection [11,14], electroporation [15,16] and lipofection [17,18].

Microinjection involves the mechanical introduction of the CRISPR/Cas9 components into the oocyte/embryo using a needle and micromanipulator. This technique is widely used and well established but requires specialized equipment and experienced personnel. One of the most promising methods for gene editing in embryos is electroporation, which allows efficient delivery of the CRISPR/Cas9 system into oocyte/embryo cells by applying an electric field that creates pores in the membrane [15,16,19]. This method has become increasingly popular due to its simplicity and speed. However, it can also present some challenges, such as the need to optimize the strength and duration of the electric field, the concentration of editing reagents and the timing of electroporation (revised by Ref. [20]).

Although improvements in these technologies have led to greater efficiency in producing genetically modified animals by editing embryos, there are still limitations to be overcome. One of these is mosaicism, which is the presence of different populations of cells with different alleles for the same gene. One strategy to reduce mosaicism is to use the CRISPR/Cas9 system to edit DNA before the zygote enters the S phase of the cell cycle. Previous studies have shown that microinjection of oocytes before IVF reduces mosaicism [14], and electroporation or lipofection of oocytes before insemination is also a good option, as our group previously has shown [15,17,21].

The aim of this study was to evaluate the efficacy of electroporation of oocytes prior to *in vitro* fertilization to generate edited gene embryos for different models and to analyze the effect of factors such as RNA guide concentration or the simultaneous use of multiple sgRNAs on efficiency, measured in terms of embryo development, mutation and mosaicism rate.

On the one hand, we have studied the use of electroporation to generate embryos deficient in the gene PLC ζ (phospholipase C zeta), which is related to the sperm protein found in the cytosol. This protein induces calcium signaling in the oocyte and plays a key role in post-fertilization activation [22,23]. On the other hand, mutations in the fused in sarcoma (FUS) gene have recently been identified as a major cause of familial amyotrophic lateral sclerosis (FALS) [24,25].

In addition to simple models (PLC ζ and FUS in this study), the electroporation of oocytes/zygotes with CRISPR/Cas9 allows the editing of multiple genes in the same organism. This allows strategies such as the generation of knockouts of 2–5 genes, which we will develop in this work. In fact, the genes TPC1 (two-pore channel 1) and TPC2 (two-pore channel 2) are related to calcium signaling in cells as the proteins that encode are involved in the regulation of calcium movement within cells. Calcium signaling plays a crucial role in various cellular processes and is essential for the proper functioning of both excitable and nonexcitable cells (Patel 2015; Ruas et al., 2015). The double KO of TPC1 and TPC2 mice has provided an interesting model to study various functional activities, such as hearing or oxytocin secretion [26,27]. Previously, our group generated TPC2KO piglets [14] and TPC1KO pig embryos [21]. Thus, the generation of the double KO TPC1/2 in pigs will be a valuable model to complement this line of research.

The modification of proteins needed for viral infection or replication will increase resistance to these viral diseases; PRRS and swine influenza are two important diseases for which genetically engineered pigs could improve resistance [28,29]. Thus, the generation of a double KO to induce resistance to these diseases will have an important impact on the swine industry and public health. In another application, such as xenotransplantation, it is necessary to edit multiple genes to eliminate glycan antigens and overexpress human transgenes to facilitate the long-term life-support function of grafts [4]. The use of electroporation to edit multigenes in a single step facilitates the development of these studies. In this study, 5 genes were edited with high efficiency, demonstrating the interest and applicability of this strategy.

2. Material and methods

2.1. Ethical issues

The study was developed in accordance with the Spanish Animal Protection Policy (RD 53/2013), which is in line with the European Union Directive 2010/63/EU on the protection of animals used in scientific experiments. This project was approved by the Ethics Committee of the University of Murcia and by the Regional Government of Murcia (Reference GENOCRISPR, A13230307).

2.2. Culture media reagents

All chemicals were purchased from Sigma−Aldrich Quimica, S.A. (Madrid, Spain) unless otherwise stated.

2.3. Design of sgRNAs

Single guide RNAs (sgRNAs) against the different target genes were designed using Braking-Cas software (BioinfoGP, CNB–CSIC, Madrid, Spain) [30] (Table 1). Both sgRNA and Cas9 protein were purchased from IDT (Integrated DNA Technologies, Leuven, Belgium), and ribonucleoprotein complexes (RNP) were prepared according to the manufacturer's instructions.

2.4. Oocyte In vitro maturation (IVM)

Cumulus-oocyte complexes (COCs) were obtained from gilt ovaries at the slaughterhouse and were processed as described previously [31]. Briefly, ovaries were transported to the laboratory in saline at 38 °C, washed once in 0.04 % cetrimide and twice in saline, both at 38 °C. COCs were collected by aspiration from follicles between 3 and 6 mm in diameter, washed in Dulbecco's PBS with 0.2 g/L polyvinyl alcohol (DPBS-PVA) and then washed again in NCSU-37 maturation medium supplemented with 10 % (v/v) porcine follicular fluid. Groups of 50–55 COCs were then cultured for *in vitro* maturation (IVM) in 500 mL NCSU-37 supplemented with 1 mM dibutyryl cAMP, 10 UI/mL eCG and 10 UI/mL hCG for 20–22 h at 38.5 °C under 5 % CO_2 and 7 % O_2 conditions, followed by another 20–22 h in NCSU-37 without dibutyryl cAMP, eCG and hCG.

After IVM, 25 μL of 0.5 % hyaluronidase was added to each well

Table 1
sgRNAs designed to knock out a range of genes using Breaking-Cas software (https://bioinfogp.cnb.csic.es/tools/breakingcas/) [30].

Target gene	sgRNA (5'->3')
PLC ζ	GTTCGGGATGACTTTAAAGGTGG
FUS	GGGACCGTGGGGGCTTCCGAGGG
TPC1	CATTCGGCACAAACGGACCATGG
TPC2	GGTGATTAATGGAGCGGTA1CTGG
CD163	TACTTCAACACGACCAGAGCAGG
TMPRSS2	CACCCGCCGTCGTCGTCAGCAGG
GGTA1	AGACGCTATAGGCAACGAAAAGG
B4GALNT2	CTGTATCGAGGAACACGCTTTGG
Pseudo B4GALNT2	CTGTATCGAGGAACACGCTTTGG
CMAH	TTGAGATTGGCAGCTTCGGCAGG
GHR	TAGTTCAGGTGAACGGCACTTGG

containing 500 μL of NCSU37 and COCs and incubated at 38.5 °C for 2 min. The mature COCs were manually decumulated by pipetting until most of the cumulus cells were removed.

2.5. CRISPR/Cas9 delivery by electroporation of in vitro matured oocytes

Prior to electroporation, decumulated oocytes were washed in Opti-MEM I Reduced Serum Media (Thermo Fisher, Waltham, MA USA). Groups of 50–100 oocytes were then transferred to a droplet containing CRISPR/Cas9 RNPs, placed on a microscope slide between 1 mm gap electrodes (45–0104, BTX, Harvard Apparatus, USA) connected to the ECM 830 Electroporation System (BTX, Harvard Apparatus, USA), and electroporated using 4 pulses at 30 V, 1 ms pulse duration and 100 ms pulse interval [15].

2.6. In vitro fertilization (IVF), in vitro embryo culture and embryo assessment

IVF was performed essentially as described previously [32]. Briefly, *in vitro* matured oocytes were washed in TALP medium supplemented with 1 mM sodium pyruvate, 0.3 % BSA and 50 mg/mL gentamycin (IVF-TALP) and transferred in groups of 50–55 oocytes to wells containing 250 mL IVF-TALP medium. The oocytes were inseminated with frozen-thawed ejaculated sperm from a fertile boar after being selected for motility by a swim-up procedure [33]. Briefly, a 0.25 mL semen straw was thawed in a water bath (30 s, 38 °C), and the semen was diluted in 2 mL sperm swim-up medium (PIG-SUM, EmbryoCloud, Murcia, Spain) at 38 °C. Sperm selection was performed by adding 1 mL of sperm swim-up medium to a conical tube, placing 1 mL of thawed diluted sperm under the medium, incubating (38 °C, 20 min, 45° angle) and removing 500 μL of the upper medium by gentle aspiration. The concentration of selected motile sperm was adjusted to 4000–6000 sperm/ml in IVF-TALP, and oocytes were inseminated with 250 μL of sperm suspension (final IVF well volume 500 μL). The gametes were cocultured at 38.5 °C, 5 % CO_2 and 7 % O_2 for 20–22 h.

After gamete coincubation, putative zygotes were cultured in NCSU-23 medium supplemented with 5 mM sodium lactate, 0.5 mM sodium pyruvate and both essential (1 % v/v) and nonessential (1 % v/v) amino acids for 24 h at 38.5 °C, 5 % CO_2 and 7 % O_2 [34]. Cleavage rates were then assessed, and 2–4 cell embryos were transferred to NCSU-23 medium supplemented with 5.5 mM glucose and essential (1 % v/v) and nonessential (1 % v/v) amino acids until 156 h post insemination. After *in vitro* culture, the blastocyst rate was determined, and mutation rates were evaluated as described below.

2.7. Blastocyst mutation analysis

The zona pellucida was digested with 0.5 % pronase (protease from Streptomyces griseus, P8811, Sigma–Aldrich, Madrid, Spain), after which the blastocysts were washed in nuclease-free water and stored individually in minimal volumes at −20 °C until analysis.

DNA extraction and PCR were performed using a Phire Animal Tissue Direct PCR Kit (Thermofisher, Waltham, MA USA). Genomic DNA was extracted according to the dilution protocol of this kit. One microlite per sample was used for 12.5 μL of PCR containing 0.5 μM primers (Table 2).

Mutation detection on the target genes was determined using a fluorescent PCR-capillary gel electrophoresis technique [14]. PCR was performed using 6-FAM-labeled FW primers.

After PCR, samples were diluted 1:100 in TE buffer, and 1 μL of the mixed samples was added to a clean 1.5 mL tube containing 11.5 μL HiDi™formamide (Thermofisher) and 0.1 μL GeneScan™ 500 LIZ Size Standard (Applied Biosystem, Thermofisher). The sample was incubated (3 min at 95 °C), immediately cooled on ice for 2 min and analyzed by capillary gel electrophoresis on a 3500 Genetic Analyzer (Applied Biosystems, Thermofisher). The details of the instrumental protocol were capillary length: 50 cm; polymer: POP7; dye set: G5; run voltage: 19.5 kV; prerun voltage: 15 kV; injection voltage: 1.6 kV; run time: 1330 s; prerun time: 180 s; injection time: 15 s; data delay: 1 s; standard size: GS500 (−250) LIZ; and size caller: SizeCallerv1.10. The results were analyzed using Gene Mapper 5 (Life Technologies).

Embryos were considered wild type (WT) if the peak obtained by capillary electrophoresis was the same size as the control peak. Other peaks that differed in size from the control peak were considered to be knockout (KO), and if more than two peaks were detected in a sample, the embryo was considered to be mosaic.

Table 2
Primer sequences for various target genes.

Target gene		Sequence (5'->3')
TPC2	Forward	AGCCCTTCTGTCCACAGTCT
	Reverse	GCTCCTTGTGGGGATAAGGC
TPC1	Forward	TGCTTGCCTCCCTTAGGACA
	Reverse	CACAGTGACATCCAGAGCAAG
PLC ζ	Forward	AGCATGAGATAGACTGCCCTC
	Reverse	TGGATTTAAGTCCTTCCTAGGGT
FUS	Forward	GGGGTTTGGGGGACACAATA
	Reverse	AAACCTGGACACCCCAAGAC
CD163	Forward	TTGTCTCCAGGGAAGGACAGG
	Reverse	AGAGTGAAAGGTGGGACTCG
TMPRSS2	Forward	CGAGGGTGATGGGGCATTT
	Reverse	GGACATCCCCAGCAGACAGA
GGTA1	Forward	AGGTTTCCCTGCTCCTGACA
	Reverse	CCCTGTCGGGAATGTTCTCAT
B4GALNT2	Forward	TGCTATTCCCATCTATGTCGCA
	Reverse	GTGCAAAGTGGTA1TTCTTGCCA
PSEUDO B4GALNT2	Forward	GCGGTCTTCTTGCCATCTCA
	Reverse	ACGAAACAGGTTTGACTTGCAT
CMAH	Forward	GGAAGGCGGAGATAAACCCTT
	Reverse	TGAGCACATTTCCTGCCAAA
GHR	Forward	GAAGCTGTGACCCAGGAAAAC
	Reverse	CACCAGCTGGGAACAAATCTC

2.8. Statistical analysis

All data analysis was performed using IBM SPSS Statistics for Windows, version 28.0. Armonk, NY: IBM Corp. Variables in all experiments were tested for normality using the Shapiro–Wilk test, and because data were not normally distributed, they were analyzed using nonparametric analysis with the Kruskal–Wallis test or the chi-squared test. When data showed significant differences ($p < 0.05$), values were compared using a Conover-Inman test for pairwise comparisons.

2.9. Experimental design

2.9.1. Efficiency in generating simple gene-edited embryos using electroporation. Effect of sgRNA concentration on system optimization using single RNA guides targeting PLC ζ and FUS genes

To optimize the electroporation delivery of sgRNAs, we assessed the effect of sgRNA concentration on the generation of PLC ζ or FUS KO embryos. sgRNAs were designed to target exon 3 of the PLC ζ gene or exon 14 of the FUS gene (Table 1).

In vitro matured oocytes were electroporated in the presence of different concentrations of sgRNA and Cas9 ribonucleoprotein complexes. The efficiency of the system was evaluated for each gene model in terms of embryo development (% blastocyst/oocytes), mutation and mosaicism rates.

2.9.2. Efficiency of the electroporation system to generate double knockout embryos in a simple step

We assessed the efficiency of the double KO models by electroporation in a simple step. These models are the double TPC1 and TPC2 genes related to cell calcium signaling and, on the other hand, a model for resistance to two important viral porcine diseases, PRRS and porcine influenza, targeting the genes CD-163 and TMPRSS2, respectively.

2.9.2.1. Model double mutation for TPC1 and TPC2. sgRNAs were designed for exon 7 of the gene TPC1 [35] and exon 9 of the gene TPC2 (Navarro-Serna et al., 2021a) (Table 1). *In vitro* matured oocytes were electroporated in the presence of 25 μg/μL of each sgRNA and 100 μg/μL of Cas9 protein. The efficiency of the system was evaluated for mutations in every gene and for double mutations.

2.9.2.2. Model double mutation for resistance to PRRS and influenza porcine virus. sgRNAs were designed for exon 7 of the CD163 gene, which confers resistance to PRRS, and exon 3 of the TMPRSS2 gene, which encodes the enzyme transmembrane protease serine 2, which is involved in the infection process of several viruses, including swine influenza (Table 1). *In vitro* matured oocytes were electroporated in the presence of 25 μg/μL of each sgRNA and 100 μg/μL of Cas9 protein. The efficiency of the system was evaluated for mutations in every gene and for double mutations.

2.9.3. Efficiency of the electroporation system in generating multiple knockout embryos in a simple step (xenotransplantation)

Single guides were designed for the four target genes (GGTA1, B4GALNT2, CMAH and GHR) (Table 1). The sgRNA for B4GALNT2 was designed to target this gene and a pseudogene (pseudo B4GALNT2). *In vitro* matured oocytes were electroporated in the presence of 25 μg/μL of each sgRNA and 200 μg/μL Cas9 protein. The efficiency of the system was evaluated for mutations in every gene and multiple mutations. The exons targeted for each guide were 3, 2, 1 and 11 for GGTA1, B4GALNT2, and CMAH Y GHR, respectively, with the objective of avoiding the presence of these codified proteins related to the hyperacute immune reaction (GGTA1, B4GALNT2, CMAH) and the growth of the animal (GHR).

3. Results

3.1. Efficiency in the generation of simple gene-edited embryos by electroporation. Effect of the sgRNA concentration. KO models for PLCzeta and FUS genes

The cleavage rate in the electroporated groups was higher ($p < 0.05$, in the FUS experiment) or tended to be higher ($p = 0.052$, in the PLC ζ experiment) than that in the control groups (Table 3), but the blastocyst rates within both genes were similar in control and electroporated oocytes ($P > 0.05$, Table 3). There was no negative effect of electroporation on the blastocyst rate, regardless of the concentration used.

For the two models studied, increasing the concentration of CRISPR/Cas9 reagents resulted in higher mutation rates ($P < 0.05$, Table 3). However, the rates of mosaicism and biallelic mutation from mutant embryos are similar in each concentration group. Therefore, to optimize the maximum blastocyst formation and mutation rate and the minimum mosaicism rate, the optimal concentration must be selected for each gene model. With this objective, we constructed an efficiency index that includes the blastocyst rate, the mutation rate, and the inverse of the mosaicism rate. This index is a quality index equivalent to the efficiency in generating edited embryos without mosaicism from the number of oocytes used in IVF, which could not be statistically analyzed with precision. According to this index, the optimal concentration for FUS was 50/25, while both concentrations used for PLC ζ offer a similar efficiency (Table 3).

3.2. Efficiency of the electroporation system to generate double knockout embryos in a simple step to generate double ko in a single step (TPC1/TPC2; PRRS/Influenza).

3.1.1. TPC1/TPC2

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 that of the control (64.7 %, $p = 0.002$) and a similar blastocyst rate (29.7 vs. 30.1 %, $p = 0.94$). When the mutation rates were evaluated separately for each gene, the mutation rates were not different for TPC1 and TPC2 (91.30 and 84.37 %, $P > 0.05$, Table 4), but the mosaicism rate was higher for TPC1 than for TPC2 ($P < 0.03$), and consequently, the percentage of biallelic mutations was lower ($P < 0.01$, Table 4).

When both genes were examined simultaneously, 79.4 % of the embryos had a double mutation in at least one allele, 11.1 % of the embryos had mutations only in TPC1, and 4.8 % of the blastocysts had no mutations at all or only in the TPC2 gene (chi-square p value = 0.016, Fig. 1a). Interestingly, the mosaicism rate was maintained in the range of 55–60 % for TPC1 and 33–38 % for TPC2 in both single- and double-edited embryos (Fig. 1b). Similarly, the biallelic mutation rate is 14–14.3 % for TPC1 and 36–67 % for TPC2.

3.1.2. PRRS/influenza resistance

In terms of embryo development, the cleavage rate was 81.5 % (75/92), and the blastocyst rate was 29.7 % (31/92). Analyzing the mutation rates for each gene, we found that the mutation for the CD163 gene occurred in 71 % of the blastocysts, with a mosaicism rate of 22.7 % and

Table 4
Mutation and mosaicism rates after CRISPR/Cas9oocyte electroporation with double sgRNA for the TPC1 and TPC2 genes.

Gene	Mutation rate (%)	Mosaicism rate (%)*	Biallelic mutation (%)#
TPC1	63/69 (91.30 %)	36/63 (57.14 %)[a]	9/63 (14.29 %)[a]
TPC2	54/64 (84.37 %)	20/54 (37.04 %)[b]	21/54 (38.89 %)[b]
P value	0.219	0.030	0.002

[a,b] Differences between groups.
*Mosaicism in mutated embryos, presence that more of 2 different alleles.
#Bilallelic mutation from mutated embryos.

Table 3
Embryo development (cleavage and blastocyst formation), mutation, mosaicism and biallelic mutation rates after CRISPR/Cas9 oocyte electroporation. The efficiency index included the blastocyst rate, the mutation rate, and the inverse of the mosaicism rate.

Gene	Cas/sgRNA concentration	n	Cleavage (%)	Blastocyst (%)	Mutation (%)	Mosaicism (%)*	Biallelic mutation (%)#	Efficiency index
PLC ζ	Control 0/0	104	63.5	23.1				
	12.5/6.25	94	77.4	18.3	41/67 (61.2 %)[a]	32/41 (78.0 %)	1/41 (2.4 %)	2.46
	25/12.5	100	76	21	44/55 (80.0 %)[b]	37/44 (84.1 %)	0 (0.0 %)	2.52
P value			***0.052***	***0.712***	***0.025***	***0.482***	***0.303***	
FUS	Control 0/0	162	64.8[x]	19.1				
	25/12.5	156	71.2[xy]	16.0	20/62 (32.3 %)[a]	6/20 (30.0 %)	2/20 (10 %)	3.61
	50/25	161	78,9[y]	19.3	38/56 (67.9 %)[b]	13/38 (34.2 %)	5/38 (13.2 %)	8.62
P value			***0.019***	***0.743***	***<0.001***	***0.745***	***0.726***	

[a,b]Differences between the groups of Cas/sgRNA concentrations in the same gene.
*Mosaicism in mutated embryos, presence that more of 2 different alleles.
#Biallelic mutation from mutated embryos.

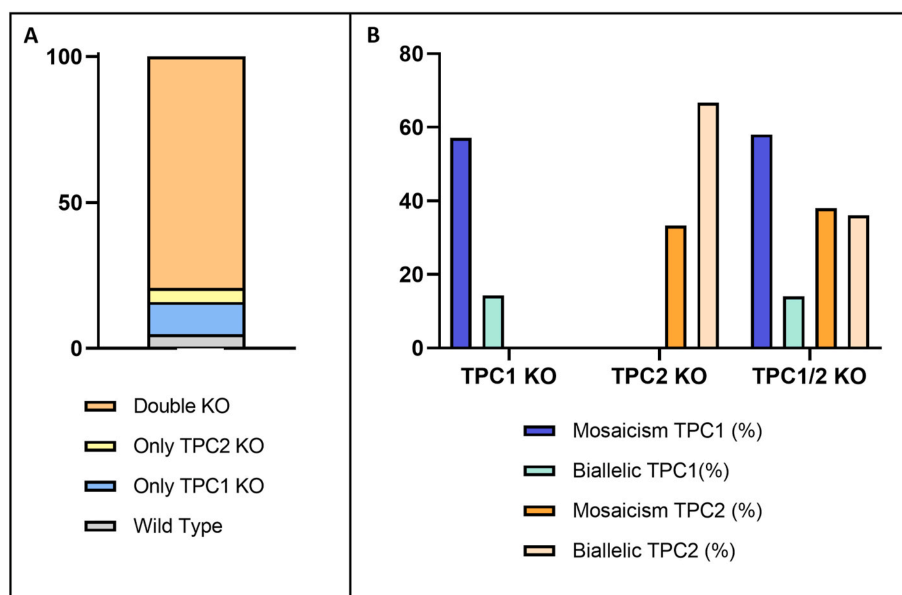

Fig. 1. A. Distribution of embryos according to mutations in the TPC1 and TPC2 genes. B. Mosaicism rate and biallelic mutation rate for the gene edited blastocyst for both TPC1 and TPC2 genes.

9.09 % of biallelic mutations, and these values were similar for the TMPRSS2 gene (Table 5, P > 0.05). When both genes were examined simultaneously, mutations in both genes occurred in 63 % of the genotyped blastocysts, while 14.8 % of the embryos had a single mutation in the TMPRSS2 gene, 7.4 % had a single mutation in the CD163 gene, and 14.8 % had no mutation at all (Fig. 2a).

As we observed previously, the mosaicism rate for TMPRSS2 was maintained in the range of 50 % in both single- and double-edited embryos (Fig. 2b). Similarly, the biallelic mutation rate for this gene is in the range of 0–5.88 %. In contrast, the proportion of mosaicism and biallelic mutations in the CD163 gene tended to be different in mono- and double-edited embryos (Fig. 2b).

3.2. *Efficiency of the electroporation system in generating multiple knockout embryos in a simple step (xenotransplantation)*

As in the previous sections, the cleavage rate was significantly higher in the electroporated group (72.50 % (58/80)) than in the control group (51.06 % (48/94), p < 0.05), while the blastocyst formation rate was similar in the control and electroporated groups (13.75 vs. 18.09 %, p > 0.05), indicating that the procedure does not affect embryo development. Regardless of the efficiency in genotyping the different genes, it was higher than 95 % for all 5 genes (P > 0.05, Table 6), while the efficiency in genotyping the other genes showed intermediate values. When the mutation rates were evaluated separately for each gene, the mutation rates were higher than 85 % for the 5 genes (Table 6, P > 0.05). In contrast, the lowest value of mosaicism was for B4GALNT2 (40.00 %), while the mosaicism for the other 4 genes showed higher values, ranging from 70 to 88 % (Table 6, p = 0.001). These high values of mosaicism determined low values of biallelic mutation ranging from 0 to 7.5 % without differences between genes (Table 6, P > 0.05).

When we analyzed the possible multiple editions in each treated embryo, we found that out of 49 embryos, 43 were able to genotype the 5 genes at the same time (global efficiency for genotyping 87,75 %). In 70 % of these embryos, we found simultaneous mutations in the 5 genes (Fig. 3).

Table 5
Mutation and mosaicism rates after CRISPR/Cas9 oocyte electroporation with double sgRNA for CD163 and TMPRSS2 genes.

Gene	Mutation rate (%)	Mosaicism rate (%)*	Biallelic mutation (%)#
CD163	22/31 (70.97 %)	5/22 (22.73 %)	2/22 (9.09 %)
TMPRSS2	22/28 (78.57 %)	10/22 (45.45 %)	1/22 (4.45 %)
P value	0.503	0.112	0.603

*Mosaicism in mutated embryos, presence that more of 2 different alleles.
#Bilallelic mutation from mutated embryos.

4. Discussion

Our research group previously optimized microinjection, electroporation, and lipofection systems for *in vitro*-matured porcine oocytes to enhance the proportion of blastocysts with biallelic mutations [14,15,17,21]. In this study, we affirmed that electroporating oocytes before IVF is an effective strategy with no adverse effects on early embryo development. As previously reported, oocyte electroporation also increased the cleavage rate, likely associated with oocyte activation, resulting in approximately 27 % parthenotes under our experimental conditions [36]. Partial generation of parthenotes does not pose a problem for the evaluation and optimization of the gene-edited embryo production system and facilitates the increase in the number of embryos to be evaluated. Parthenotes were used to evaluate the mutation rate after Crispr/Cas treatment for multiple targets [37].

Moreover, electroporation did not have a negative effect on the blastocyst rate, as previously reported for electroporation of zygotes [16] and oocytes [15,17,21], while the voltage of the pulse was equal to or less than 30 V/mm [38] and less than 5 pulses [15,39].

As shown in Experiment 1, the sgRNA/Cas concentration affects mutation and mosaicism rates in a dose-dependent manner, thereby affecting system efficiency. Previous studies have investigated Cas protein concentration [40] and sgRNA and Cas protein concentration on electroporation system efficiency (Navarro-Serna et al., 2022a; Navarro-Serna et al., 2022b). However, the optimal concentration varies for different genes. Fortunately, electroporation simplifies concentration optimization because it requires minimal equipment and allows numerous oocytes to be processed in a single session.

Later, in the second experiment, we confirmed the generation of double mutations for some models by using 2 different sgRNAs; 79 % of the electroporated embryos showed double mutations in the TPC1 and TPC2 genes, and 63 % of the embryos for the model of simultaneous

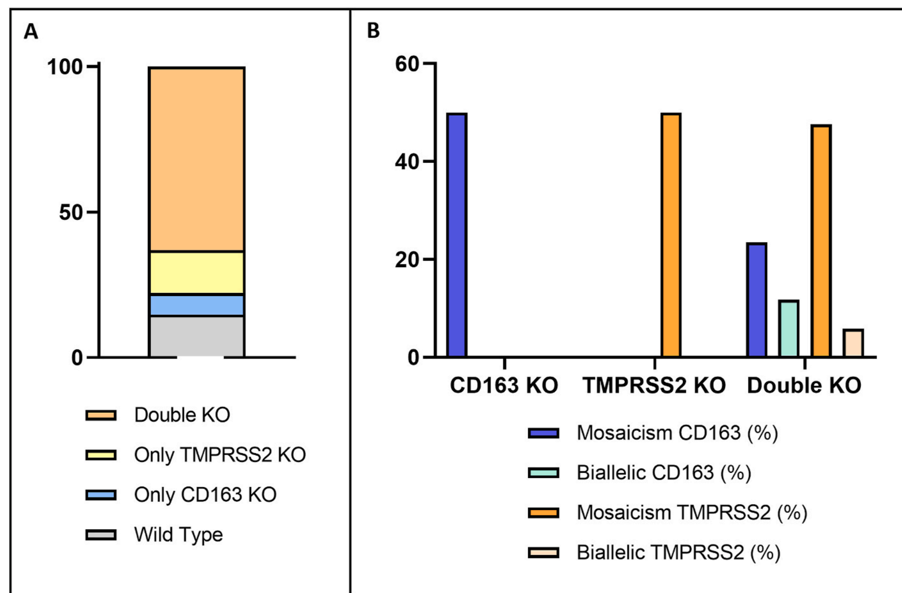

Fig. 2. A. Distribution of embryos according to mutations in the CD163 and TMPRSS2 genes. B. Mosaicism rate and biallelic mutation rate for the gene edited blastocyst for both CD163 and TMPRSS2 genes in the different embryos.

Table 6
Mutation and mosaicism rates after CRISPR/Cas9 oocyte electroporation with 5 sgRNAs for the GGTA11, CMAH, B4GALNT2, Pseudo B4GALNT2 and GHR genes.

Gene	Genotyping efficiency (%)	Mutation rate (%)	Mosaicism rate (%)*	Biallelic mutation (%)#
GGTA11	95.91 %	43/47 (91.49 %)	38/43 (88.37 %[a])	2/43 (4.65 %)
CMAH	95.91 %	40/47 (85.11 %)	28/40 (70.00 %[a])	3/40 (7.50 %)
B4GALNT2	100 %	43/49 (87.76 %)	18/43 (41.86 %[b])	0/43 (0 %)
Pseudo B4GALNT2	97.96 %	42/48 (87.50 %)	37/42 (88.10 %[a])	2/42 (4.76 %)
GHR	95.91 %	43/47 (91.49 %)	35/43 (81.40 %[b])	3/43 (6.98 %)
P value	0.676	0.840	<0.001	0.516

[a,b] Differences between groups.
*Mosaicism in mutated embryos, presence that more of 2 different alleles.
#Bilallelic mutation from mutated embryos.

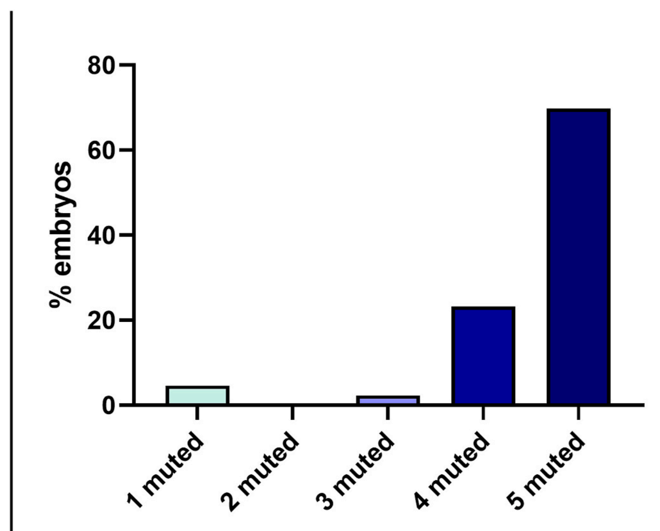

Fig. 3. Distribution of the percentage of embryos with different numbers of mutated genes after effective genotyping of 5 genes related to xenotrasplantation (GGTA1, B4GALNT2, pseudo B4GALNT2, CMAH and GHR).

resistance to pig viral diseases such as PRRS and porcine influenza. This proportion is equivalent to the multiplication of the efficiencies in mutation of the single guide. These results suggested that the mutation rate of a gene (and in less measure the mosaicism and biallelic mutation rate) is fairly fixed for the sgRNA in use and the concentration of sgRNA and Cas protein, and if more probes are added at the same time, the mutation rate is similar to what you would do alone, as previously reported using pig zygote electroporation [41]. This makes it much easier to optimize systems for producing edited embryos without altering embryo development.

We evaluated the similarity or differences in mutation rates with TPC1, TPC2, and CD163 from previous work using a single gene (Table 7). We observed that in this study, the mutation rates were higher when double sgRNA was used, which was related to the use of twice the concentration of Cas protein, as previously reported for the MSTN gene [40]. The mutation rates in this study are higher than when we used a single probe with the same concentration for the same gene target (TPC1, TPC2 and CD163) [14,17,33].

The efficiency of the system to edit in a single step several (up to 5) genes related to xenotransplantation by oocyte electroporation opens a valuable application in this field, equalling or improving the results reported by other researchers using SCNT [42–45], zygote electroporation [46,47] or in combination with microinjection [48].

In conclusion, although some technical aspects still need to be resolved, the electroporation technique could become a primary method for the one-step generation of multiple gene modifications in pigs to

Table 7
Mutation and mosaicism rates after CRISPR/Cas9 oocyte electroporation with double sgRNA or single edition.

Gene	Mutation rate (%)	Mosaicism rate (%)*	Biallelic mutation (%)♯
TPC1 (with TPC2)	63/69 (91.30 %)	36/63 (57.14 %)	9/63 (14.29 %)
TPC1 [21]	9/51 (76.47 %)	(13/39) (33.3 %)	4/39 (10.26 %)
P value	**0.024**	**0.019**	**0.553**
TPC2 (with TPC1)	54/64 (84.37 %)	20/54 (37.04 %)	21/54 (38.89 %)
TPC2 [14]	20/42 (47.6 %)	3/20 (15.0 %)	10/20 (50 %%)
P value	**<0.001**	**0.069**	**0.389**
CD163 (with TMPRSS2)	22/31 (70.97 %)	5/22 (22.73 %)	2/22 (9.09 %)
CD 163 [17]	15/50 (30 %)	3/15 (20 %)	3/15 (20 %)
P value	**<0.001**	**0.843**	**0.340**

improve the design of human disease models, virus-resistant pigs and/or pig-to-human xenotransplantation, as previously reported [47].

5. Conclusions

The results of this study demonstrate that electroporation prior to *in vitro* fertilization (IVF) is a viable and efficient strategy for increasing the proportion of blastocysts with biallelic mutations in porcine embryos. This approach does not adversely affect early embryonic development. The concentration of sgRNA and Cas protein plays a significant role in the mutation and mosaicism rates and affects the overall efficiency of the gene editing system. Electroporation led to the processing of numerous oocytes in a single session using less expensive equipment. Double mutations can be efficiently generated using two different sgRNAs, with a high proportion of embryos having double mutations for specific gene targets. Electroporation offers the potential to edit multiple genes simultaneously in a single step, which is particularly valuable for xenotransplantation research, a critical requirement for improving the compatibility of porcine organs for human transplantation.

In conclusion, electroporation is a versatile and efficient technique for generating genetically modified porcine embryos for various gene targets, offering significant advantages for biomedical and agricultural research, xenotransplantation and disease resistance studies.

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CRediT authorship contribution statement

Sergio Navarro-Serna: Conceptualization, Investigation, Methodology, Writing – review & editing. **Celia Piñeiro-Silva:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Irene Fernández-Martín:** Investigation, Methodology. **Martxel Dehesa-Etxebeste:** Investigation, Methodology. **Adolfo López de Munain:** Conceptualization, Funding acquisition, Project administration. **Joaquín Gadea:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Writing – original draft, Writing – review & editing.

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