UNIVERSIDAD DE MURCIA

Escuela de Doctorado

TESIS DOCTORAL

Optimización de la generación de cerdos modificados genéticamente

Optimization of the generation of genetically modified pigs

AUTOR/A Celia Piñeiro Silva

DIRECTOR/ES Joaquín Jerónimo Gadea Mateos

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Index

Summary (TESEO) 1

Resumen (TESEO) 5

Resumen 9

Introduction 17

Justification of the scientific unity of the Doctoral Thesis 31

Hypotheses and Objectives 35

Unified summary of the research 39

Chapter 1. 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 47

Chapter 2. Optimizing oocyte electroporation for genetic modification of porcine embryos: Evaluation of the parthenogenetic activation 51

Chapter 3. Production of genetically modified porcine embryos by lipofection of *in vitro* matured oocytes using the CRISPR/Cas9 system 55

Chapter 4. Optimization of lipofection protocols for CRISPR/Cas9 delivery in porcine *zona pellucida* intact oocytes: A study of coincubation duration and reagent efficacy 59

Conclusions 63

Future directions 67

References 71

Summary (TESEO)

This thesis is presented as a compendium of four scientific publications that contribute to a line of research focused on the gene editing of porcine embryos using electroporation and lipofection techniques.

The objective of this thesis was to optimize the methods of gene editing in porcine embryos using the CRISPR/Cas9 system and electroporation and lipofection techniques. Improving the efficiency of these techniques will enable their application in biomedicine and animal production, including the generation of disease models, viral disease resistance models, and advances in xenotransplantation.

The studies were conducted using a standard procedure of *in vitro* production of porcine embryos. Different CRISPR/Cas9 gene editing protocols were applied and the efficiency of electroporation and lipofection was evaluated in terms of embryo development and mutation parameters. Genotyping was performed by fluorescent-PCR and capillary gel electrophoresis to verify the genetic modifications in porcine embryos, and their development up to the blastocyst stage was assessed.

It was shown that electroporation prior to *in vitro* fertilization (IVF) allows efficient generation of porcine embryos with single, double and multiple mutations. The mutation rate does not depend on the number of guides per treatment, but rather on the specific guide used. The concentration of single guide RNA (sgRNA) and Cas9 protein plays a critical role in the efficiency of the gene editing system, affecting both the mutation rate and the degree of mosaicism. In addition, the combination of electroporation and IVF enhances early embryonic development by increasing oocyte activation and the proportion of oocytes with a single pronucleus at 18 hours post-insemination, resulting in increased parthenote formation without altering the blastocyst rate. In terms of technical optimization, Opti-MEM was found to be a better electroporation medium than Duplex Buffer for embryo development. In addition, the timing of the electroporation procedure relative to IVF does not affect embryo development or parthenogenetic activation, but it does affect the mutation rate, which is more efficient when performed 7 hours after IVF.

In addition, it was confirmed that genetically modified porcine embryos could be generated by lipofection of *zona pellucida*-intact oocytes IVF, with an optimal liposome-RNP concentration of 5% v/v. No significant differences were found between the commercial lipofectamines CRISPRMAX™ and 3000 in terms of mutation rate and

embryo development. However, the optimal co-incubation time with lipofectamine CRISPRMAX™ was found to be 8 hours under our experimental conditions.

The results of this work make a significant contribution to the fields of reproductive biotechnology and gene editing, with applications in both the biomedical and livestock sectors.

Resumen (TESEO)

La presente tesis doctoral se presenta como un compendio de cuatro publicaciones científicas que contribuyen a una línea de investigación centrada en la edición genética de embriones porcinos mediante técnicas de electroporación y lipofección.

El objetivo de esta tesis fue optimizar las metodologías de edición genética en embriones porcinos mediante el uso del sistema CRISPR/Cas9 y de las técnicas de electroporación y lipofección. La mejora de la eficiencia de estas técnicas permitirá su aplicación en los campos de la biomedicina y la producción animal, incluyendo la generación de modelos de enfermedades, la resistencia a enfermedades virales y el avance en xenotrasplantes.

Los estudios se realizaron fundamentalmente siguiendo un procedimiento estándar de producción *in vitro* de embriones porcinos. Se aplicaron diferentes protocolos de edición genética con CRISPR/Cas9 y se evaluó la eficiencia de la electroporación y la lipofección en términos de desarrollo embrionario y parámetros de mutación. Se realizó el genotipado de los embriones mediante PCR fluorescente y electroforesis capilar en gel para verificar las modificaciones genéticas en los embriones porcinos y se evaluó su desarrollo hasta la fase de blastocisto.

Se demostró que la electroporación antes de la fecundación *in vitro* (FIV) permite generar embriones porcinos con mutaciones simples, dobles y múltiples de manera eficiente. La tasa de mutación no depende del número de guías por tratamiento, sino de la guía específica utilizada, y la concentración de ARN guía (sgRNA) y de la proteína Cas9 juega un papel crucial en la eficiencia del sistema de edición genética, afectando tanto a la tasa de mutación como al grado de mosaicismo. Además, la combinación de la electroporación con la FIV favorece el desarrollo embrionario temprano, ya que aumenta la activación ovocitaria y la proporción de ovocitos con un solo pronúcleo a las 18 horas post-inseminación, lo que incrementa la formación de partenotes sin alterar la tasa de blastocisto. En términos de optimización técnica, se identificó que el Opti-MEM es mejor medio de electroporación que el Duplex Buffer en términos de desarrollo embrionario. Asimismo, el momento de realización del procedimiento de electroporación con respecto a la FIV no influye en el desarrollo embrionario ni en la activación partenogénica, pero sí afecta a la tasa de mutación, siendo más eficiente si se realiza 7 horas después de la FIV.

Por otro lado, se comprobó que es posible generar embriones porcinos modificados genéticamente mediante lipofección de ovocitos con zona pelúcida intacta durante la FIV, con una concentración óptima de liposomas-RNP del 5 % v/v. No se encontraron

diferencias significativas entre las lipofectaminas comerciales CRISPRMAX™ y 3000 en términos de tasa de mutación y desarrollo embrionario, aunque se determinó que el tiempo de co-incubación óptimo con la lipofectamina CRISPRMAX™ es de 8 horas en nuestras condiciones experimentales.

Los resultados de esta tesis contribuyen significativamente al campo de la biotecnología reproductiva y la edición genética, con aplicaciones en los ámbitos biomédico y ganadero.

Resumen

La generación de cerdos modificados genéticamente está ganando importancia tanto en el campo de la biomedicina como en el de la agricultura. Por esta razón, se están generando cerdos editados genéticamente con diversas aplicaciones como investigación en xenotrasplantes, modelos de enfermedades humanas y producción ganadera.

El uso del sistema CRISPR/Cas9 ha facilitado la generación de estos animales mediante dos métodos principales: la transferencia nuclear de células somáticas (SCNT) y la modificación directa de embriones. La SCNT es una técnica compleja que implica la enucleación de un ovocito y su fusión con una célula somática modificada genéticamente. Por otro lado, se pueden introducir los elementos del sistema CRISPR/Cas9 directamente en el ovocito/zigoto mediante distintos métodos. El primero en ser desarrollado fue la microinyección, que consiste en la inyección del ovocito/ usando un micromanipulador. Al ser una técnica compleja y que requiere el uso de equipamiento específico, se están desarrollando técnicas más sencillas como la electroporación y la lipofección.

La electroporación consiste en la generación de poros en la membrana del ovocito gracias a su exposición a un campo eléctrico. Esta técnica es más rápida y reduce además el daño mecánico asociado a la microinyección. En cambio, este campo eléctrico puede producir la activación de los ovocitos sin la penetración del espermatozoide, generando así partenotes. Durante este proceso es necesario optimizar diferentes parámetros como son el número, duración, voltaje y polaridad de los pulsos, la concentración del sistema CRISPR/Cas9, etc. ya que cada uno influye en la eficiencia de la técnica.

Por último, la lipofección consiste en la encapsulación de las moléculas de interés en liposomas, que se pueden fusionar con o ser endocitadas por la célula. Así, la lipofección es más rápida y sencilla y no requiere de equipamiento específico. Los primeros estudios que utilizaron la lipofección en ovocitos o embriones porcinos eliminaban previamente la *zona pellucida* (ZP) de la célula. Mientras que la eliminación de la ZP facilita la incorporación de moléculas mediante lipofección, es un procedimiento invasivo que afecta negativamente al desarrollo embrionario, complica el manejo de los embriones y promueve su agregación.

Por todo lo anterior, esta tesis doctoral se basa en el compendio de cuatro publicaciones con el objetivo general de optimizar las técnicas de electroporación y lipofección.

Los objetivos específicos de cada uno de los capítulos fueron: evaluar la eficacia de electroporación de ovocitos previamente a la FIV, examinar el efecto de la electroporación

en la formación de pronúcleos y partenogénesis, explorar el uso de la lipofección en ovocitos con ZP como método alternativo y optimizar diferentes parámetros como la concentración, tipo de reactivo y tiempo de coincubación.

Metodológicamente, los cuatro estudios se basaron en el sistema convencional de producción *in vitro* de embriones porcinos. Los complejos cúmulo-ovocito fueron recogidos de ovarios de cerdas prepúberes de matadero y madurados *in vitro* por 40-44 h. Después de la maduración, los ovocitos fueron fecundados con semen de machos fértiles descongelado y seleccionado mediante *swim-up*. Después de la fecundación *in vitro* (FIV), los presuntos zigotos fueron cultivados hasta día 6 post-inseminación (pi). La tasa de primera división se evaluó en día 2 pi y la tasa de formación de blastocisto en día 6 pi. Los blastocistos se congelaron individualmente y se genotiparon mediante PCR fluorescente y electroforesis capilar en gel para analizar los tipos de mutación, niveles de mosaicismo y eficiencia general. Este flujo de trabajo nos permite una evaluación del desarrollo embrionario y de mutación, asegurando robustez en las metodologías utilizadas en los cuatro estudios.

En cada capítulo, ARNs guía (ARNg) se diseñaron utilizando el software Breaking-Cas disponible en CNB-CSIC (Oliveros *et al*., 2016). El ARNg y la proteína Cas9 fueron adquiridos en Integrated DNA Technologies (IDT, Coralville, IA, United States) y la ribonucleoproteína (RNP) fue sintetizada de acuerdo a las especificaciones comerciales.

La electroporación se realizó mayoritariamente antes de la FIV en medio Opti-MEM I Reduced Serum Media (Thermo Fisher, Waltham, MA USA) en un portaobjetos entre dos electrodos con una separación de 1 mm (45–0104, BTX, Harvard Apparatus, USA) conectados al ECM 830 Electroporation System (BTX, Harvard Apparatus, USA), utilizando 4 pulsos de 30 V y 1 ms de duración con 100 ms de intervalo.

La lipofección se realizó al mismo tiempo que la FIV utilizando Lipofectamina CRISPRMAX^{TM} Cas9 (Thermo Fisher, Waltham, USA) siguiendo las recomendaciones comerciales.

En primer lugar, en el **Capítulo 1** se utilizó la electroporación para generar embriones mono, doble o múltiple KO. Los ovocitos fueron electroporados con los componentes del sistema CRISPR/Cas9 a diferentes concentraciones inmediatamente antes de la FIV. Hasta 10 ARNg diferentes fueron diseñadas y utilizadas en diferentes combinaciones: modelos de guía única (genes PLCζ y FUS), modelos de doble guía (TPC1/TPC2 y

TMPRSS2/CD163) y modelos de guía múltiple (GGTA1/B4GALNT2/Pseudo B4GALNT2/CMAH/GHR).

En todos los experimentos observamos de forma general que la tasa de primera división fue más alta en los grupos electroporados en comparación con el grupo control (no electroporado), mientras que la tasa de blastocisto era similar. En los modelos de guía única la tasa de mutación (PLCζ: 80,0%; FUS 67,9%), la concentración óptima (PLCζ: 25 ng/µl Cas9:12,5 ng/µl ARNg; FUS: 50 ng/µl:25 ng/µl, respectivamente) y los niveles de mosaicismo (PLCζ: 84,1%; FUS: 34,2%) variaban en función de la guía. En los modelos de doble guía la mayoría de los embriones tenían la doble mutación deseada (TPC2/TPC1: 79,4%; TMPRSS2/CD163: 63,0%). Estas proporciones correspondían al producto de la mutación independiente de cada guía, sugiriendo que la tasa de mutación es inherente de la guía. En los modelos de guía múltiple la tasa de mutación para cada gen fue mayor del 85% con el 70% de los embriones con mutación en los cinco genes objetivo. A pesar de esto, el mosaicismo fue mayor del 70% para todas las guías. Este estudio demuestra que se pueden generar embriones mono, doble y múltiple KO mediante electroporación con una alta eficiencia, a pesar de los altos niveles de mosaicismo.

Este aumento en la tasa de primera división sugiere que los pulsos eléctricos pueden haber activado los ovocitos independientemente de la interacción con los espermatozoides. Por esto, en el **Capítulo 2** investigamos como los blastocistos obtenidos después de la electroporación y FIV provenían de la penetración del espermatozoide (embrión verdadero) o de la activación ovocitaria (partenote). Para distinguirlos, utilizamos semen de un macho TPC2 KO con una deleción de 11 pares de bases. Los embriones con esta mutación se clasificaron como embriones verdaderos (ya que la mutación proviene del ADN del espermatozoide), mientras que aquellos sin la deleción fueron clasificados como partenotes. Además, a 18 h pi una parte de los presuntos zigotos fueron fijados y teñidos con Hoechst para evaluar la penetración y activación.

Se realizaron tres experimentos. En el primero se evaluó la activación partenogénica de la electroporación comparando un grupo control fecundado, un grupo electroporado no inseminado (E) y un grupo electroporado e inseminado (E-FIV). En el segundo experimento se compararon dos medios de electroporación (Opti-MEM -Opti- y Duplex Buffer -DB) utilizando los mismos grupos experimentales (control, Opti-E-FIV, Opti-E, DB-E-FIV y DB-E). En el último experimento se investigó el tiempo en el que se realiza la electroporación en relación con el inicio de la FIV, realizando la electroporación en tres

momentos diferentes: una hora antes (E-FIV-t-1), justo antes (E-FIV-t0) y siete horas después (E-FIV-t7), además del grupo control. Además, en este experimento se utilizó el sistema CRISPR/Cas9 para el gen CAPN3.

En el primer experimento observamos un incremento en la activación a 18 h pi y un aumento de la tasa de primera división en los grupos electroporados en comparación con el control (86,6-91,3% vs 54,8% y 63,3-67,3% vs 49,1%, respectivamente, $p<0,05$), pero no se encontraron diferencias en la tasa de blastocisto (24,9-31,0%). La tasa de partenogénesis fue significativamente mayor en E-FIV en comparación con el control (30,2% vs 6,8%, $p<0,05$), una tendencia que se repite en los siguientes experimentos. En el segundo experimento observamos que el DB tiene un efecto negativo en el desarrollo embrionario con menor tasa de primera división y de blastocisto en comparación con los grupos Opti-MEM. La tasa de partenogénesis fue mayor en los grupos electroporados que en el control (DB-E-FIV: 60,0% y Opti-E-FIV: 48,5% vs 0%, $p<0,05$), sin diferencias entre los medios estudiados. En el último experimento, la electroporación aumentó la activación y la tasa de primera división sin diferencias en la tasa de blastocisto. La tasa de partenogénesis fue menor en el grupo control (2,44%) aunque fue similar en todos los grupos electroporados (51,6-63,9%) mientras que la tasa de mutación fue mayor en el grupo t7 (81,3% vs 54,6-57,5%). En general, la tasa de mutación fue mayor en los partenotes (71,9%) que en los embriones verdaderos (54,8%).

Estos dos primeros capítulos demostraron que la electroporación es un método rápido y eficiente para testar ARNg. A pesar de esto, la activación partenogénica de los ovocitos supone un problema en la generación de animales vivos por lo que es necesaria la investigación de los parámetros de electroporación para conseguir mejor tasa de mutación minimizando el mosaicismo y la activación partenogénica.

Por esta razón hemos investigado en los siguientes capítulos el uso de la lipofección en ovocitos porcinos. En estudios previos solamente se han utilizado ovocitos y zigotos sin zona pelúcida (ZP) para generar embriones mutantes, en cambio, la eliminación de la ZP puede afectar negativamente en el proceso.

En el **Capítulo 3** se investigó la viabilidad de la lipofección en ovocitos con ZP para generar embriones modificados genéticamente, utilizando la Lipofectamina CRISPRMAX^{TM} e incubando los ovocitos con los liposomas durante toda la FIV. Se evaluaron dos factores: la concentración de complejos liposoma-RNP (5% y 10% v/v) y

la concentración de RNP en los liposomas (50 ng/µl Cas9:25 ng/µl ARNg -1x- y 100 ng/µl:50 ng/µl -2x), manteniendo un grupo electroporado como control positivo de mutación y un grupo sin tratamiento como control negativo.

Nuestros resultados mostraron que la tasa de primera división y de blastocisto fueron más bajas en el grupo 10% v/v (30,45% y 13,41%, respectivamente) en comparación con los otros grupos, sin diferencias entre ellos. La tasa de mutación fue similar en todos los grupos tratados, pero la eficiencia fue menor en el grupo 10% v/v (electroporados: 6,38%; 5%: 5,52% vs 10%: 2,02%, $p<0,05$). Además, la concentración de RNP en los liposomas no afectó al desarrollo embrionario o a los parámetros de mutación, con resultados similares en cuanto a eficiencia de los grupos 1x, 2x y electroporados (1,75%, 3,60% y 3,75%, respectivamente). Esto sugiere que la lipofección se puede utilizar para introducir el sistema CRISPR/Cas9 en ovocitos con ZP, pero la concentración de liposomas puede afectar negativamente al proceso.

Por último, en el **Capítulo 4** se evaluaron dos factores adicionales para la lipofección: el tipo de reactivo y la duración de la co-incubación con lipofectamina y espermatozoides.

En el primer experimento, los ovocitos fueron incubados durante toda la FIV con Lipofectamina CRISPRMAX™ o Lipofectamina 3000 utilizando dos ARNg contra los genes CAPN3 y CD163, manteniendo un grupo electroporado como control positivo y un grupo sin tratamiento como control negativo. En el segundo experimento se evaluaron diferentes duraciones de co-incubación ovocito-espermatozoide-liposomas (4, 8 y 24 h), con un grupo sin tratamiento como control negativo.

No se encontraron diferencias significativas entre grupos lipofectados en términos de tasa de blastocisto (12-16%), tasa de mutación (35-55%) o eficiencia (3,5-7%). Los niveles de mosaicismo y número de alelos fueron diferentes entre ARNg, pero no entre grupos, indicando que son parámetros guía-dependientes. En cuanto al tiempo de co-incubación, la tasa de primera división fue menor en los grupos 4 h (42,6%) y 24 h (49,44%) en comparación con el control (61,54%, $p<0,05$). Esta disminución puede deberse al reducido tiempo de incubación con los espermatozoides en el grupo 4 h y por la posible toxicidad de la lipofectamina en el grupo 24 h. No se encontraron diferencias en la tasa de blastocisto (21,02-29,71%), pero el grupo 8 h mostró mayor tasa de mutación (49,02%) y eficiencia (14,36%), sugiriendo que es el tiempo de co-incubación óptimo entre los estudiados.

Estos resultados demostraron que la lipofección es un método viable para generar cerdos modificados genéticamente sin la necesidad de la eliminación de la ZP. Esta técnica es más simple que la microinyección, sin requerir equipamiento o personal especializado, permitiendo la lipofección simultánea de cientos de ovocitos sin manipulación. A pesar de esto, su eficiencia es todavía baja y necesita más optimización.

En conclusión, esta tesis se centra en la optimización de la generación de embriones porcinos modificados genéticamente utilizando dos técnicas: la electroporación y la lipofección. Hemos investigado sistemáticamente varios factores que influyen en la eficiencia de las técnicas, identificando aspectos clave que afectan al desarrollo embrionario y a la eficiencia de edición global.

Ambas técnicas se utilizaron de forma exitosa para generar embriones modificados, demostrando su potencial como alternativas a la microinyección. A pesar de ello, cada método conlleva retos específicos que necesitan ser solventados para mejorar la reproducibilidad y la eficiencia y es esencial continuar trabajando en la optimización, mejorando la precisión de la edición y minimizando la toxicidad, para maximizar la aplicabilidad de estas técnicas para modificación genética en cerdos a gran escala.

Introduction

In agriculture and biomedicine, the porcine species (*Sus scrofa*) has become increasingly important. On the one hand, it is the second most consumed meat in the world after poultry, according to the Food and Agriculture Organization of the United Nations (FAO, 2024). On the other hand, their similarities to humans in terms of size, physiology and anatomy makes this species an excellent model for mimicking human diseases and for other biomedical purposes (reviewed by Navarro-Serna *et al*., 2022c). For this reason, genetically modified pigs are being produced for various applications in these fields, including xenotransplantation research (Fischer *et al*., 2020; Hein *et al*., 2020), mimicking human diseases (Koppes *et al*., 2020; Tanihara *et al*., 2018) and livestock production, which includes improving meat production (Hirata *et al.,* 2021b; Tanihara *et al*., 2016) and resistance to viral diseases (Chen *et al*., 2019; Yang *et al*., 2018).

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR Associated Protein 9 (Cas9) genome editing technology has had a major impact on the production of mutant animals, including pigs (Hai *et al*., 2014; Whitworth *et al*., 2014). This system consists of an endonuclease (Cas9) and a guide RNA (gRNA). The gRNA guides the Cas9 protein to the target site in the genome and the endonuclease creates a double-strand break (DSB) in the DNA (**Figure 1**). Once this break occurs, the cell can repair it through two different molecular pathways: non-homologous end joining (NHEJ) or homology-directed repair (HDR). In most cases, the NHEJ pathway is used for repair. During this repair, small insertions or deletions (indels) can occur, which, if it is not a multiple of three, will alter the reading frame and cause the premature appearance of a stop codon. This results in a knock-out (KO). If a donor DNA is inserted along the CRISPR/Cas9 system, the repair can occur via the HDR pathway. In this case, the foreign DNA sequence is introduced, which can be a change of a few base pairs (bp) or an entire gene, resulting in a knock-in (KI) (Jinek *et al*., 2012; Petersen, 2017; Whitelaw *et al*., 2016).

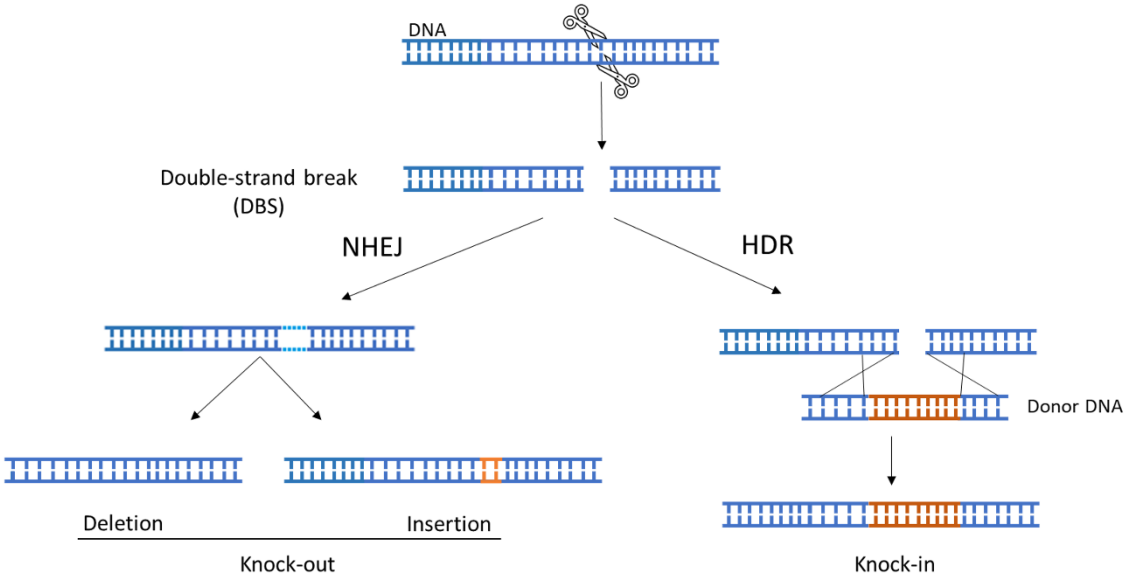

Figure 1. Mechanism of action of the CRISPR/Cas9 system. (Image from Navarro-Serna *et al*., 2022c).

There are two main methods to produce a genetically modified pig: somatic cell nuclear transfer (SCNT) and the direct introduction of the CRISPR/Cas9 system compounds into the oocyte/embryo (reviewed by Navarro-Serna *et al*., 2022c).

SCNT, also known as cloning, involves the enucleation of a mature oocyte and the fusion of this enucleated oocyte with a somatic cell. This cell is genetically modified and selected previously, so that the nucleus of the resulting embryo will be the same as that of the somatic cell. This technique has the advantage that the genotype of the offspring is known, and it is useful for complex modifications because they are easier to perform in cultured cells. In addition, SCNT facilitates the production of multiple genetically identical pigs, which is particularly valuable for studies requiring uniform genetic backgrounds in biomedical and agricultural research (Nesiyama *et al*., 2025). On the other hand, this technique is complex and involves different procedures, it requires trained personnel and specific equipment (e.g., a micromanipulator and a fusion device) and the overall efficiency is limited (Beaujean *et al*., 2015). Nevertheless, genetically modified pigs have been produced by SCNT in the fields of basic science (Lai *et al*., 2016; Shi *et al*., 2020), livestock production (Luo et al., 2019; Wang et al., 2015) and biomedicine (Dorado et al., 2019; Fischer *et al*., 2020).

The CRISPR/Cas9 system components can be introduced directly into oocytes or early embryos (zygotes) in a single step, so that all cells derived from that single cell are expected to be edited. Microinjection is the most commonly used method to deliver CRISPR/Cas9 components into the oocyte/zygote. However, this technique requires specialized equipment (e.g., a micromanipulator), trained personnel, and is slow to perform (it requires microinjection of oocytes/embryos one at a time) making it technically challenging (Navarro-Serna *et al*., 2021). To address these challenges, alternative methods such as electroporation and lipofection are being developed that offer simpler and less expensive approaches with the same level of efficiency in gene editing (Hirata *et al*., 2021b; Navarro-Serna *et al*., 2022a; Tanihara *et al*., 2016).

1. Electroporation

Electroporation was initially used to activate oocytes and zygotes after fertilization (Funahashi *et al*., 1993; Sun *et al*., 1992) and after fusion of somatic cells with enucleated oocytes in cloning procedures (Tao *et al*., 2000). Over time, this technique has evolved beyond activation purposes and is now widely used to deliver various molecules, including DNA, RNA and proteins, into spermatozoa, oocytes and embryos and somatic cells (Colleoni *et al*., 2005; da Silva *et al*., 2018; Frangoul *et al*., 2021). Electroporation is a technique in which oocytes or zygotes are exposed to an electric field that causes the temporary formation of pores in the membrane of the cells. These pores allow the components of the CRISPR/Cas9 system present in the surrounding electroporation media to enter the cytoplasm (**Figure 2**) (Colleoni *et al*., 2005; Lin and Van Eenennaam, 2021). The main advantage over the previously described techniques is its simplicity, as it requires only a stereomicroscope, an electroporator and an electroporation slide. More than 50-100 oocytes/zygotes can be electroporated simultaneously, making this technique faster than microinjection.

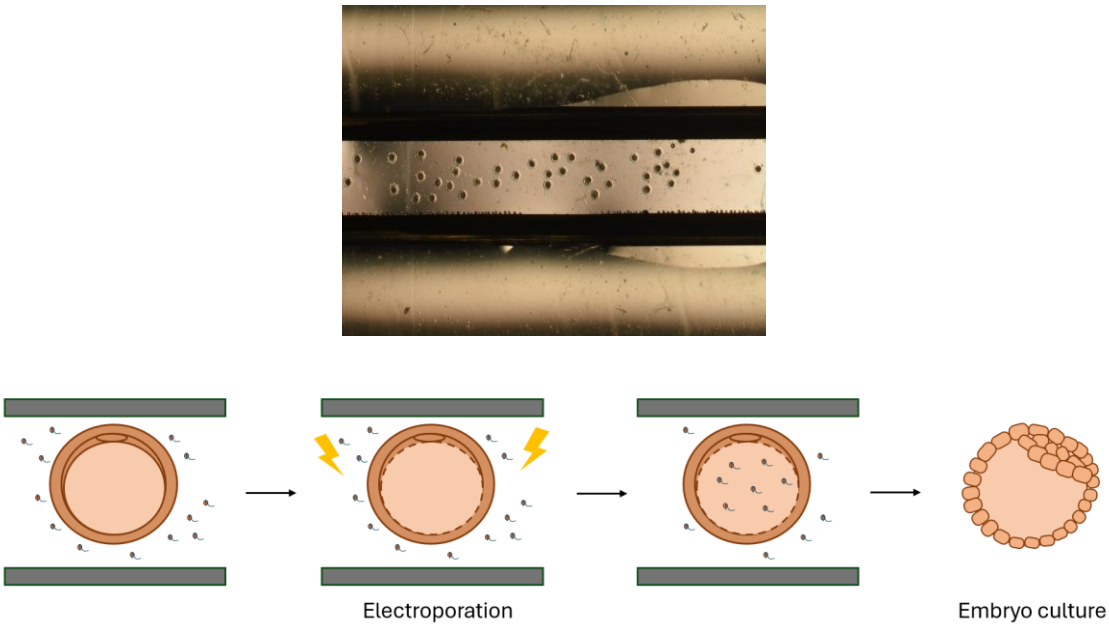

Figure 2. Schematic representation of electroporation of oocytes/zygotes. (Image modified from Navarro-Serna *et al.*, 2022c).

The first study to develop this technique using porcine embryos was reported by Tanihara *et al.* in 2016, generating a KO pig for the MSTN gene (Tanihara *et al*., 2016). Up to the current date, only eight KO live pig models (including single, double and triple modifications) and one model with a point mutation by HDR have been generated (Tanihara *et al*., 2023b, 2023a, 2021, 2020b, 2020a, 2019, 2018, 2016). However, the use of this method in porcine oocytes or embryos is not well understood and its use has been limited to a small number of groups worldwide. Research on porcine electroporation has mainly focused on the protocol optimization, including electroporation time, pulse duration and voltage, number of pulses, etc.

It has been shown that the **voltage** of the pulses plays a critical role in determining the efficiency of the electroporation technique. In somatic cells, increasing the voltage increases the density of the pores generated (Krassowska and Filev, 2007). In porcine oocytes and embryos, the electroporation is usually performed at voltages in the range of 25 to 30 V/mm (Navarro-Serna *et al*., 2022a; Nishio *et al*., 2018). The optimization of the voltage can determine the general outcomes of the procedure, as high voltage promotes a better entry of the CRISPR/Cas9 system compounds into the oocyte or embryo by increasing membrane permeability (Fernández *et al*., 2024), but it can affect embryo

development such as decreasing cleavage and blastocyst rates and increasing apoptosis (Nishio *et al*., 2018).

In regard to pulse **polarity**, both unipolar and bipolar pulses can be used in electroporation protocols. Unipolar pulses consist of electrical pulses of a single polarity, whereas bipolar pulses consist of two consecutive unipolar pulses of opposite polarity (Novickij *et al*., 2022). Nishio *et al*. compared the use of unipolar and bipolar pulses when other electroporation parameters (voltage and pulse duration and number) were held constant and found that bipolar pulses had a detrimental effect on blastocyst formation (Nishio *et al.*, 2018).

Other important factors for the electroporation technique are the **number** and the **duration** of the pulses, which determine the number and size of the pores generated and subsequently affect the molecular uptake and cell viability. In general, shorter pulses allow faster regeneration of the cell membrane, and increasing the number of pulses increases the density of the pores. In porcine oocytes, the number of pulses has a direct effect on molecular uptake (Navarro-Serna *et al*., 2022a), but its effect on embryo development remains unclear. Navarro-Serna *et al.* found that the cleavage rate is higher in electroporated groups (receiving 2, 4, or 6 pulses of 1 ms at 30V/mm) than in the control but the blastocyst rate remains similar, whereas Hirata *et al.* found that cleavage and blastocyst rate were lower in electroporated groups (receiving 3, 5 or 7 pulses of 1 ms at 30 V/mm) (Hirata *et al*., 2019; Navarro-Serna *et al*., 2022a). In porcine zygotes, increasing the number and duration of the pulses was found to affect embryo development but increased mutation efficiency (Hirata *et al*., 2019; Tanihara *et al*., 2016).

The **developmental stage** at which electroporation is performed has a significant impact on the efficiency of the technique. Electroporation can be performed in oocytes before *in vitro* fertilization (IVF) or in zygotes at different times after IVF. Tanihara *et al.* examined different electroporation times after IVF (11, 13, 19 and 23 h) and found that the blastocyst rate was higher when electroporation was performed 13 h after IVF, while the mutation rate was similar for all groups (Tanihara *et al*., 2016). Hirata *et al.* compared the electroporation of *in vitro* matured oocytes before IVF with zygotes 13 h after IVF and found that even though the oocyte group had a lower cleavage rate, the blastocyst and mutation rates remained similar for both cell types (Hirata *et al*., 2019). Later, Le *et al.* investigated the electroporation at different cleavage stages comparing one-cell embryos (12 h post IVF) with two-cell embryos (24 h post IVF). They observed that the blastocyst

rate tended to be higher in the two-cell embryo group, but the mutation efficiency was not affected by the developmental stage (Le *et al*., 2021).

Other critical factors are those related to the **CRISPR/Cas9 system** such as Cas9 type (mRNA or protein), reagent concentration and sgRNA design. Cas 9 protein is normally used in electroporation procedures. Tanihara *et al.* found that the mutation rate is higher with Cas9 protein than mRNA due to its smaller size which can facilitate the cellular uptake (Tanihara *et al*., 2016). Reagent concentration can be critical as excessive levels can increase cytotoxicity, decrease embryo development and increase mosaicism, while low levels can reduce gene editing efficiency; generally, 50 ng/µl is recommended for Cas9 protein and 25 ng/µl for sgRNA (Le *et al*., 2020; Park *et al*., 2024). In this context, the specific sequence of the sgRNA can also influence the efficiency of the procedure, depending on the target gene, the genomic context and the experimental conditions. Several studies have shown that sgRNAs targeting the same gene can result in different mutation efficiencies and developmental rates (Tanihara et al., 2023b, 2020b, 2020a, 2018).

Finally, different **electroporation media** can be used to perform this technique. In general, two commercial media are used: Opti-MEM (Tanihara *et al*., 2016) and Nuclease-Free Duplex Buffer (DB) (Nguyen *et al*., 2024). Their composition differs as Opti-MEM media is a modification of Eagle's Minimal Essential Medium, buffered with HEPES and sodium bicarbonate, and supplemented with hypoxanthine, thymidine, sodium pyruvate, L-glutamine, insulin, transferrin, trace elements and growth factors, with a $CaCl_2$ concentration of 99.9 mg/L (Navarro-Serna *et al*., 2022b) while DB is a buffer containing 30 mM HEPES and 100 mM potassium acetate (Tanihara et al., 2023b), but, up to our knowledge, there is no comparative study between them.

For all of the above, there are several key factors that need to be optimized when starting an electroporation procedure. In **Chapter 1** we investigated the possibility of producing genetically modified pig embryos by electroporation of oocytes prior to IVF for different models. We used up to 10 different sgRNAs to target different genes alone or in combination of several sgRNAs. When using only one sgRNA, we tested different concentrations of ribonucleoprotein (RNP; Cas9 protein and sgRNA) for two models (FUS and PLCζ genes). We found that the optimal concentration was different for each gene, so optimization of this parameter is needed when using a new guide. Next, when using two sgRNAs simultaneously (TPC1/TPC2 genes and CD163/TMPRSS2 genes), we

observed that the mutation rate of each guide is independent of the number of guides, as the double mutation rate is equal to the multiplication of each single mutation rate. Finally, we were able to generate mutant embryos for 5 different genes in the same electroporation treatment (GGTA11, CMAH, B4GALNT2, pseudo-B4GALNT2 and GHR) as a model for xenotransplantation.

It has been shown that exposure of mature oocytes to an electric field can lead to their activation, inducing the formation of parthenotes (Koo *et al*., 2008; Procházka *et al*., 1992) and triggering activation-related effects such as pronuclear formation and exocytosis of cortical granules (Funahashi *et al*., 1995; Wang *et al*., 1998). However, electrical stimulation in combination with IVF has not been studied in depth. A few studies suggest that the oocytes can be activated either by the sperm or the electrical pulse, which can prevent the formation of male pronuclei (Funahashi *et al*., 1995, 1993; Wang *et al*., 1998).

For these reasons, in **Chapter 2** we focused on the possibility of parthenogenetic activation of the oocytes during the process of electroporation to generate genetically modified embryos. First, we compared three experimental groups: a control group (only inseminated), an electroporated and inseminated group and an only electroporated group. We observed that the cleavage was higher in the electroporated groups (with or without IVF), but the blastocyst rate remained similar. The parthenote rate was significantly higher in the electroporated and inseminated group in comparison with the control group (30.2 vs 6.8%). In the second experiment, we compared two commercial electroporation buffers (Opti-MEM and Duplex Buffer) with the same experimental groups as before. There were differences between buffers in embryo development, with the Opti-MEM being the best option, but no difference was found in parthenote activation, and it was significantly higher than the control. Finally, we analyzed the time when the electroporation procedure was performed in relation to IVF (1 h before, at the same time and 7 h after). Again, the blastocyst rate did not differ from the control, but the parthenote rate was higher in all electroporated groups, without differences between the times (51.6-63.9%).

This high incidence of parthenogenetic activation during the electroporation process can be a problem if the final objective is to generate a genetically modified animal, since only the true embryos are able to develop. For that reason, we analyzed the use of the lipofection technique to generate genetically modified embryos.

2. Lipofection

Lipofection is a method commonly used in cells to deliver foreign molecules into the cytoplasm. It involves the encapsulation of the molecules in vesicles called liposomes, which consist of a lipid bilayer that protects the cargo while facilitating their delivery into target cells (Felgner *et al*., 1987). These liposomes can be endocytosed or fuse with the cell membrane to release the molecules, depending on the liposome and cell type (Gandek *et al*., 2023). Liposomes were originally designed to transfer DNA (Felgner *et al*., 1987), but they have been modified to encapsulate and deliver other compounds, such as those of the CRISPR/Cas9 system (reviewed by Yin *et al*., 2023). The procedure implies the encapsulation of the cargo (Cas9 protein and sgRNA) into the vesicles and subsequent coincubation of the liposomes with the target cells (**Figure 3**). This allows the use of a large number of oocytes/embryos without the need for handling.

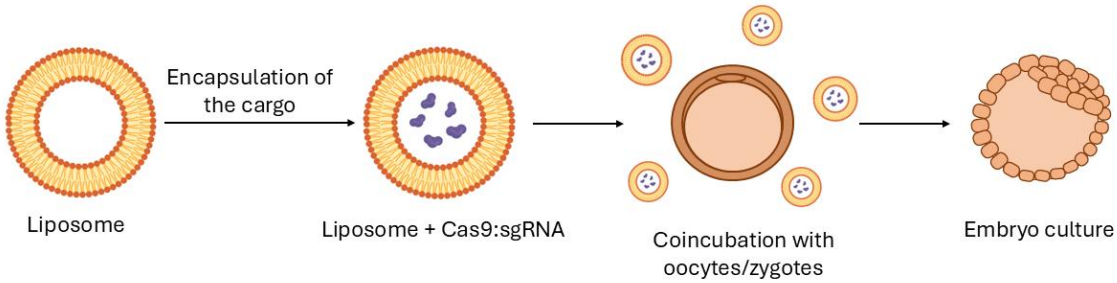

Figure 3. Schematic representation of lipofection of oocytes/zygotes.

Despite its wide use in cells, lipofection of porcine oocytes and embryos has not been studied in depth. Hirata *et al.* reported the first use of this technology in porcine zygotes. They used liposomes to introduce the components of the CRISPR/Cas9 system into *in vitro* produced embryos that had previously denuded to remove the *zona pellucida* (ZP) (Hirata *et al*., 2021b, 2021a). To date, only one KO pig model has been generated using this method, with a monoallelic mutation for the MSTN gene (Hirata *et al*., 2021b).

These authors suggest that ZP may block liposome entry in pigs. In an interesting study, Takebayashi *et al.* compared the use of lipofection to generate genetically modified embryos in ZP-intact and ZP-free zygotes. They found only monoallelic mutations when

ZP-free embryos were lipofected, and no mutation was found in ZP-intact embryos (Takebayashi *et al*., 2022). The process of removing the ZP from the zygote, which is an aggressive procedure, can have a detrimental effect on embryo development, implies more complicated embryo handling, and may facilitate embryo aggregation, which can induce mosaicism (Hirata *et al*., 2021a). To overcome this issue, some authors have explored the single-culture of ZP-free embryos in 25-well plates with better blastocyst formation than in large groups in 4-well plates (Lin *et al*., 2024).

The studies of lipofection of porcine oocytes and zygotes focused on its optimization. Different factors such as types of carriers, concentration, time of coincubation and developmental stage have been studied.

The **carrier** and the **concentration** of the reagents can determine the efficiency of the process. Different studies have investigated the use of different commercial lipofectamines and other types of carriers such as: lipofectamine 2000, lipofectamine 3000, lipofectamine CRISPRMAX^{TM}, jetCRISPR, TransIT-X2, ProteoCarry… Interestingly, the mutation rate is similar for all the compounds, but the blastocyst rate can vary (Lin *et al*., 2024, 2023). The concentration of the liposomes has an influence on embryo development and mutation rate. When using jetCRISPR, increasing the concentration did not affect the blastocyst rate, but increased the mutation rate (Hirata *et al*., 2021b).

Another important factor is the **developmental stage** of the oocyte/embryo at the beginning of the coincubation. The lipofection protocol can be performed in oocytes or in zygotes after IVF. When it is performed in oocytes, the gametes and liposomes are usually co-incubated during the entire treatment period. Hirata *et al.* compared the lipofection treatment in oocytes (for 5 h at the time of IVF) and in zygotes (for 5 h at 24 or 29 h post-insemination -pi) and found that the mutation rate was higher when lipofecting zygotes (Hirata *et al*., 2021a). Also, different hours post-insemination were tested (5, 10, 15 h pi), showing that when treatment starts at 5 h pi there is no mutation in the resulting blastocysts (Lin *et al*., 2021).

Regarding the **coincubation time**, different durations were tested (2.5, 5, 10 and 20 h). It was shown that the mutation rate and the blastocyst rate decreased when the time of coincubation increased, showing a possible toxic effect of the treatment (Lin *et al*., 2021).

For all these reasons, it is necessary to optimize the conditions for each protocol to balance embryo development and quality with mutation efficiency.

The use of lipofection to generate genetically modified pigs will be improved by the use of ZP-intact oocytes/embryos. In **Chapter 3** we investigated the use of Lipofectamine CRISPRMAX™ to generate mutant embryos by lipofection of ZP-intact oocytes. We lipofected porcine *in vitro* matured ZP-intact oocytes during the IVF process and optimized the protocol with respect to the concentration of lipofectamine-ribonucleoprotein (RNP) complexes and the concentration of RNP in the liposomes. We were able to generate mutant embryos for the CD163 gene using this method. The CD163 gene encodes the binding protein for the porcine respiratory and reproductive syndrome (PRRS) virus. The KO animals for CD163 are known to be resistant to this disease, which causes important economic losses in the swine industry worldwide (Yang *et al*., 2018).

First, we used two different concentrations of lipofectamine-RNP complexes (5% vs. 10% v/v) and compared them with the standard method in the laboratory, which was electroporation. We found that the increment on this concentration had a detrimental effect on embryo development and on mutation rate, but the 5% group had similar effects to the electroporation technique. Then, using the 5% v/v concentration, we increased the RNP concentration within the liposomes (50 ng/µl Cas9 protein:25 ng/µl sgRNA vs 100 ng/µl Cas9 protein:50 ng/µl sgRNA) and found that there was no effect on either embryo development or on mutation rates of the RNP concentration within the liposomes.

Chapter 4 focused on the comparison of two commercial lipofectamines and on the time of coincubation with the liposomes. First, we compared the Lipofectamine CRISPRMAX™ and the Lipofectamine 3000 using two different sgRNAs: one targeting the CD163 gene (the same as in Chapter 3) and another targeting the CAPN3 gene. CAPN3 deficiency is related to the limb-girdle muscular dystrophy recessive 1 (LGMDR1), an autosomal recessive myopathy described in humans (usually in children and young adults) that is characterized by progressive weakening of the shoulder, pelvic and proximal limb muscles (Lasa-Elgarresta *et al*., 2019).

In the first experiment, we compared the two commercial lipofectamines using the same protocol as described in Chapter 3, co-incubating the ZP-intact oocytes and the liposomes throughout the IVF process (18-20 h) at a 5% v/v concentration. The resulting embryos were genotyped. We found that blastocyst, mutation and mosaicism rates and the overall

efficiency were similar for both of the two groups, independently of the guide used. In the second experiment, we focused on the lipofectamine CRISPRMAX™ and the CD163 guide. We initiated the treatment at the beginning of IVF and evaluated three different coincubation times: 4, 8, 24 h. We found that the blastocyst rate is not affected by the treatment, but the mutation rate is higher with 8 h of coincubation in comparison to 24 h (49.0% vs. 27.0%), and the best efficiency is achieved with 8 h (14.4%).

In conclusion, this Doctoral Thesis is a continuous work with the main objective of optimizing the conditions of production of genetically modified pig embryos, focusing on the electroporation and lipofection techniques, an easier alternative to microinjection and SCNT.

Our study highlights the potential of electroporation as a viable alternative for generating genetically modified porcine embryos and demonstrates its efficiency in delivering CRISPR/Cas9 components and generating targeted mutations. We successfully optimized key parameters, including sgRNA concentration and number of simultaneous gene modifications, providing a framework for further advancements in gene editing techniques. In addition, our findings of unintended parthenogenetic activation of oocytes during electroporation highlight the need for further investigation of the interaction between electrical stimulation and fertilization processes. Future research should focus on refining electroporation protocols to increase mutation efficiency while minimizing unwanted activation effects

On the other hand, lipofection represents a promising alternative for genetic modification of porcine embryos, especially when applied to ZP-intact oocytes during the IVF process. Our results demonstrate that Lipofectamine CRISPRMAX™ can efficiently deliver CRISPR/Cas9 components to generate mutant embryos for genes of interest with comparable results to electroporation. Optimization of key parameters, including lipofectamine-RNP concentration and co-incubation time, showed that a 5% v/v concentration and an 8 h incubation time yielded the highest mutation efficiency while maintaining embryo viability. Future applications should focus on further refining the protocol to increase mutation rates and reduce mosaicism while ensuring that embryo development is not compromised.

Justification of the scientific unity of the Doctoral Thesis

The present doctoral thesis is submitted as a compendium of four scientific publications, all of which contribute to a coherent and unified line of research centered on the genetic modification of porcine embryos through electroporation and lipofection techniques. The studies included in this thesis collectively address critical aspects of gene editing methodologies and their applications in biomedicine and animal production. The common scientific objectives, methodologies, and overarching themes of these studies establish the necessary unity to justify their inclusion in a single doctoral dissertation.

All four publications focus on optimizing gene editing techniques in porcine embryos, specifically using the CRISPR/Cas9 system. The central aim is to improve the efficiency and applicability of these genetic modifications for purposes including disease resistance, xenotransplantation and biomedical research. The studies investigated different aspects of the process, such as the optimization of electroporation conditions, the differentiation between parthenogenetic activation and true fertilization, and the comparison between electroporation and lipofection as delivery methods for CRISPR/Cas9 components.

Each study represents a logical progression in the development of efficient gene editing protocols: Chapter **1** (Navarro-Serna, Piñeiro-Silva *et al*., 2024, *Theriogenology*) demonstrates the effectiveness of electroporation before *in vitro* fertilization (IVF) to generate genetically modified embryos with single, double, and multiple knock-out (KO) modifications relevant to biomedical and agricultural applications. Chapter **2** (Piñeiro-Silva *et al*., 2024, *Theriogenology*) explores the effects of electroporation on oocyte activation, addressing the challenge of differentiating true embryos from parthenotes and optimizing the conditions to maximize genetic modification efficiency. Chapter **3** (Piñeiro-Silva *et al*., 2023, *Animals*) presents an alternative to electroporation by evaluating lipofection as a non-invasive gene delivery method, comparing its efficiency with electroporation in terms of embryo development and mutation rates. Chapter **4** (Piñeiro-Silva *et al.*, 2024, *Theriogenology*) further refines the lipofection methodology developed in previous studies.

The four studies included in this thesis constitute a unified and cohesive body of work, demonstrating a progressive and systematic approach to refining gene editing methodologies in porcine embryos. The scientific continuity, shared objectives, and methodological consistency justify their inclusion as a compendium of publications forming a single doctoral thesis.

Hypotheses and Objectives

Hypotheses

The main hypothesis of this Doctoral Thesis was that the conditions for generation of genetically modified pig embryos with the CRISPR/Cas9 system by electroporation and lipofection can be optimized.

The specific hypotheses were:

- Electroporation procedures can be used to produce single, double and multiple KO pig embryos (Chapter 1).
- Electroporation can influence parthenogenetic activation of oocytes (Chapter 2).
- It is possible to produce genetically modified pig embryos by lipofection of oocytes without the need to remove the ZP as an alternative to electroporation (Chapter 3 and 4).
- The concentration of lipofectamine-RNP complexes and RNP can influence the embryo development and mutation rates during lipofection of ZP-intact oocytes (Chapter 3).
- The type of lipofectamine and the coincubation time of lipofectamine and oocytes can influence embryo development and mutations rates during lipofection of ZP-intact oocytes (Chapter 4).

Objectives

The main objective of this research was to optimize the methods for CRISPR/Cas9 gene editing in porcine embryos, with a focus on electroporation and lipofection. The overall goal was to improve the efficiency and applicability of these techniques for biomedical and animal production purposes, including the development of disease models, viral disease resistance, and advances in xenotransplantation. Through a series of studies, the research evaluated different electroporation and lipofection protocols, analyzed embryo development outcomes, and assessed the impact of different experimental conditions on mutation rates and embryo viability.

The specific objectives were:

1. Analyze the possibility of producing single, double and multiple KO pig embryos by electroporation (Chapter 1).

2. Study the implications of the concentration of sgRNA and Cas9 and the number of guides on the outcome of the electroporation technique (Chapter 1).
3. Analyze the implications of the electroporation procedure in parthenogenetic activation of the oocytes (Chapter 2).
4. Study the use of two commercial electroporation media (Chapter 2).
5. Investigate if the moment when the electroporation is performed in relation to the beginning of the IVF influences parthenote activation, embryo development and mutation parameters (Chapter 2).
6. Produce genetically modified pig embryos by lipofection of ZP-intact oocytes as an alternative to electroporation and optimize the concentration of lipofectamine-RNP complexes and only RNP (Chapter 3).
7. Investigate the use of two commercial lipofectamines in the production of genetically modified pig embryos by lipofection of ZP-intact oocytes (Chapter 4).
8. Analyze the duration of coincubation in the production of genetically modified pig embryos by lipofection of ZP-intact oocytes (Chapter 4).

Unified summary of the research

Methodologically, all the four studies were based on the conventional system for *in vitro* porcine embryo production. Cumulus-oocyte complexes were recovered from the ovaries of slaughtered prepubertal gilts and subsequently matured *in vitro* for 40-44 h. Following maturation, the oocytes were inseminated with frozen-thawed sperm from fertile boars selected using the swim-up procedure. After *in vitro* fertilization (IVF), the putative zygotes were cultured until day 6 post-insemination (pi). Cleavage rates were assessed on day 2 pi, while blastocyst formation rates were evaluated on day 6 pi, when individual blastocysts were frozen and genotyped using fluorescent-PCR capillary gel electrophoresis to analyze mutation types, levels of mosaicism, and overall gene-editing efficiency. This workflow allowed for a comprehensive evaluation of embryo development and genetic modifications, ensuring a robust assessment of the methodologies employed across the four studies.

In each chapter, single guide RNAs (sgRNAs) were designed for various target genes using the Breaking-Cas software available from CNB-CSIC (Oliveros *et al*., 2016). Both the sgRNAs and Cas9 protein were purchased from Integrated DNA Technologies (IDT, Coralville, IA, United States) and the ribonucleoprotein (RNP) was generated as commercially specified.

Electroporation was mainly performed before IVF in Opti-MEM I Reduced Serum Media (Thermo Fisher, Waltham, MA USA) 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) using 4 pulses at 30 V, 1 ms pulse duration and 100 ms pulse interval.

Lipofection was mainly performed at the same time as IVF using Lipofectamine CRISPRMAX™ Cas9 (Thermo Fisher, Waltham, USA) as commercially specified.

In **Chapter 1**, electroporation was used to generate single, double or multiple knock-out (KO) embryos. Oocytes were electroporated with the components of the CRISPR/Cas9 system (sgRNA and Cas9 protein) at different concentrations immediately before IVF. Up to 10 different sgRNAs were designed and used in diverse combinations: single-guide models targeting PLCζ and FUS genes, two-guide models targeting TPC1/TPC2 and TMPRSS2/CD163 genes, and multiple-guide models targeting 5 genes related to xenotransplantation (GGTA1/B4GALNT2/Pseudo B4GALNT2/CMAH/GHR).

We found that the cleavage rate was higher in the electroporated groups compared to the non-electroporated control group, while the blastocyst rate remained similar. When examining single-guide models, the mutation rate (PLCζ: 80.0%; FUS 67.9%), the optimal CRISPR/Cas9 components concentrations (PLCζ: 25 ng/µl Cas9:12.5 ng/µl sgRNA; FUS: 50 ng/µl:25 ng/µl, respectively) and the mosaicism levels (PLCζ: 84.1%; FUS: 34.2%) varied depending on the guide. When analyzing two-guide models, most embryos had carried the desired double mutation (TPC2/TPC1: 79.4%; TMPRSS2/CD163: 63.0%). These proportions corresponded to the product of the individual mutation efficiencies of each guide, suggesting that the mutation rate is inherent to each specific sgRNA. When examining the multiple-guide model, the mutation rate for each gene exceeded 85.1%, with 70% of the genotyped embryos carrying mutations in all five target genes. However, the mosaicism was higher than 70% for all the guides. This study demonstrates that single, double and multiple KO porcine embryos can be efficiently generated by electroporation with high efficiency, although with a high incidence of mosaicism.

This increment in cleavage rate suggests that the electrical pulses may have activated the oocytes independently of sperm interaction. Therefore, in **Chapter 2**, we investigated whether blastocysts obtained after electroporation and subsequent IVF resulted from sperm penetration (real embryos) or oocyte activation (parthenotes).

To distinguish between them, we used semen from a TPC2 KO boar carrying an 11 base pair (bp) deletion. Embryos with this mutation in one TPC2 allele were classified as true embryos (since the mutation was present on sperm DNA), while those without the deletion were classified as parthenotes. In addition, at 18 h pi a portion of putative zygotes were fixed and stained with Hoechst to evaluate penetration and activation parameters.

Three experiments were performed. The first evaluated the parthenogenetic activation potential of electroporation by comparing a control IVF group, an electroporated but not inseminated group (E) and an electroporated and inseminated group (E-IVF). The second experiment compared two different electroporation media with different chemical composition (Opti-MEM -Opti- and Duplex Buffer -DB) using the same experimental groups (control, Opti-E-IVF, Opti-E, DB-E-IVF and DB-E). The third experiment investigated whether the timing of electroporation relative to IVF affected the parthenogenetic activation. Electroporation was performed at three time points: one hour

before (E-IVF-t-1), just before (E-IVF-t0) and seven hours after (E-IVF-t7), along with a control group. In this experiment, the CRISPR/Cas9 system targeted the CAPN3 gene.

In the first experiment, we observed increased oocyte activation at 18 h pi and higher cleavage rate at 48 h pi in both electroporated groups in comparison to the control (86.6-91.3% vs 54.8% and 63.3-67.3% vs 49.1%, respectively, $p<0.05$). However, no difference was found in blastocyst rates (24.9-31.0%). The parthenote rate was significantly higher in the E-IVF group than in the control (30.2% vs 6.8%, $p<0.05$), a trend that was consistent across subsequent experiments. In the second experiment, DB media had a detrimental effect on embryo development, resulting in lower cleavage and blastocyst rates compared to the control and the Opti-MEM groups. The parthenote rate was significantly higher in both electroporated groups than in the control (DB-E-IVF: 60.0% and Opti-E-IVF: 48.5% vs 0%, $p<0.05$), but did not differ between media types. In the last experiment, the electroporated groups showed again increased activation and cleavage rates, with no difference in the blastocyst rate. The parthenote rate was lower in the control group (2.44%) but remained similar across electroporation time points compared to IVF (51.6-63.9%). The mutation rate was higher in the t7 group (81.3% vs 54.6-57.5%), and overall, the mutation rate was higher in parthenotes (71.9%) than in real embryos (54.8%).

These two chapters demonstrated that electroporation is a fast and efficient method for testing sgRNAs. However, the parthenogenetic activation of oocytes poses a challenge when aiming to generate live animals. It will be necessary to further investigate the factors affecting electroporation efficiency to achieve higher mutation rates while minimizing issues related to mosaicism and parthenogenetic activation.

For that reason, we investigated the use of lipofection to introduce CRISPR/Cas9 components into porcine oocytes and zygotes. To our knowledge, previous studies have used only ZP-free oocytes and zygotes to generate genetically modified embryos. However, the removal of the ZP may negatively affect embryo development and handling.

In **Chapter 3**, we investigated the feasibility of using lipofection in ZP-intact oocytes to generate genetically modified porcine embryos. Lipofectamine CRISPRMAX™ was used, and oocytes were incubated with liposomes throughout the IVF process.

We evaluated two key factors: the concentration of the liposome-RNP complexes (5% and 10% v/v) and the concentration of RNP within the liposomes (50 ng/µl Cas9:25 ng/µl

sgRNA -1x- and 100 ng/µl:50 ng/µl -2x). An electroporated group was used as a positive control of mutation while an untreated group served as negative control.

Our results showed that cleavage and blastocyst rates were lower in the 10% v/v group (30.45% and 13.41%, respectively) compared to the other groups, while no differences were observed between the 5% v/v and control groups. Mutation rates were comparable in all treated groups, but the efficiency was significantly reduced in the 10% v/v group (electroporated: 6.38%; 5%: 5.52% vs 10%: 2.02%, $p<0.05$). In addition, the concentration of RNP in the liposomes did not affect embryo development or mutation parameters, with similar results in the efficiency of the 1x and 2x and electroporated group (1.75%, 3.60% and 3.75%, respectively). These results suggest that while lipofection can be used to introduce CRISPR/Cas9 components into ZP-intact porcine oocytes, higher concentrations of liposomes may negatively affect embryo development and mutation efficiency.

In **Chapter 4**, we evaluated two additional factors for the lipofection treatment: the reagent used and the duration of coincubation with lipofectamine and sperm.

In the first experiment, ZP-intact oocytes were incubated throughout the IVF process with either Lipofectamine CRISPRMAX™ or Lipofectamine 3000. Two different sgRNAs targeting CAPN3 and CD163 genes were tested. An electroporated group was used as a positive control, while an untreated group served as negative control. In the second experiment, we evaluated different durations of sperm, oocyte and liposome coincubation (4, 8 and 24 h), with an additional untreated group as a negative control.

No significant differences were observed between the lipofected groups in terms of blastocyst rate (12-16%), mutation rate (35-55%) or overall efficiency (3.5-7%). Mosaicism levels and the number of alleles varied significantly between sgRNAs, but not between groups, indicating that these parameters are sgRNA dependent. Regarding the duration of coincubation, cleavage rate was lower in the 4 h (42.6%) and 24 h groups (49.44%) in comparison with the control (61.54%, $p<0.05$). This decrease may be due to the short time of coincubation with the sperm in the 4 h group and the possible lipofectamine-induced toxicity in the 24 h group. However, no differences in blastocyst rate were found among the coincubation times (ranging from 21.02% to 29.71%). Notably, the 8 h coincubation group exhibited the highest mutation rate (49.02%) and

overall efficiency (14.36%), suggesting that this duration may be optimal for maximizing gene editing efficiency while maintaining embryo viability.

These results demonstrated that lipofection is a viable method for generating gene edited pigs, eliminating the need for ZP removal to obtain genetically modified embryos. This technique is simpler than microinjection, does not require specialized equipment, and allows simultaneous lipofection of hundreds of oocytes without handling. However, its efficiency remains still low and needs further optimization.

In conclusion, this thesis focused on optimizing the generation of gene-edited pig embryos using two main methods: electroporation and lipofection. We systematically investigated various factors that influence the efficiency of these techniques, identifying key aspects that affect embryo development, mutation rates and overall editing efficiency.

Both electroporation and lipofection successfully produced gene-edited embryos, demonstrating their potential as viable alternatives to traditional microinjection. However, each method presents specific challenges that must be addressed to improve reproducibility and efficiency. Further optimization, including refining delivery conditions, improving editing accuracy, and minimizing potential toxicity, is essential to maximize the applicability of these techniques for large-scale genetic modification in pigs.

Chapter 1

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

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

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Abstract

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.

PhD student's contributions to the work

Generation of KO embryos for the different models. Genotyping of the embryos including DNA extraction, PCR and analysis. Drafting and revision of the manuscript.

Chapter 2

Optimizing oocyte electroporation for genetic modification of porcine embryos: Evaluation of the parthenogenetic activation

Optimizing oocyte electroporation for genetic modification of porcine embryos: Evaluation of the parthenogenetic activation

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PhD student's contributions to the work

Design of the experiments. *In vitro* production of embryos. Electroporation, staining, genotyping and analysis of the results. Elaboration of figures, drafting and revision of the manuscript.

Abstract

In reproductive biology, understanding the effects of novel techniques on early embryo development is of paramount importance. To date, the effects of electrical activation on oocytes prior to in vitro fertilization (IVF) are not well understood. The aim of this studywas to investigate the effects of oocyte electroporation prior to IVF on embryo development and to differentiate between true embryos and parthenotes by using a TPCN2 knock-out (KO) male to evaluate the presence of the KO allele in the resulting blastocysts. The study consisted of three experiments. The first one examined oocyte electroporation with and without subsequent IVF and found that electroporated oocytes had higher activation rates, increased occurrence of a single pronucleus, and no effect on sperm penetration. Cleavage rates improved in electroporated oocytes, but blastocyst rates remained constant. Genotype analysis revealed a significant increase in the proportion of parthenotes in the electroporated groups compared to the IVF control (30.2 % vs. 6.8 %). The second experiment compared two electroporation media, Opti-MEM and Nuclease-Free Duplex Buffer (DB). DB induced higher oocyte degeneration rates, and lower cleavage and blastocyst rates than Opti-MEM, while parthenogenetic formation remained consistent (60.0 and 48.5 %). In the third experiment, the timing of electroporation relative to IVF was evaluated (1 h before IVF, immediately before IVF and 7 h after IVF). Electroporation immediately before IVF resulted in higher activation rates and different pronuclear proportions compared to the other timing groups. The penetration rate was higher in the immediate electroporation group, and cleavage rate improved in all electroporated groups compared to the control. Blastocyst rates remained constant. Genotyping revealed no significant differences in parthenote proportions among the timing groups, but these were higher than the control (56.25 %, 63.89 %, 51.61 %, 2.44 %, respectively), and showed higher mutation rates when electroporation was performed 7 h after IVF. Overall, this comprehensive study sheds light on the potential of electroporation for creating genetically modified embryos and the importance of media selection and timing in the process, the best media being the Opti-MEM and the more efficient timing regarding mutation rate, 7 h post-IVF, even when the parthenote formation did not differ among electroporated groups. Further studies are needed to reduce the parthenogenetic activation while maintaining high mutation rates to optimize the use of this procedure for the generation of gene-edited pig embryos by oocyte/zygote electroporation.

Chapter 3

Production of genetically modified porcine embryos via lipofection of *zona-pellucida*-intact oocytes using the CRISPR/Cas9 system

Production of genetically modified porcine embryos via lipofection of zona-pellucida-intact oocytes using the CRISPR/Cas9 system

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PhD student's contributions to the work

Design of the experiments. *In vitro* production of embryos. Lipofection, genotyping and analysis of the results. Elaboration of figures, drafting and revision of the manuscript.

Abstract

The generation of genetically modified pigs has an important impact thanks its applications in basic research, biomedicine, and meat production. Cloning was the first technique used for this production, although easier and cheaper methods were developed, such as the microinjection, electroporation, or lipofection of oocytes and zygotes. In this study, we analyzed the production of genetically modified embryos via lipofection of zona-pellucida-intact oocytes using Lipofectamine™ CRISPRMAX™ Cas9 in comparison with the electroporation method. Two factors were evaluated: (i) the increment in the concentration of the lipofectamine–ribonucleoprotein complexes (LRNPC) (5% vs. 10%) and (ii) the concentration of ribonucleoprotein within the complexes (1xRNP vs. 2xRNP). We found that the increment in the concentration of the LRNPC had a detrimental effect on embryo development and a subsequent effect on the number of mutant embryos. The 5% group had a similar mutant blastocyst rate to the electroporation method (5.52% and 6.38%, respectively, $p > 0.05$). The increment in the concentration of the ribonucleoprotein inside the complexes had no effect on the blastocyst rate and mutation rate, with the mutant blastocyst rate being similar in both the 1xRNP and 2xRNP lipofection groups and the electroporation group (1.75%, 3.60%, and 3.57%, respectively, $p > 0.05$). Here, we showed that it is possible to produce knock-out embryos via lipofection of zona-pellucida-intact porcine oocytes with similar efficiencies as with electroporation, although more optimization is needed, mainly in terms of the use of more efficient vesicles for encapsulation with different compositions.

Chapter 4

Optimization of lipofection protocols for CRISPR/Cas9 delivery in porcine *zona pellucida* intact oocytes: A study of coincubation duration and reagent efficacy

Optimization of lipofection protocols for CRISPR/Cas9 delivery in porcine zona pellucida intact oocytes: A study of coincubation duration and reagent efficacy

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Design of the experiments. *In vitro* production of embryos. Lipofection, staining, genotyping and analysis of the results. Elaboration of figures, drafting and revision of the manuscript.

Abstract

A priority to facilitate the application of lipofection to generate genetically modified porcine embryos and animals will be the use of zona pellucida (ZP)-intact oocytes and zygotes. Recently, our group produced genetically modified embryos by lipofection of ZP-intact oocytes during in vitro fertilization (IVF). This study investigates the effect of two commercial lipofection reagents, Lipofectamine 3000 and Lipofectamine CRISPRMAX, on embryo development and mutation efficiency in ZP-intact porcine oocytes. We compared these reagents with the electroporation method and a control group using two sgRNAs targeting the CAPN3 and CD163 genes. The detrimental effects on cleavage rates were observed in both lipofection treatments compared to the control and electroporated groups. However, blastocyst rates were higher in the Lipofectamine 3000 group than in the electroporated group for both genes. Mutation parameters varied by target gene, with Lipofectamine 3000 achieving higher mutation rates for CD163, while all groups were similar for the CAPN3 gene. Overall efficiency was similar for both lipofectamines, confirming their feasibility for use. In addition, we evaluated the effect of coincubation time (4, 8, and 24 h) on IVF outcomes, embryo development, and mutation parameters. Results indicated that an 8-h coincubation period optimized fertilization and mutation efficiency without significant toxic effects. This study demonstrates that lipofection with either Lipofectamine 3000 or CRISPRMAX during IVF is an effective method for generating genetically modified porcine embryos without the need for specialized equipment or trained personnel, with efficiencies similar to or greater than electroporation. This study also highlights the importance of optimizing reagent selection and coincubation times. There is no difference between Lipofectamine 3000 and CRISPRMAXTM in terms of embryo development and mutation efficiency, and under our experimental conditions, the optimal coincubation time with lipofectamine is 8 h.

Conclusions

1. It is possible to generate single, double and multiple KO pig embryos by electroporation before IVF.
2. The mutation rate depends on the guide and not on the number of guides per treatment. The concentration of sgRNA and Cas9 protein plays a significant role in the mutation and mosaicism rates and affects the overall efficiency of the gene editing system.
3. Oocyte electroporation in combination with IVF enhances early embryo development (cleavage rate) by increasing oocyte activation and increasing the proportion of 1 PN oocytes, resulting in increased parthenote formation, with similar blastocyst rate.
4. Opti-MEM is better than Duplex Buffer as electroporation buffer in terms of embryo development.
5. The timing of the electroporation procedure in relation to IVF does not influence embryo development or the parthenote activation, but it affects the mutation rate, being the best option 7h post-IVF.
6. It is possible to generate genetically modified pig embryos by lipofection of ZP-intact oocytes during IVF and the optimal liposome-RNP concentration is 5% v/v.
7. There is no difference between Lipofectamine CRISPRMAX™ and Lipofectamine 3000 in terms of mutation rate and embryo development.
8. The best coincubation time with Lipofectamine CRISPRMAX™ is 8 h, under our experimental conditions.

Future directions

The use of electroporation and lipofection technologies offers a promising and simplified approach to generate genetically modified pigs. These techniques provide an alternative to traditional microinjection, potentially increasing efficiency and scalability. However, significant limitations remain, particularly in reducing mosaicism, optimizing gene editing efficiency, and preventing unintended effects such as parthenogenetic activation. Addressing these challenges is critical to refine these methods and advancing their application in biomedical and agricultural research.

One of the major concerns with electroporation is its tendency to induce parthenogenetic activation in oocytes, which compromises embryo development and viability. Refinement of electroporation parameters (including voltage intensity, pulse duration, and interval settings) will be critical to maximize mutation rates while minimizing embryonic damage and parthenogenetic activation. To overcome this, it is essential to assess the key factors influencing parthenogenetic activation and to develop targeted strategies to mitigate this problem. Mechanistic studies should investigate the molecular pathways involved in this process and potentially identify chemical inhibitors that can suppress unwanted activation while maintaining genome editing efficiency.

Lipofection, although a promising non-invasive gene delivery method, requires further optimization to increase its efficiency as a viable alternative to microinjection. A deeper understanding of the interactions between liposomes and oocytes is necessary to improve transfection efficiency and minimize cytotoxic effects. Future studies should explore a broader range of transfection carriers, such as polymeric nanocarriers, gold-based nanoparticles, or hybrid material-based platforms, which could provide better stability, controlled release, and enhanced cellular uptake of CRISPR/Cas9 components.

In addition to improved delivery methods, advances in CRISPR technologies could further improve the efficiency and safety of genome editing in pigs. While Cas9 is the most widely used nuclease, newer CRISPR variants offer advantages in reducing off-target effects and increasing editing precision. For example, Cas12a (Cpf1) is a compelling alternative as it exhibits greater specificity and facilitates homology-directed repair (HDR) more effectively than Cas9. In addition, novel CRISPR-based approaches such as base editing and prime editing allow precise genetic modification without inducing double-strand breaks (DSBs), thereby reducing the risks associated with error-prone repair mechanisms. These alternative editing strategies could be particularly valuable for introducing specific point mutations relevant to biomedical applications.

The integration of these advances will provide valuable insights into the development of genetically modified pigs in a more efficient and controlled manner. However, to validate the efficacy and practicality of these genome editing methods, it is essential to conduct *in vivo* studies by transferring gene-edited embryos into recipient sows. Monitoring pregnancy rates, fetal development, and postnatal survival will provide critical data on the long-term viability, health, and stability of genetic modifications. In addition, assessment of potential off-target effects and evaluation of genetic integrity across generations will be fundamental to ensuring the safety and reproducibility of these techniques.

By refining electroporation and lipofection protocols, incorporating next-generation CRISPR technologies, and conducting rigorous *in vivo* evaluations, these methods could significantly advance the field of swine genome engineering. Ultimately, these improvements will facilitate the generation of genetically modified pigs for both biomedical research, including organ transplantation models, and agricultural applications, such as disease-resistant livestock.

References

Beaujean, N., Jammes, H., Jouneau, A., 2015. Nuclear reprogramming: Methods and protocols: Second edition, Nuclear Reprogramming: Methods and Protocols: Second Edition, Methods in Molecular Biology. Springer New York, New York, NY. https://doi.org/10.1007/978-1-4939-1594-1

Chen, J., Wang, H., Bai, J., Liu, W., Liu, X., Yu, D., Feng, T., Sun, Z., Zhang, L., Ma, L., Hu, Y., Zou, Y., Tan, T., Zhong, J., Hu, M., Bai, X., Pan, D., Xing, Y., Zhao, Y., Tian, K., Hu, X., Li, N., 2019. Generation of Pigs Resistant to Highly Pathogenic-Porcine Reproductive and Respiratory Syndrome Virus through Gene Editing of CD163. Int J Biol Sci 15, 481–492. https://doi.org/10.7150/ijbs.25862

Colleoni, S., Donofrio, G., Lagutina, I., Duchi, R., Galli, C., Lazzari, G., 2005. Establishment, differentiation, electroporation, viral transduction, and nuclear transfer of bovine and porcine mesenchymal stem cells. Cloning Stem Cells 7, 154–166. https://doi.org/10.1089/clo.2005.7.154

da Silva, Z., de Souza, A.P., Pandolfi, J.R.C., da Fonseca, F.N., da Veiga Lima-Rosa, C.A., Marques, M.G., 2018. Comparison between electroporation and polyfection in pig sperm: efficiency and cell viability implications. Zygote 26, 286–293. https://doi.org/10.1017/S0967199418000205

Dorado, B., Pløen, G.G., Barettino, A., Macías, A., Gonzalo, P., Andrés-Manzano, M.J., González-Gómez, C., Galán-Arriola, C., Alfonso, J.M., Lobo, M., López-Martín, G.J., Molina, A., Sánchez-Sánchez, R., Gadea, J., Sánchez-González, J., Liu, Y., Callesen, H., Filgueiras-Rama, D., Ibáñez, B., Sørensen, C.B., Andrés, V., 2019. Generation and characterization of a novel knockin minipig model of Hutchinson-Gilford progeria syndrome. Cell Discov 5, 16. https://doi.org/10.1038/s41421-019-0084-z

FAO, 2024. Food Outlook – Biannual report on global food markets, Food Outlook. FAO, Rome.

Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M., Danielsen, M., 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc Natl Acad Sci USA 84, 7413. https://doi.org/10.1073/PNAS.84.21.7413

Fernández, J.P., Petersen, B., Hassel, P., Lucas Hahn, A., Kielau, P., Geibel, J., Kues, W.A., 2024. Comparison Between Electroporation at Different Voltage Levels and

Microinjection to Generate Porcine Embryos with Multiple Xenoantigen Knock-Outs. Int J Mol Sci 25, 11894. https://doi.org/10.3390/ijms252211894

Fischer, K., Rieblinger, B., Hein, R., Sfriso, R., Zuber, J., Fischer, A., Klinger, B., Liang, W., Flisikowski, K., Kurome, M., Zakhartchenko, V., Kessler, B., Wolf, E., Rieben, R., Schwinzer, R., Kind, A., Schnieke, A., 2020. Viable pigs after simultaneous inactivation of porcine MHC class I and three xenoreactive antigen genes GGTA1, CMAH and B4GALNT2. Xenotransplantation 27. https://doi.org/10.1111/xen.12560

Frangoul, H., Altshuler, D., Cappellini, M.D., Chen, Y.-S., Domm, J., Eustace, B.K., Foell, J., de la Fuente, J., Grupp, S., Handgretinger, R., Ho, T.W., Kattamis, A., Kernytsky, A., Lekstrom-Himes, J., Li, A.M., Locatelli, F., Mapara, M.Y., de Montalembert, M., Rondelli, D., Sharma, A., Sheth, S., Soni, S., Steinberg, M.H., Wall, D., Yen, A., Corbacioglu, S., 2021. CRISPR-Cas9 Gene Editing for Sickle Cell Disease and β-Thalassemia. N Engl J Med 384, 252–260. https://doi.org/10.1056/NEJMoa2031054

Funahashi, H., Stumpf, T.T., Cantley, T.C., Kim, N.H., Day, B.N., 1995. Pronuclear formation and intracellular glutathione content of in vitro-matured porcine oocytes following in vitro fertilisation and/or electrical activation. Zygote (Cambridge, England) 3, 273–281. https://doi.org/10.1017/s0967199400002677

Funahashi, H., Stumpf, T.T., Terlouw, S.L., Day, B.N., 1993. Effects of electrical stimulation before or after in vitro fertilization on sperm penetration and pronuclear formation of pig oocytes. Mol Reprod Dev 36, 361–367. https://doi.org/10.1002/MRD.1080360312

Gandek, T.B., van der Koog, L., Nagelkerke, A., 2023. A Comparison of Cellular Uptake Mechanisms, Delivery Efficacy, and Intracellular Fate between Liposomes and Extracellular Vesicles. Adv Healthc Mater 12, e2300319. https://doi.org/10.1002/adhm.202300319

Hai, T., Teng, F., Guo, R., Li, W., Zhou, Q., 2014. One-step generation of knockout pigs by zygote injection of CRISPR/Cas system. Cell Research 24, 372–375. https://doi.org/10.1038/cr.2014.11

Hein, R., Sake, H.J., Pokoyski, C., Hundrieser, J., Brinkmann, A., Baars, W., Nowak-Imialek, M., Lucas-Hahn, A., Figueiredo, C., Schuberth, H.J., Niemann, H., Petersen, B., Schwinzer, R., 2020. Triple (GGTA1, CMAH, B2M) modified pigs expressing an SLA

class Ilow phenotype—Effects on immune status and susceptibility to human immune responses. Am J Transplant 20, 988–998. https://doi.org/10.1111/ajt.15710

Hirata, M., Tanihara, F., Wittayarat, M., Hirano, T., Nguyen, N.T., Le, Q.A., Namula, Z., Nii, M., Otoi, T., 2019. Genome mutation after introduction of the gene editing by electroporation of Cas9 protein (GEEP) system in matured oocytes and putative zygotes. In Vitro Cell Dev Biol Anim 55, 237–242. https://doi.org/10.1007/s11626-019-00338-3

Hirata, M., Wittayarat, M., Namula, Z., Anh Le, Q., Lin, Q., Takebayashi, K., Thongkittidilok, C., Tanihara, F., Otoi, T., 2021a. Lipofection-Mediated Introduction of CRISPR/Cas9 System into Porcine Oocytes and Embryos. Animals (Basel) 11, 578. https://doi.org/10.3390/ani11020578

Hirata, M., Wittayarat, M., Namula, Z., Le, Q.A., Lin, Q., Takebayashi, K., Thongkittidilok, C., Mito, T., Tomonari, S., Tanihara, F., Otoi, T., 2021b. Generation of mutant pigs by lipofection-mediated genome editing in embryos. Sci Rep 11, 23806. https://doi.org/10.1038/S41598-021-03325-5

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., Charpentier, E., 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337, 816–821. https://doi.org/10.1126/science.1225829

Koo, O.J., Jang, G., Kwon, D.K., Kang, J.T., Kwon, O.S., Park, H.J., Kang, S.K., Lee, B.C., 2008. Electrical activation induces reactive oxygen species in porcine embryos. Theriogenology 70, 1111–1118. https://doi.org/10.1016/j.theriogenology.2008.06.031

Koppes, E.A., Redel, B.K., Johnson, M.A., Skvorak, K.J., Ghaloul-Gonzalez, L., Yates, M.E., Lewis, D.W., Gollin, S.M., Wu, Y.L., Christ, S.E., Yerle, M., Leshinski, A., Spate, L.D., Benne, J.A., Murphy, S.L., Samuel, M.S., Walters, E.M., Hansen, S.A., Wells, K.D., Lichter-Konecki, U., Wagner, R.A., Newsome, J.T., Dobrowolski, S.F., Vockley, J., Prather, R.S., Nicholls, R.D., 2020. A porcine model of phenylketonuria generated by CRISPR/Cas9 genome editing. JCI Insight 5. https://doi.org/10.1172/jci.insight.141523

Krassowska, W., Filev, P.D., 2007. Modeling Electroporation in a Single Cell. Biophys J 92, 404–417. https://doi.org/10.1529/biophysj.106.094235

Lai, S., Wei, S., Zhao, B., Ouyang, Z., Zhang, Q., Fan, N., Liu, Z., Zhao, Y., Yan, Q., Zhou, X., Li, L., Xin, J., Zeng, Y., Lai, L., Zou, Q., 2016. Generation of knock-in pigs

carrying Oct4-tdTomato reporter through CRISPR/Cas9-mediated genome engineering. PLoS ONE 11, e0146562. https://doi.org/10.1371/journal.pone.0146562

Lasa-Elgarresta, J., Mosqueira-Martín, L., Naldaiz-Gastesi, N., Sáenz, A., López de Munain, A., Vallejo-Illarramendi, A., 2019. Calcium Mechanisms in Limb-Girdle Muscular Dystrophy with CAPN3 Mutations. Int J Mol Sci 20, 4548. https://doi.org/10.3390/ijms20184548

Le, Q.A., Hirata, M., Nguyen, N.T., Takebayashi, K., Wittayarat, M., Sato, Y., Namula, Z., Nii, M., Tanihara, F., Otoi, T., 2020. Effects of electroporation treatment using different concentrations of Cas9 protein with gRNA targeting Myostatin (MSTN) genes on the development and gene editing of porcine zygotes. Anim Sci J 91, e13386. https://doi.org/10.1111/asj.13386

Le, Q.A., Tanihara, F., Wittayarat, M., Namula, Z., Sato, Y., Lin, Q., Takebayashi, K., Hirata, M., Otoi, T., 2021. Comparison of the effects of introducing the CRISPR/Cas9 system by microinjection and electroporation into porcine embryos at different stages. BMC Res Notes 14. https://doi.org/10.1186/S13104-020-05412-8

Lin, J.C., Van Eenennaam, A.L., 2021. Electroporation-Mediated Genome Editing of Livestock Zygotes. Front Genet 12, 546. https://doi.org/10.3389/FGENE.2021.648482/BIBTEX

Lin, Q., Le, Q.A., Takebayashi, K., Thongkittidilok, C., Wittayarat, M., Hirata, M., Tanihara, F., Otoi, T., 2021. Timing and duration of lipofection-mediated CRISPR/Cas9 delivery into porcine zygotes affect gene-editing events. BMC Res Notes 14, 1–6. https://doi.org/10.1186/S13104-021-05800-8

Lin, Q., Takebayashi, K., Torigoe, N., Liu, B., Namula, Z., Hirata, M., Nagahara, M., Tanihara, F., Otoi, T., 2024. Evaluation of culture methods and chemical reagent combinations on CRISPR/Cas9 gene editing systems by lipofection in pig zygotes. In Vitro Cell Dev Biol Anim. https://doi.org/10.1007/s11626-024-00908-0

Lin, Q., Takebayashi, K., Torigoe, N., Liu, B., Namula, Z., Hirata, M., Tanihara, F., Nagahara, M., Otoi, T., 2023. Comparison of chemically mediated CRISPR/Cas9 gene editing systems using different nonviral vectors in porcine embryos. Anim Sci J 94, e13878. https://doi.org/10.1111/asj.13878

Luo, L., Wang, S., Zhu, L., Fan, B., Liu, T., Wang, L., Zhao, P., Dang, Y., Sun, P., Chen, J., Zhang, Y., Chang, X., Yu, Z., Wang, H., Guo, R., Li, B., Zhang, K., 2019. Aminopeptidase N-null neonatal piglets are protected from transmissible gastroenteritis virus but not porcine epidemic diarrhea virus. Sci Rep 9, 13186–13186. https://doi.org/10.1038/s41598-019-49838-y

Navarro-Serna, Dehesa-Etxebeste, M., Piñeiro-Silva, C., Romar, R., Lopes, J.S., López de Munaín, A., Gadea, J., 2022a. Generation of Calpain-3 knock-out porcine embryos by CRISPR-Cas9 electroporation and intracytoplasmic microinjection of oocytes before insemination. Theriogenology 186, 175–184. https://doi.org/10.1016/J.THERIOGENOLOGY.2022.04.012

Navarro-Serna, Piñeiro-Silva, C., Luongo, C., Parrington, J., Romar, R., Gadea, J., 2022b. Effect of Aphidicolin, a Reversible Inhibitor of Eukaryotic Nuclear DNA Replication, on the Production of Genetically Modified Porcine Embryos by CRISPR/Cas9. Int J Mol Sci 23, 2135. https://doi.org/10.3390/IJMS23042135

Navarro-Serna, Piñeiro-Silva, C., Romar, R., Parrington, J., Gadea, J., 2022c. Generation of Gene Edited Pigs, in: Kumar Yata, V., Kumar Mohanty, A., Lichtfouse, E. (Eds.), Sustainable Agriculture Reviews 57: Animal Biotechnology for Livestock Production 2. Springer, pp. 71–130. https://doi.org/10.1007/978-3-031-07496-7_3

Navarro-Serna, S., Hachem, A., Canha-Gouveia, A., Hanbashi, A., Garrappa, G., Lopes, J.S., París-Oller, E., Sarriás-Gil, L., Flores-Flores, C., Bassett, A., Sánchez, R., Bermejo-Álvarez, P., Matás, C., Romar, R., Parrington, J., Gadea, J., 2021. Generation of Nonmosaic, Two-Pore Channel 2 Biallelic Knockout Pigs in One Generation by CRISPR-Cas9 Microinjection before Oocyte Insemination. CRISPR J 4, 132–146. https://doi.org/10.1089/crispr.2020.0078

Nesiyama, T.N.G., Sangalli, J.R., De Bem, T.H.C., Recchia, K., Martins, S.M.M.K., de Andrade, A.F.C., Ferst, J.G., Almeida, G.H.D.R., Marques, M.G., Dória, R.G.S., Carregaro, A.B., Feliciano, M.A.R., Miglino, M.A., Bressan, F.F., Perecin, F., da Silveira, J.C., Smith, L.C., Bordignon, V., Meirelles, F.V., 2025. Swine clones: potential application for animal production and animal models. Anim Reprod 22, e20240037. https://doi.org/10.1590/1984-3143-AR2024-0037

Nguyen, T.-V., Do, L.T.K., Lin, Q., Nagahara, M., Namula, Z., Wittayarat, M., Hirata, M., Otoi, T., Tanihara, F., 2024. Programmed cell death-1-modified pig developed using electroporation-mediated gene editing for in vitro fertilized zygotes. In Vitro Cell Dev Biol Anim. https://doi.org/10.1007/s11626-024-00869-4

Nishio, K., Tanihara, F., Nguyen, T.V., Kunihara, T., Nii, M., Hirata, M., Takemoto, T., Otoi, T., 2018. Effects of voltage strength during electroporation on the development and quality of in vitro-produced porcine embryos. Reprod Domest Anim 53, 313–318. https://doi.org/10.1111/RDA.13106

Novickij, V., Rembiałkowska, N., Szlasa, W., Kulbacka, J., 2022. Does the shape of the electric pulse matter in electroporation? Front Oncol 12, 958128. https://doi.org/10.3389/fonc.2022.958128

Park, I., Navarro-Serna, S., Pinho, R.M., Berger, T., Maga, E.A., Gadea, J., Kim, S.K., Ross, P.J., 2024. Electroporation of CRISPR/Cas9 Targeting Neurogenin 3 (NGN3) in Porcine Embryos and Its Effects on Mosaicism and Off-Target Effects by Next Generation Sequencing (NGS). Re:GEN Open 4, 9–20. https://doi.org/10.1089/regen.2023.0021

Petersen, B., 2017. Basics of genome editing technology and its application in livestock species. Reprod Domest Anim 52, 4–13. https://doi.org/10.1111/rda.13012

Procházka, R., Kanka, J., Sutovský, P., Fulka, J., Motlík, J., 1992. Development of pronuclei in pig oocytes activated by a single electric pulse. J Reprod Fertil 96, 725–734. https://doi.org/10.1530/jrf.0.0960725

Shi, X., Tang, T., Lin, Q., Liu, H., Qin, Y., Liang, X., Cong, P., Mo, D., Liu, X., Chen, Y., He, Z., 2020. Efficient generation of bone morphogenetic protein 15-edited Yorkshire pigs using CRISPR/Cas9. Biol Reprod 103, 1054–1068. https://doi.org/10.1093/biolre/ioaa138

Sun, F.Z., Hoyland, J., Huang, X., Mason, W., Moor, R.M., 1992. A comparison of intracellular changes in porcine eggs after fertilization and electroactivation. Development 115, 947–956. https://doi.org/10.1242/dev.115.4.947

Takebayashi, K., Wittayarat, M., Lin, Q., Hirata, M., Yoshimura, N., Torigoe, N., Nagahara, M., Do, L.T.K., Tanihara, F., Otoi, T., 2022. Gene editing in porcine embryos using a combination of electroporation and transfection methods. Reprod Domest Anim https://doi.org/10.1111/RDA.14184

Tanihara, F., Hirata, M., Namula, Z., Do, L.T.K., Yoshimura, N., Lin, Q., Takebayashi, K., Sakuma, T., Yamamoto, T., Otoi, T., 2023a. Pigs with an INS point mutation derived from zygotes electroporated with CRISPR/Cas9 and ssODN. Front Cell Dev Biol 11, 884340. https://doi.org/10.3389/fcell.2023.884340

Tanihara, F., Hirata, M., Namula, Z., Wittayarat, M., Do, L., Lin, Q., Takebayashi, K., Hara, H., Nagahara, M., Otoi, T., 2023b. GHR-mutant pig derived from domestic pig and microminipig hybrid zygotes using CRISPR/Cas9 system. Mol Biol Rep 1–9. https://doi.org/10.1007/s11033-023-08388-3

Tanihara, F., Hirata, M., Nguyen, N.T., Le, Q.A., Hirano, T., Takemoto, T., Nakai, M., Fuchimoto, D.I., Otoi, T., 2018. Generation of a TP53-modified porcine cancer model by CRISPR/Cas9-mediated gene modification in porcine zygotes via electroporation. PLoS ONE 13, e0206360. https://doi.org/10.1371/journal.pone.0206360

Tanihara, F., Hirata, M., Nguyen, N.T., Le, Q.A., Wittayarat, M., Fahrudin, M., Hirano, T., Otoi, T., 2019. Generation of CD163-edited pig via electroporation of the CRISPR/Cas9 system into porcine in vitro-fertilized zygotes. Anim Biotechnol 1–8. https://doi.org/10.1080/10495398.2019.1668801

Tanihara, F., Hirata, M., Nguyen, N.T., Sawamoto, O., Kikuchi, T., Doi, M., Otoi, T., 2020a. Efficient generation of GGTA1-deficient pigs by electroporation of the CRISPR/Cas9 system into in vitro-fertilized zygotes. BMC Biotechnol 20, 40. https://doi.org/10.1186/s12896-020-00638-7

Tanihara, F., Hirata, M., Nguyen, N.T., Sawamoto, O., Kikuchi, T., Otoi, T., 2021. One-Step Generation of Multiple Gene-Edited Pigs by Electroporation of the CRISPR/Cas9 System into Zygotes to Reduce Xenoantigen Biosynthesis. Int J Mol Sci 22, 2249. https://doi.org/10.3390/IJMS22052249

Tanihara, F., Hirata, M., Thi Nguyen, N., Anh Le, Q., Hirano, T., Otoi, T., 2020b. Generation of viable PDX1 gene-edited founder pigs as providers of nonmosaics. Mol Reprod Dev 87, 471–481. https://doi.org/10.1002/mrd.23335

Tanihara, F., Takemoto, T., Kitagawa, E., Rao, S., Do, L.T.K., Onishi, A., Yamashita, Y., Kosugi, C., Suzuki, H., Sembon, S., Suzuki, S., Nakai, M., Hashimoto, M., Yasue, A., Matsuhisa, M., Noji, S., Fujimura, T., Fuchimoto, D. ichiro, Otoi, T., 2016. Somatic cell

reprogramming-free generation of genetically modified pigs. Sci Adv 2, e1600803. https://doi.org/10.1126/sciadv.1600803

Tao, T., Macháty, Z., Abeydeera, L.R., Day, B.N., Prather, R.S., 2000. Optimisation of porcine oocyte activation following nuclear transfer. Zygote 8, 69–77. https://doi.org/10.1017/s0967199400000848

Wang, K., Ouyang, H., Xie, Z., Yao, C., Guo, N., Li, M., Jiao, H., Pang, D., 2015. Efficient Generation of Myostatin Mutations in Pigs Using the CRISPR/Cas9 System. Sci Rep 5, 16623. https://doi.org/10.1038/srep16623

Wang, W.-H., Abeydeera, L.R., Prather, R.S., Day, B.N., 1998. Functional analysis of activation of porcine oocytes by spermatozoa, calcium ionophore, and electrical pulse. Mol Reprod Dev 51, 346–353. https://doi.org/10.1002/(SICI)1098-2795(199811)51:3<346::AID-MRD15>3.0.CO;2-0

Whitelaw, C.B.A., Sheets, T.P., Lillico, S.G., Telugu, B.P., 2016. Engineering large animal models of human disease. J Pathol 238, 247–256. https://doi.org/10.1002/path.4648

Whitworth, K.M., Lee, K., Benne, J.A., Beaton, B.P., Spate, L.D., Murphy, S.L., Samuel, M.S., Mao, J., O'Gorman, C., Walters, E.M., Murphy, C.N., Driver, J., Mileham, A., McLaren, D., Wells, K.D., Prather, R.S., 2014. Use of the CRISPR/Cas9 system to produce genetically engineered pigs from in vitro-derived oocytes and embryos. Biol Reprod 91. https://doi.org/10.1095/biolreprod.114.121723

Yang, H., Zhang, J., Zhang, X., Shi, J., Pan, Y., Zhou, R., Li, G., Li, Z., Cai, G., Wu, Z., 2018. CD163 knockout pigs are fully resistant to highly pathogenic porcine reproductive and respiratory syndrome virus. Antivir Res 151, 63–70. https://doi.org/10.1016/j.antiviral.2018.01.004

Yin, X., Harmancey, R., McPherson, D.D., Kim, H., Huang, S.-L., 2023. Liposome-Based Carriers for CRISPR Genome Editing. Int J Mol Sci 24, 12844. https://doi.org/10.3390/ijms241612844