UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Generation of genetically modified pigs by CRISPR-Cas9 for experimental models

Generación de cerdos modificados genéticamente mediante CRISPR-Cas9 como modelos experimentales

D. Sergio Navarro Serna

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D. Joaquín Jerónimo Gadea Mateos, Catedrático de Universidad del Área de Fisiología Veterinaria en el Departamento de Fisiología, AUTORIZA:

La presentación de la Tesis Doctoral titulada "Generación de cerdos modificados genéticamente mediante CRISPR-Cas9 como modelos experimentales", realizada por D. Sergio Navarro Serna, bajo mi inmediata dirección y supervisión, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

En Murcia, a 31 de Mayo de 2022

UNIVERSIDAD DE
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Dª. Raquel Romar Andrés, Profesora Titular de Universidad del Área de Fisiología Veterinaria en el Departamento de Fisiología, AUTORIZA:

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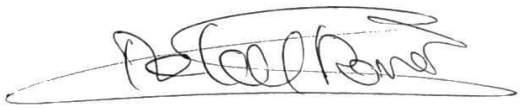

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Nombre y apellidos del doctorando/a / Ph.D. student name and surname: Sergio Navarro Serna
Centro de origen / Institution of origin: Escuela Internacional de Doctorado de la Universidad de Murcia
Programa de Doctorado / Doctoral Programme: Doctorado en Biología y Tecnología de la Salud Reproductiva
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Nombre del Centro de Educación Superior o Instituto de Investigación / Name of the host institution: School of Veterinary Medicine, University of California Davis
Departamento/Centro / Department/Centre: Department of Animal Science/ School of Veterinary Medicine
Dirección del Centro / Address: 1 Shields Ave, Davis, CA 95616, EE.UU.
Localidad y país / City and country: Davis / Estados Unidos de América

3. DATOS DEL TUTOR/INVESTIGADOR RESPONSABLE DE LA ESTANCIA / RESEARCH SUPERVISOR AT HOST INSTITUTION

Nombre y apellidos del tutor/investigador / Research supervisor name and surname: Pablo Juan Ross
DNI/Pasaporte nº. / I.D. / Passport nº:USA 522606401
E-mail: pross@ucdavis.edu
Departamento/Centro al que pertenece / Department/Centre: Department of Animal Science / University of California Davis

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El abajo firmante certifica que el doctorando/a arriba mencionado/a ha realizado una estancia en este Centro bajo mi supervisión en las siguientes fechas: desde el 20 de 09 de 2019 hasta el 19 de 12 de 2019, cumpliendo los objetivos planteados para la estancia.

Durante la estancia se han realizado técnicas producción de embriones porcinos partenogenicos, embriones porcinos in vitro y recogida de embriones producidos *in vivo* para ser editados genéticamente con CRISPR/Cas9 mediante electroporación y posteriormente transferidos a receptoras mediante transferencia quirúrgica.

También se realizaron técnicas de biología molecular y bioinformática para evaluar mosaicismo y ediciones off-target en embriones editados mediante CRISPR/Cas9.

The person who sign this document certify that the Ph.D. student above-mentioned has visited this institution under my supervision in the following dates: from 20 of 09 of 2019 to 19 of 12 of 2019, fulfilling the objectives planned for the visiting research.

In this time, techniques of parthenotes embryo production, *in vitro* embryo production and *in vivo* embryo recollection were performed to do genetic edition using CRISPR/Cas9 technology by electroporation. Some of these embryos were transferred to recipients by surgery embryo transfer.

In this period, techniques of molecular biology and bioinformatics were used to evaluate mosaicism and off-target edition on CRISPR/Cas9 genetically edited embryos

En/In Davis a 19 de Diciembre de 2019

Firmado y sellado / Signed and stamped*. Dr.: Pablo Juan Ross

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A mis padres

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This Doctoral Thesis by a compendium of publications includes one book chapter that will be published by Springer Nature editorial in July 2022 and three articles published in different journals included in the Journal Citation Reports (Web of Science). The four contributions are the following:

1.**Navarro-Serna, S.**; Piñeiro-Silva, C.; Romar, R.; Parrington, J.; Gadea, J. **Generation of gene edited pigs**. Book: Sustainable Agriculture Reviews 57. Animal Biotechnology for Livestock Production 2. 2022. Editors: Vinod Kumar Yata, Ashok Kumar Mohanty and Eric Lichtfouse. Series: Sustainable Agriculture Reviews. Series Ed.: Lichtfouse, Eric. Series ISBN 9783031074950. Publisher: Springer Cham. https://link.springer.com/book/9783031074950.

According to Bibliometric Indicators for Publishers project (http://bipublishers.es/), in 2014 Springer editorial published a total of 66 books in the field of Agricultural Sciences with 1000 book chapters that received 1576 citations, with a value of 1.83 citations for Field Normalized Citation Score. This publisher has an Activity Index of 1.48. Higher values than one means more specialization in the given field.

2.**Navarro-Serna, S.**; Hachem, A.; Canha-Gouveia, A.; Hanbashi, A.; Garrappa, G.; Lopes, J.S.; Paris-Oller, E.; Bermejo-Álvarez, P.; Matas, C.; Romar, R.; Parrington, J.; Gadea, J. Generation of Nonmosaic, Two-Pore Channel 2 Biallelic Knockout Pigs in One Generation by CRISPR-Cas9 Microinjection Before Oocyte Insemination. **The CRISPR Journal. 2021;** 4: 1-16. https://doi.org/10.1089/crispr.2020.0078.

Impact Factor: 6.071. Journal Citation Reports (Clarivate, 2021).
Category: Genetics & Heredity; position 24 from 175; Q1.
Citations: 4 Scopus, 4 Google scholar (data on 08th June 2022).

3. **Navarro-Serna, S.**; Dehesa-Etxebeste, M.; Piñeiro-Silva, C.; Romar, R.; Lopes, J.S.; López de Munaín, A.; Gadea, J. Generation of Calpian-3 knock-out porcine embryos by CRISPR-Cas9 electroporation and intracytoplasmic microinjection of oocytes before insemination. **Theriogenology. 2022**; 186: 175-184. https://doi.org10.1016/j.theriogenology.2022.04.012.

   Impact Factor: 2,740. Journal Citation Reports (Clarivate, 2021).

   Category: Veterinary Sciences; position 21 from 146; Q1.

Citations: 0 Scopus, 0 Google scholar (data on 08th June 2022).

4. **Navarro-Serna, S.**; Piñeiro-Silva, C.; Luongo, C.; Parrington, J.; Romar, R.; Gadea, J. Effect of Aphidicolin, a Reversible Inhibitor of Eukaryotic Nuclear DNA Replication, on the Production of Genetically Modified Porcine Embryos by CRISPR/Cas9. **International Journal of Molecular Sciences. 2022**, 23, 2135. https://doi.org/10.3390/ijms23042135.

Impact Factor: 5.924. Journal Citation Reports (Clarivate, 2021).

Category: Biochemistry & Molecular Biology; position 67 from 295; Q1.

Citations: 1 Scopus, 1 Google scholar (data on 08th June 2022)..

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SUMMARY/RESUMEN

SUMMARY

Gene editing consists of the modification of the genomic sequence by insertions, deletion, or base pairs substitution. The development of programmable endonucleases like Zing-finger nucleases (ZFNs), transcription activator like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR-associated (Cas) supposed an important advance in the efficiency of gene editing. The use of this technology to produce gene edited animals is widely developed and has different applications such as studying the role of specific genes for basic science, improving agricultural production, or using animals for biomedicine applications (xenotransplantation, disease models or animals for bioreactors).

The pig (*Sus scrofa domesticus*) is one of the most important animals in the meat industry worldwide. In addition, the physiological similarity between pigs and human allows use this animal as a good candidate for biomedical applications. Despite the suitability of pig as an experimental model, *in vitro* embryo production in this species is still inefficient due to the high incidence of polyspermic fertilization and suboptimal embryo development, being this an important limitation for the fast development of gene-edited pigs. For this reason, one of the main objectives of this Thesis was to optimize the conditions for the generation of knock-out (KO) pig embryos by different methodologies to achieve the higher efficiency of the system, in terms of blastocyst formation and mutation rates. On the other hand, the production of gene-edited embryos has an important limitation, the high incidence of mosaicism, thus being another central objective of this study to produce embryos with the lowest mosaicism incidence to later obtain pigs with the desired mutation and maximum efficiency.

All the studies shown in this Doctoral Thesis were based on the use of a conventional system of *in vitro* embryo production (IVP) involving the use of immature cumulus-oocyte complexes (COCs) from ovaries obtained from prepubertal gilts slaughtered for meat consumption. This approach increases the availability of a huge number of COCs per week avoiding the ethical issues derived from the use of animals for experimentation. Once collected, the use of immature COCs implies the need of *in vitro* oocyte maturation (IVM) and fertilization (IVF), followed by an embryo culture procedure. In this experimental work, frozen-thawed boar semen is routinely used for IVF after being selected by a swim-up procedure that had been previously optimized too. With this IVP system the blastocyst rate, considered as the percentage of blastocysts obtained per total number of inseminated oocytes, ranged from 21 to 40 % in the control

groups of the different studies being over the regular data for porcine species. Therefore, besides avoiding ethical issues, this IVP system allowed to produce a high number of embryos per work session.

Different genotyping methodologies have been employed throughout this study. Initially, High-resolution melt (HRM) analysis was used to analyse the two-pore channel 2 (TPC2) gene in the pig cells following transfection of the single guide RNA (sgRNA) expression constructs. Later, mutation detection was performed using the fluorescent PCR-capillary gel electrophoresis technique. Samples were considered wild type when the peak obtained by capillary electrophoresis was the same size as the control peak. Other peaks of different sizes concerning the control peak were considered to be KO, and when more than two peaks were detected in a sample, this sample was evaluated as a mosaic. Fluorescent PCR-capillary gel electrophoresis technique offers a fast and simple method to evaluate both mutation and mosaicism with similar cost to Sanger sequencing, in which only mutation can be detected. As a limitation, fluorescent PCR-capillary electrophoresis does not allow knowing the sequence, for which it is necessary to perform sequencing.

In the first study (chapter 2) the system of microinjection was optimized by varying two main factors: the time of microinjection and the use of Cas9 as RNA or ribonucleoprotein (RNP). After determining that in our IVP system, DNA replication first begins around 10 hours post-insemination (hpi), we decided to evaluate the effect of timing of CRISPR-Cas9 microinjection before insemination, 5-6 hpi (before pronucleus formation), and 10-12 hpi (after pronucleus formation), and when DNA replication had been completed. Also, we investigated the possible effects of injecting Cas9 protein compared with Cas9 mRNA. When Cas9 was delivered as mRNA, blastocyst yield was significantly lower (P=0.05) in the groups microinjected after insemination compared with embryos microinjected before insemination, but all groups were similar to the control group. The mutation rate was similar in all groups, being around 30-42%, whereas the mosaicism rate was 0%, 9%, and 21% for embryos microinjected before insemination, at 5-6 hpi and 10-12 hpi, respectively. On the other hand, when Cas9 was delivered as protein, blastocyst yield was lower in embryos microinjected before insemination and 5-6 hpi compared with non-injected embryos (P=0.01). No differences were observed between embryos microinjected at 10-12 hpi and the control group. Mutation rates using Cas9 protein did not differ between groups but were higher compared with those microinjected with Cas9 mRNA. Mosaicism rate was lower in the group microinjected before insemination (15%) compared with those injected afterwards (33% and 57%, for

5-6 hpi and 10-12 hpi respectively), but the differences were only significant for the comparison with the 10-12 hpi group (P=0.02).

Due to the better efficiency in producing biallelic KO embryos, the condition of RNP injection before insemination was selected to produce TPC2 KO piglets. Therefore, *in vitro*-produced embryos microinjected with RNP before insemination followed by embryo transfer allowed the production of two females and one male with frame shift mutations in both alleles of the TPC2 gene, which will be used to generate a homozygous TPC2 KO pig line for the study of the effects of TPC2 functional deficit on pathophysiological processes in pigs.

In the two next chapters, electroporation and microinjection were used. In chapter 3, after the optimization of electroporation conditions (4 pulses of electroporation) and changes to the ratio of sgRNA:Cas9, the efficiency of electroporation was tested in comparison to microinjection to analyse different strategies to generate CAPN3 KO pig embryo that could be models of LGMDR1 human disease. Regarding mutation parameters and blastocyst rate, no differences were found between either the methods (electroporation *vs.* microinjection) or the combination of guides.

In the last chapter, the objective was to evaluate whether the application of aphidicolin makes it possible to improve the gene-editing system by reducing the mosaicism without affecting the quality and quantity of genetically modified embryos obtained. For this, sgRNAs against TPCN1 were used. After electroporation, the use of aphidicolin induced a reduction of the blastocyst rate, with a tendency (P=0.06) to reduce the mosaicism rate. The mutation rate was higher in groups with a higher RNP concentration, and no differences were found following the use of aphidicolin. Finally, the possible effect of the use of aphidicolin was explored using microinjection conditions established in chapter 2 and electroporation conditions in chapter 3. The negative effect of aphidicolin on the blastocyst development was confirmed, especially when microinjection was used and a positive effect in avoiding the mosaicism was observed. Therefore, overall, the rate of non-mosaic KO embryos produced with or without aphidicolin was similar. Therefore, under the tested conditions in these experiments, the use of aphidicolin did not show any advantage.

In conclusion, after the studies carried out in the different chapters of this Thesis, an efficient and optimized system has been established to generate gene-edited embryos and pigs that could be used for human disease models.

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RESUMEN

La edición génica consiste en la modificación de la secuencia de ADN mediante inserciones, deleciones o sustituciones de pares de bases. A mediados de los años 80, las modificaciones génicas en embriones porcinos comenzaron a realizarse mediante la inserción aleatoria de ADN exógeno mediante la microinyección pronuclear. Posteriormente, se utilizaron otros enfoques para introducir ADN extraño en embriones de cerdo, como el uso de retrovirus, vectores lentivirales, transferencia de ADN mediada por espermatozoides o el uso de transposones. Estos enfoques tenían una relevancia y utilidad limitadas porque el ADN extraño se insertaba aleatoriamente en el genoma del huésped y no permitía la edición específica de genes endógenos, por lo que se necesitaban otros enfoques. El desarrollo de endonucleasas programables tales como las endonucleasas con dedos de zinc (ZFNs), las nucleasas de actividad similar a activador de transcripción (TALENs) y las repeticiones palindrómicas cortas agrupadas y regularmente interespaciadas (CRISPR)-Cas9 ha supuesto un importante avance en la eficiencia de la edición génica. Todos ellos están compuestos principalmente por dos dominios: un dominio de unión al ADN que reconoce la secuencia de ADN diana y un dominio de escisión que produce una rotura de doble cadena (DSB) en la secuencia de ADN diana. El uso de esta tecnología para producir animales modificados genéticamente se encuentra ampliamente desarrollada, presentando diversas aplicaciones como el estudio de genes para el desarrollo de ciencia básica, la mejora de la producción ganadera mediante la mejora de la canal o generar animales resistentes a enfermedades infecciosas y el uso de animales para aplicaciones biomédicas tales como el uso de animales para xenotrasplantes, modelos de enfermedad y biorreactores.

El cerdo (*Sus scrofa domesticus*) es uno de los animales más importantes de la industria alimentaria. Además, la similitud fisiológica entre éste y el ser humano permite que el cerdo sea un buen candidato como modelo para aplicaciones biomédicas. A pesar de la idoneidad del cerdo como modelo experimental, la producción de embriones *in vitro* en esta especie aún es ineficiente debido a la alta incidencia de la polispermia en los procesos de fecundación *in vitro* y el bajo rendimiento en el desarrollo *in vitro* de embriones, siendo esta una limitación importante para el rápido desarrollo de los cerdos editados genéticamente. Por esta razón, uno de los principales objetivos de esta tesis fue optimizar las condiciones para generar cerdos *knock-out* (KO) por diferentes metodologías para alcanzar una alta tasa de blastocistos con un alto rendimiento de

mutación. Por otro lado, también encontramos limitaciones respecto a la generación de embriones editados genéticamente, la alta incidencia de mosaicismo (presencia de más de dos alelos distintos en un mismo organismo), siendo también otro de los principales objetivos de la tesis intentar reducir el mosaicismo para posteriormente obtener animales mutados con la máxima eficiencia posible.

En el capitulo 1, se realizó una revisión bibliográfica en la que se recopila el desarrollo y uso de la edición génica y concretamente de la tecnología CRISPR-Cas9 en la especie porcina además de revisar en profundidad las aplicaciones de modelos porcinos, los últimos avances en la producción de cerdos modificados genéticamente considerando las ventajas y las limitaciones, así como los nuevos enfoques con respecto a esta tecnología. Posteriormente se ha continuado con los capítulos de contenido experimental.

Todos los estudios mostrados en esta tesis se basan en el uso convencional de un sistema de producción *in vitro* de embriones (IVP), el cual incluye el uso de complejos cúmulo-ovocito (COCs) procedentes de ovarios de cerdas prepúberes destinadas a matadero, lo cual permite obtener un gran número de COCs, evitando así los problemas éticos derivados del uso de animales de experimentación. Una vez recolectados, los COCs se llevan a madurar *in vitro*, posteriormente se fecundan *in vitro* (IVF) y se realiza el cultivo embrionario. En este trabajo experimental se usaron muestras de semen de verraco congeladas-descongeladas para la IVF tras ser seleccionados por swim-up previamente optimizado. Con este sistema de IVP, la tasa de blastocistos, considerada como el número de blastocistos obtenidos respecto al total de ovocitos inseminados, se encuentra entre el 21-40% en los grupos control, siendo un dato medio observado en la especie porcina. Por lo tanto, además de evitar problemas éticos, este sistema IVP permitió producir una gran cantidad de embriones por sesión de trabajo.

En este trabajo se han empleado diversas metodologías para el genotipado. Inicialmente, se utilizó el análisis de alta resolución de fusión (HRM) para analizar el gen *two pore channel 2* (TPC2) en las células de cerdo después de la transfección de las construcciones de (guías simples de RNA) sgRNA. Posteriormente, la mutación fue detectada mediante PCR con cebadores fluorescentes seguida de una electroforesis capilar, considerándose nativas las muestras con un pico similar al pico control y editado cuando presentaba picos diferentes al control. Cuando fueron detectados más de dos picos por muestras, correspondiente a la presencia de más de dos alelos diferentes, ésta fue considerada mosaico. El uso de PCR con cebadores fluorescentes seguida de una electroforesis capilar como estrategia ofrece un método rápido y sencillo para detectar y evaluar tanto mutación como mosaicismo. El coste de este procedimiento es

similar a la secuenciación por Sanger, en el cual solo la mutación puede ser detectada. Como limitación, la PCR con cebadores fluorescentes seguida de electroforesis capilar no permite conocer la secuencia, siendo necesario una posterior secuenciación.

En el primer estudio, correspondiendo al capítulo 2, optimizamos el sistema de microinyección variando dos factores; el tiempo de microinyección y el uso de Cas9 como RNA o ribonucleoproteína (RNP). Después de determinar que en nuestro sistema de IVP la replicación del ADN comenzaba aproximadamente tras 10 horas post inseminación (hpi), se decidió evaluar el efecto del tiempo de microinyección de CRISPR-Cas9 antes de la inseminación, 5-6 hpi (antes de la formación de pronúcleos sin haber tenido lugar la primera replicación de ADN del embrión) y 10-12 hpi (después de la formación de pronúcleos y cuando la replicación del ADN se ha completado). También investigamos el posible efecto de la inyección de Cas9 como proteína respecto a ARN. Cuando Cas9 fue liberado como RNA, la tasa de blastocisto fue significativamente menor (P=0,05) en los grupos microinyectados después de la inseminación en comparación con los embriones microinyectados antes de la inseminación, pero todos los grupos fueron similares respecto al control. En cuanto a la tasa de mutación, ésta fue similar en todos los grupos, rondando el 30-42%, mientras el mosaicismo fue del 0%, 9% y 21% en los embriones microinyectados antes de la inseminación, 5-6 hpi y 10-12 hpi respectivamente. Por otro lado, cuando Cas9 fue liberado como proteína, la tasa de blastocisto fue menor en los embriones microinyectados antes de la inseminación y 5-6 hpi comparados con los no microinyectados (P=0.01). No se observaron diferencias entre embriones microinyectados a las 10-12 hpi y los controles. La tasa de mutación no fue diferente entre los distintos tiempos, pero si fue superior a los microinyectados con ARN. El mosaicismo fue menor en el grupo microinyectado antes de la inseminación (15%) comparado con los otros grupos (33% y 57% en 5-6 hpi y 10-12 hpi respectivamente), pero las diferencias fueron solo significativas cuando se compararon con el grupo 10-12 hpi (P=0.02).

En este capitulo se utilizaron guías para generar embriones KO para TPC2, los cuales son proteínas de canales permeables a cationes ubicadas en las superficies de los orgánulos endolisosomales. Los canales TPC se encuentran en muchos mamíferos tales como los humanos, ratones y cerdos y presentan dos isoformas funcionales TPCN1 (con el que trabajamos en el capítulo 3) y TPC2. Se ha demostrado que las proteínas TPC se encuentran involucradas en una variedad de procesos fisiopatológicos importantes, incluido el desarrollo embrionario, la diferenciación celular, la regulación de la autofagia en corazón y músculo esquelético, la secreción de insulina en células β-

pancreáticas, la progresión del cáncer, la reacción acrosómica en espermatozoides, la síntesis de melanina en los melanosomas y en la infección de virus como el ébola y el SARS-CoV-2.

Debido a la mejor eficiencia en la producción de embriones KO bialélicos cuando se utilizaban las condiciones de inyección de RNP antes de la inseminación, este método fue utilizado para producir cerdos KO para TPC2. Por lo tanto, embriones producidos *in vitro* con estas condiciones fueron transferidos, permitiendo la producción de dos hembras y un macho con mutaciones en ambos alelos de TPC2, los cuales fueron utilizados para generar una línea de cerdos TP2 KO para el estudio del déficit funcional de TPC2 en procesos fisiopatológicos.

En los dos capítulos siguientes, la electroporación y la microinyección fueron usadas. En el capítulo 3, se realizó una optimización de las condiciones de electroporación variando las condiciones de pulso, siendo 4 pulsos a 30 V y 1 ms de duración de pulso las condiciones óptimas alcanzadas para nuestro sistema de desarrollo embrionario. Además de las condiciones de pulsos de electroporación, también se cambió la ratio de sgRNA frente a Cas9, siendo más eficiente la mutación cuando se duplicaba la concentración de sgRNA manteniendo una concentración de Cas9 constante.

Después de optimizar las condiciones de electroporación (4 pulsos) y cambiar la ratio de sgRNA frente a Cas9, la eficiencia de la electroporación fue comparada respecto a microinyección analizando distintas estrategias para generar embriones de cerdos KO para CAPN3, los cuales son un modelo para la enfermedad humana distrofia muscular de cinturas autosómica recesiva de tipo R1 (LGMDR1). La enfermedad LGMDR1, anteriormente conocida como LGMD2A, es una miopatía autosómica recesiva descrita en humanos que está causada por una deficiencia de CAPN3. Actualmente, no existe un tratamiento efectivo para LGMDR1, lo que resulta en una pérdida de la capacidad de andar dentro de los 20 años posteriores al inicio de la enfermedad en la mayoría de los pacientes. La posibilidad de utilizar un modelo porcino para esta enfermedad abrirá nuevas estrategias de tratamiento para las distrofias actualmente en desarrollo. Para generar este modelo, por un lado, se usaron dos combinaciones distintas de guías cuyo objetivo fue generar una deleción en el exón 1 y eliminar el codón de inicio de CAPN3 y por otro lado se intentó generar una mutación en el exón 22 con el objetivo de generar una mutación concreta que causa LGMDR1. Respecto a los parámetros de mutación y la tasa de blastocisto, no se observaron diferencia entre ambos métodos (electroporación *vs*. microinyección) o la combinación de las diferentes guías.

En el último capítulo, se testó el uso de afidicolina en el proceso de edición génica, siendo ésta un fármaco que inhibe de forma reversible la replicación del ADN en células de mamífero y cuyos resultados ya habían sido reportados en cultivo *in vitro* de líneas celulares de la especie porcina para la sincronización de células y su posterior uso en procesos como la transferencia nuclear de células somáticas. Por tanto, el objetivo de este capítulo fue evaluar si la aplicación de afidicolina hacía posible mejorar el sistema de edición génica reduciendo el mosaicismo, mediante la inhibición temporal o ralentización del comienzo de la replicación del ADN en embriones porcinos sin afectar a la calidad y cantidad de embriones modificados obtenidos. Para esto, una sgRNA contra el gen TPCN1 fue usada. En este experimento, tras la fecundación y posterior electroporación, la incubación con afidicolina durante un periodo de 14 horas causó una reducción de la tasa de blastocisto, con una tendencia (P=0.06) de reducción de la tasa de mosaicismo. Además de incubar con afidicolina los ovocitos fueron electroporados con las condiciones de electroporación descritas en el capítulo 3 y con dos concentraciones de RNP, como resultado se observó que la mutación fue mayor en los grupos con una concentración mayor de RNP y no se observaron diferencias dependientes del uso de afidicolina. Finalmente, el posible efecto del uso de afidicolina fue estudiado en las condiciones de microinyección establecidas en el capítulo 2 y las condiciones de electroporación del capítulo 3. En ésta última experiencia, el efecto negativo del uso de afidicolina en el desarrollo embrionario fue confirmada, especialmente cuando los embriones fueron microinyectados, pero el efecto de reducir el mosaicismo también fue confirmado, observándose una disminución en presencia de afidicolina respecto a su ausencia. Sin embargo, en general, la tasa final de embriones KO sin presencia de mosaicismo respecto al número inicial de ovocitos inseminados producidos con o sin afidicolina fue similar. Por lo tanto, bajo las condiciones probadas en estos experimentos, el uso de afidicolina no mostró ninguna ventaja.

En conclusión, tras los estudios realizados en los distintos capítulos de esta tesis doctoral se ha establecido un sistema eficiente y optimizado para generar embriones y cerdos editados genéticamente que podrían usarse para modelos de enfermedades humanas.

INTRODUCTION

The pig (*Sus scrofa domesticus*) represents an important animal for the meat industry worldwide, being the second most consumed meat source in the world in 2020 after poultry (FAO, 2021). On the other hand, the similarity between pigs and humans in physiology, anatomy, body size, and metabolic profile allows the porcine species to be used as a human experimental model for biomedical research such as human disease models, xenotransplantation, and bioreactors (Hryhorowicz et al., 2020; Niu et al., 2021; Hou et al., 2022). Since its domestication, 9000 years ago from the wild boar (*Sus scrofa*) (Giuffra et al., 2000), humans have been selecting for breeding those porcine variants with desired characteristics for food production and animal husbandry. This genetic selection has most recently allowed a significant increase in pig production in comparison with that obtained a few decades ago (Koketsu and Iida, 2020; Peltoniemi et al., 2020). However, classical breeding and genetic selection techniques in pigs are slow processes with a high economic and time cost (Bichard and David, 1985) that might be overpassed by using genetic engineering (Yang and Wu, 2018).

In the middle 80's, gene modifications in porcine embryos began to be carried out by random insertion of exogenous DNA by pronuclear microinjection (Brem et al., 1985; Hammer et al., 1985). Later on, other approaches were used to introduce foreign DNA into pig embryos such as the use of retroviruses (Petters et al., 1987), lentiviral vectors (Hofmann et al., 2003), sperm-mediated DNA transfer (Lavitrano et al., 1997; Pereyra-Bonnet et al., 2008; Garcia-Vazquez et al., 2009) or the use of transposons (Garrels et al., 2011). These approaches had limited relevance and usefulness because the foreign DNA was randomly inserted into the host genome not allowing the specific editing of endogenous genes, so other approaches were needed. In recent decades, techniques have been developed for gene editing such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and components in the clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9. All of them are composed principally of two domains: a DNA binding domain that recognises the target DNA sequence and a cleavage domain that produces a double-strand break (DSB) in the target DNA sequence (Petersen, 2017).

Gene editing consists of the modification of the genomic sequence with high accuracy and specificity by inserting or deleting nucleic pairs of bases. Although ZFNs (Hauschild et al., 2011) and TALENs (Carlson et al., 2012) were used to generate knock-out (KO) pigs by somatic cell nuclear transfer (SCNT), highly efficient gene editing results were not obtained until the development of programmable endonucleases such as CRISPR-Cas9, which allowed to generate KO and knock-in (KI) organisms in a simple and faster way (Hai et al., 2014; Whitworth et al., 2014).

The first genetically modified pigs were produced by the CRISPR-Cas9 system in 2014, a KO pig model of the human von Willebrand disease (Hai et al., 2014), and a KO pig model for CD163 and CD1D were reported (Whitworth et al., 2014). Since then, the generation of a large number of genetically modified pigs for various purposes has been reported. **In chapter 1 of this Doctoral Thesis**, we review in deep the applications of pig edited gene model produced with the latest advances in the production of genetically modified pigs with a particular focus on the use of CRISPR-Cas9 gene editing in the porcine species, to produce genetically modified pigs, and with a consideration of advantages and limitations, as well as new approaches regarding this technology (Navarro-Serna et al. 2022: *Generation of gene-edited pigs. Book: Sustainable Agriculture Reviews 57. Animal Biotechnology for Livestock Production 2.* https://link.springer.com/book/9783031074950). This review will be published as a book chapter in July 2022 by the prestigious editorial Springer Nature. In this first chapter, we will describe initially the history of the generation of gene-modified pigs, analyse the application of different methodologies in the historic period before the use of programmable endonucleases and later by describing the bases of the endonucleases, and finally, we will expose the methodology to generate gene-edited pigs.

In this sense, the production of genetically modified animals can be carried out directly in oocytes/embryos (Hai et al., 2014; Onuma et al., 2017) or somatic cells with a subsequent nuclear transfer in enucleated oocytes (Whitworth et al., 2014; Zhou et al., 2015). In both cases, the development and application of assisted reproductive techniques are transcendental to produce genetic edited organisms. The *in vitro* production of porcine embryos is still inefficient, due to the high incidence of polyspermy in *in vitro* fertilization (IVF) process (Coy and Aviles, 2010) being the causes of polyspermy attributed, among others, to an inefficient *in vitro* maturation of the oocyte and/or the capacitation status of spermatozoa during the gametes co-incubation (Funahashi and Romar, 2004). In any of the cases, the efficiency of production of viable porcine embryos is still nowadays much lower than other livestock species, such as bovine, therefore any additional gamete manipulation, process, or toxic compounds to which the embryo may be subjected, would further limit the performance of *in vitro* production techniques in this species. The main harmful procedure to which a genetically modified embryo can be subjected is the delivery of CRISPR-Cas9 in the cytoplasm.

Intracytoplasmic microinjection of CRISPR-Cas9 is the main method reported to introduce CRISPR/Cas9 (revised by (Navarro-Serna et al., 2020), nevertheless electroporation (Tanihara et al., 2016; Lin and Van Eenennaam, 2021) and lipofection of oocytes and embryos (Hirata et al., 2021a; Hirata et al., 2021b) were also developed as

a simpler methodology without expensive equipment and sophisticated techniques. Once produced, gene-edited embryos can be transferred to recipient animals and the final efficiency of the embryo transfer of gene-edited embryos is affected by diverse factors such as the control of the oestrous cycle, the synchrony of oestrus between donors and recipients, the donor age, and the use of surgical or non-surgical transfer techniques (Youngs, 2001; Schmidt et al., 2010; Martinez et al., 2013; Shi et al., 2015; Nohalez et al., 2017).

Another important question arises when working with gene-edited embryos: what is the most reliable procedure to detect mutations? In the literature, it can be found different methods to detect DNA modifications, which change depending on the final information that is wanted to be obtained: simple detection of mutations, identification of the mutant sequence, or evaluation of mosaicism. The simplest method is to perform PCR and then agarose gel electrophoresis. This method is used to evaluate large insertions or deletions in the DNA. Other methods allow not only to detect mutations in the sample but also to detect the number of bases that are inserted or deleted in the alleles as well as the number of alleles in a sample. The fluorescent PCR-capillary gel electrophoresis technique is accurate enough to differentiate one base-pair difference between alleles, so this technique can indicate the presence or absence of a frameshift in the coding sequence of the gene (Ramlee et al., 2017). Although electrophoresis-based techniques offer a huge amount of useful information, their use makes it not possible to know the sequences of alleles detected. For knowing this, it is necessary to perform sequencing and Sanger sequencing is the most reported method in the literature for the detection of mutations.

In addition to the low embryo production yield, there are other limitations in the generation of the gene-edited organism related to gene editing being mosaicism the main problem in the generation of gene-edited organisms by embryo editing (Sato et al., 2015). Mosaicisms consist of the presence of more than two alleles of one gene in the same organism due to the activity of CRISPR-Cas9 after the first DNA replication in the zygote (Navarro-Serna et al., 2020). This represents a problem since organisms with mosaic genotypes cannot be used for the experimental model, although they can be used to produce a second generation if the germ line has desired mutations (Lamas-Toranzo et al., 2019; Navarro-Serna et al., 2020). Despite the high specificity of CRISPR-Cas9, a mutation in sequences like the target region may be possible, causing the so-called off-target mutations. Off-target mutations are not frequent and have not been reported yet in livestock animals edited by CRISPR-Cas9 (revised by (Navarro-Serna et al., 2020)). However, this possibility must be considered since off-target mutation could

mask the phenotype of the edited animals.

The ability to edit genes allows insertion of exogenous sequences (knock-in; KI) or elimination of gene function (knock-out; KO) which might have unlimited applications for use in basic science, agriculture, and biomedicine (revised by (Gadea and Garcia Vazquez, 2010; Perisse et al., 2021). The applications of the gene-edited pigs in the biomedicine field include xenotransplantation, and bioreactors to produce different bioproducts and models of human diseases (Bertolini et al., 2016; Niu et al., 2021; Yue et al., 2021; Hou et al., 2022). In these areas, the production of genetically modified pigs makes it possible to investigate areas of interest such as gene expression, protein structure, intracellular mechanisms, and gene functions (Whitelaw et al., 2016; Wells and Prather, 2017). In terms of agricultural applications, the CRISPR-Cas9 system can be used to create animals with an improved carcass composition, decrease input requirements, animals with improved milk production, or animals with resistance to specific diseases (Burkard et al., 2017; Li et al., 2018; Zhang et al., 2018; Wang et al., 2020). Nevertheless, there are severe restrictions on consumption of food products derived from genetically modified organisms (GMOs) (Mir et al., 2022). This topic will be also revised in chapter 1.

The use of gene editing in organisms to achieve genetic advantages in a short period has been highly controversial. After the application of gene editing technologies and the development of programmable endonucleases that are as easy and cheap to use as CRISPR-Cas9, it was necessary to create legislation to regulate the use of this technology and to evaluate the ethical issues of the applications (Garas et al., 2015; Chneiweiss et al., 2017). Currently, genetically modified organisms are covered by the same regulations as transgenic organisms (Lamas-Toranzo et al., 2017; Wasmer, 2019). This legislation also includes organisms with simple genetic modifications generated by genetic engineering, such as mutations that affect a single base. However, these mutations cannot be distinguished from organisms bred by conventional techniques, such as those that arise from random mutagenesis (Wasmer, 2019).

In conclusion, in the mentioned book chapter and a previous revision article not directly included in this thesis (Navarro-Serna et al., 2020), we have revised in deep the state of the art, analysed the different aspects of the methodology, possible applications, and the ethical and legal issues of the use of the gene-edited pigs. This detailed bibliographic revision helped to design the experimental work that was sorted into three additional chapters with the global aims of i) optimizing the conditions to generate gene-edited pigs by CRISPR-Cas9; ii) increasing the percentage of efficient gene-edited

embryos to be transferred to receptors, and iii) obtaining viable genetically modified pigs through this technique.

In the **first experimental study (Chapter 2)** it was evaluated the best time for microinjection into the fertilized egg and the best molecular form of Cas9, to reduce mosaicism, to establish an acceptable mutation rate to be able to produce TPC2 KO embryos with the final goal of generating live TPC2 KO pigs. This first study was published in 2021 with open access in The CRISPR Journal (*Navarro-Serna, S.; Hachem, A.; Canha-Gouveia, A.; Hanbashi, A.; Garrappa, G.; Lopes, J.S.; Paris-Oller, E.; Bermejo-Álvarez, P.; Matas, C.; Romar, R.; Parrington, J.; Gadea, J. Generation of Nonmosaic, Two-Pore Channel 2 Biallelic Knockout Pigs in One Generation by CRISPR-Cas9 Microinjection Before Oocyte Insemination. Crispr J. 2021, 4, 1–16, doi:10.1089/crispr.2020.0078*).

The first step in this chapter was the development of efficient single guide RNAs (sgRNAs) and their testing in *in vivo*-derived embryos. For this analysis, *in vivo*-produced zygotes were microinjected with different sgRNAs and one of them was selected according to the mutation rate obtained (35.7% of the embryos microinjected). After that, it was evaluated the optimal time for microinjection into the fertilized egg and the best molecular form of Cas9 to reduce mosaicism, establish an acceptable mutation rate, and be able to produce TPC2 KO embryos. *In vitro*-matured oocytes were microinjected before or after insemination (5-6 and 10-12 hours post insemination; hpi) with the sgRNA selected previously in combination with mRNA Cas9 or Cas9 ribonucleoprotein (RNP). The best results were obtained with the microinjection of Cas9 RNP before the *in vitro* fertilization, yielding 22.7% blastocyst rate, 47.6% mutation rate and 15% mosaicism.

Once the conditions of microinjection were optimized, embryos were transferred to female recipients to produce TPC2 KO piglets. When foetuses and piglets were obtained, tissues were analysed to detect mutations in the TPC2 gene. Up to 5 foetuses and 12 piglets were obtained following embryo transfer, 1 of the foetuses (20%) and 3 of the piglets (25%) were non-mosaic biallelic TPC2 KOs. No off-target mutations were observed in any animal. This is the first reference, to our knowledge, of the generation of TPC2 KO pigs. These animals will be used for exploring the calcium cell signalling function in different organs and tissues and they will become a valuable model for different biomedical fields such as neurophysiology (Martucci and Cancela, 2022), cardiology (Capel et al., 2015), metabolism (Grimm et al., 2014; Arredouani et al., 2015),

or virology (Sakurai et al., 2015) thus facilitating and enlarging future research and TPC targeting for therapeutical purposes (Gerndt et al., 2020).

In the second study (Chapter 3), the objective was to optimize porcine oocyte electroporation delivery of sgRNAs for the generation of muscle-specific calcium-activated neutral protease 3 (calpain 3, CAPN3) KO embryo, maximizing embryo quality and mutation rate in comparison to microinjection. LGMDR1, previously known as LGMD2A, is an autosomal recessive myopathy described in humans that is caused by a deficiency of CAPN3 (Lasa-Elgarresta et al., 2019). There is no effective treatment for LGMDR1, resulting in a loss of ambulation within 20 years after disease onset in most patients (Lasa-Elgarresta et al., 2019). The possibility of using a pig model for this disease will open new strategies of treatment for dystrophias now in development (Lim et al., 2022).

This study was published in 2022 with open access in the Journal Theriogenology (*Navarro-Serna, S.; Dehesa-Etxebeste, M.; Piñeiro-Silva, C.; Romar, R.; Lopes, J.S.; López de Munaín, A.; Gadea, J. Generation of Calpian-3 knock-out porcine embryos by CRISPR-Cas9 electroporation and intracytoplasmic microinjection of oocytes before insemination. Theriogenology. 2022;186:175-184. https://doi.org10.1016/j.theriogenology.2022.04.012*).

Here, in a first step, it was evaluated the effect of the number of pulses on the capacity to deliver macromolecules and on further embryo development (blastocyst rate). Results showed that the electroporation using 4 or 6 pulses, at 30 V, 1 ms pulse duration and 100 ms pulse interval were the combination that offered the best results. Once it was confirmed that the electroporation conditions allowed macromolecule to cross through the zona pellucida (ZP) and oolemma, the next step was to evaluate the effect of these conditions on the generation of KO embryos using a strategy with two different sgRNAs. Regarding mutation parameters, the percentage of embryos with at least one deletion was significantly higher in the microinjection group than in the electroporation group (40% *vs.* 19%), and the blastocyst rate was lower for the 6-pulses group than in others. When the concentration of sgRNA for electroporation was doubled (from 12.5 to 25 ng/mL) no differences were found in the deletion or biallelic deletion rates between electroporation and microinjection groups.

Then, having optimized the electroporation conditions (4 pulses of electroporation) and changes to the ratio of sgRNA:Cas9, the efficiency of electroporation was tested in comparison to microinjection to analyse different strategies

to generate CAPN3 KO pig embryo that could be used as animal models of Limb girdle muscular dystrophy type R1 (LGMDR1) human disease. Regarding mutation parameters and blastocyst rate, no differences were found between either the methods (electroporation *vs*. microinjection) or the sgRNA combination.

In conclusion, the production of porcine KO embryos by oocyte electroporation is easier and faster than by microinjection and it does not require expensive equipment. We defined the optimal electroporation conditions to reach similar rates of KO embryos compared with the tedious microinjection approach, which led to the generation of the best strategy for producing porcine LGMDR1 models.

In the third and final study (Chapter 4), the objective was to reduce mosaicism ensuring that the CRISPR-Cas9 system edits the DNA before the zygote enters the S phase, when DNA replication occurs, by using a reversible inhibitor of the DNA replication. In this case, the possible strategy would be to slow down or pause DNA replication to increase the time over which the CRISPR-Cas9 system acts without leading to the presence of more than two alleles. For this study, aphidicolin, a reversible inhibitor of eukaryotic nuclear DNA replication that blocks the cell cycle at the pre-S phase (Zhang et al., 2012) was used. As far as we know, there is no published reference about the direct use of aphidicolin in porcine oocytes/embryos for inhibiting DNA replication. Additionally, there is no literature about its employment in reducing mosaicism after CRISPR-Cas9 treatment. Both aspects were explored in this study for the first time and the results were recently published, 2022, with open access at the International Journal of Molecular Sciences (*Navarro-Serna, S.; Piñeiro-Silva, C.; Luongo, C.; Parrington, J.; Romar, R.; Gadea, J. 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. 2022, 23, 2135, doi:10.3390/ijms23042135)*.

On this occasion, the first step was to determine the right aphidicolin concentration that allows the reversible inhibition of DNA replication and then to evaluate the likely detrimental effects of the different concentrations on embryo development. It was observed that incubation of zygotes with aphidicolin at different concentrations (from 0.15 to 10 µM) reduces the degree of DNA replicated at 20 hpi with significant differences to control embryos (0 µM aphidicolin). The reduction in DNA replication increased as the aphidicolin concentration did. Furthermore, all zygotes in all groups recovered their DNA replication level after being incubated for 4 h in absence of aphidicolin, confirming the

reversibility of this inhibitor in porcine species. On the other hand, the blastocyst rate was affected by aphidicolin, being significantly lower in groups incubated with 1-10 µM aphidicolin with respect to the control group. However, the use of 0.5 µM aphidicolin did not produce a toxic effect, so this concentration was used for the subsequent experiments.

Subsequently, the objective was to evaluate whether the application of aphidicolin makes it possible to improve the gene-editing system by reducing mosaicism levels without affecting the quality and quantity of genetically modified embryos obtained. For this, we used sgRNAs against TPCN1 and embryos were subjected to different concentrations of CRISPR with the optimized electroporation conditions that had been previously established in chapter 3. Results showed that after electroporation, the use of aphidicolin decreases the blastocyst rate with a tendency (P=0.06) to reduce the mosaicism rate. The mutation rate was higher in groups with a higher RNP concentration, and no differences were found following the use of aphidicolin.

Finally, the possible effect of the use of aphidicolin was explored using microinjection conditions established in chapter 2 and electroporation conditions. The negative effect of aphidicolin on the blastocyst development was confirmed, especially when microinjection was used, and the positive effect of avoiding the mosaicism was confirmed. Therefore, overall, the rate of non-mosaic KO embryos produced with or without aphidicolin was similar. Therefore, under our experimental conditions, the use of aphidicolin was not advantageous. However, we think the use of aphidicolin might be a useful strategy to improve results in other models/labs under other conditions, where the percentage of mosaicism is different or once the embryo development is improved for porcine blastocysts or using aphidicolin differently (with different concentration and incubation time with oocytes) since we have observed the toxicity of aphidicolin is both concentration and time-dependent.

In summarizing, this Doctoral Thesis presented as a compendium of four publications is not an accumulation of independent experimental works but rather aims to have a scientific unity in the study of the optimization of gene-edited pig generation systems. Step by step, the different and varied factors that may influence the final efficiency of the system have been analysed and studied. From the type of cell to modify (zygote *vs*. oocyte) to the most suitable moment of application, the type of Cas9 to be used, the concentration and delivery methodology, and many other factors. The complexity and multidisciplinary of this type of study imply the collaborative work of many

researchers from different laboratories with expertise in specific techniques. This obvious reality, which is observed when looking at the number and diversity of the authors of the publications, should be evaluated as a positive factor since it is the only way to make quality leaps in scientific progress.

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CHAPTER 1

LITERATURE REVIEW:

Generation of gene-edited pigs

Navarro-Serna, S.; *Piñeiro-Silva, C.; Romar, R.; Parrington, J.; Gadea, J.; 2022. Generation of gene edited pigs. Book: Sustainable Agriculture Reviews 57. Animal Biotechnology for Livestock Production 2. Editors: Vinod Kumar Yata, Ashok Kumar Mohanty and Eric Lichtfouse. Series: Sustainable Agriculture Reviews. Series Ed.: Lichtfouse, Eric. Series ISSN 2210-4410. Publisher: Springer Cham. https://link.springer.com/book/9783031074950.*

Abstract

Background:

The porcine species (Sus scrofa domesticus) has had a great impact as a sourceof food worldwide, but also it is a very useful animal for biomedical applications. Genetic engineering involves modifying the sequence of DNA. Nowadays, genetic engineering is a common procedure in many laboratories, in part due to the development of simple, accessible, cheap, and effective programmable endonucleases such as those in the CRISPR/Cas9 approach. A key advantage of gene editing is the possibility of producing pigs with desired characteristics.

Mayor Advances:

Here we review the latest advances in the production of genetically modified pigs with a particular focus on the use of CRISPR/Cas9 gene editing in the porcine species, as a way to produce genetically modified pigs, and with a consideration of advantages and limitations, as well as new approaches, with regard to this technology.

HYPOTHESIS AND OBJECTIVES

Hypothesis

The main hypothesis of this Doctoral Thesis was to analyse the possible improvement efficiency in the generation of knock-out genetically modified porcine embryos by CRISPR-Cas9.

Among the main problems that can be found in gene editing in embryos is mosaicism, so to reduce this problem, we hypothesised that if we edit the target gene as soon as possible before DNA replication (Chapter 2) or use a reversible inhibitor of eukaryotic nuclear DNA replication (Chapter 4), we could reduce the mosaicism rate. Therefore, for this purpose, we tried to introduce the CRISPR-Cas9 system into mature oocytes immediately before *in vitro* fertilization and even used Aphidicolin as a DNA replication inhibitor.

On the other hand, about the methodology, microinjection is the most widely used method to introduce CRISPR-Cas9 into embryos. However, other methods such as electroporation are simpler and faster (Tanihara et al., 2016). We hypothesized that if we can achieve similar rates using electroporation than microinjection, in terms of embryo quality, mutation and mosaicism. Using this technique, we could increase the number of embryos that we can manipulate per session or work. This would allow obtaining more embryos for transfers and greater capacity to test sgRNAs in less time and in a more comfortable way. This hypothesis was explored in chapters 3 and 4.

Objectives

The main objective of this Doctoral Thesis was to achieve and optimize the conditions to generate gene-edited pigs by CRISPR-Cas9 i) increasing the percentage of efficient gene-edited embryos to be transferred to receptors and ii) obtaining viable genetically modified pigs through this technique.

In chapter 2 our main objective in this study was to establish the optimum conditions for the generation of TPC2 KO pig embryos and subsequently piglets using CRISPR-Cas9, by evaluating the best time for microinjection into the fertilized egg and the best molecular form of Cas9, to reduce mosaicism, establish an acceptable mutation rate, and be able to produce TPC2 KO embryos of sufficient quality to generate live TPC2 KO pigs.

In chapter 3, the objective was to optimize porcine oocyte electroporation for the generation of CAPN3 KO embryo, maximizing embryo quality and mutation rate in

comparison to microinjection; and evaluate different strategies to generate LGMDR1 porcine models.

In chapter 4, the objective was to reduce mosaicism ensuring that the CRISPR/Cas9 system edits the DNA before the zygote enters the S phase, when DNA replication occurs, using a reversible inhibitor of the DNA replication. TPCN1 KO pig embryos were generated with the optimized microinjection conditions established in chapter 2 and electroporation conditions established in chapter 3.

CHAPTER 2

Generation of nonmosaic, two-pore channel 2 biallelic knockout pigs in one generation by CRISPR-Cas9 microinjection before oocyte insemination.

***Navarro-Serna, S**.; Hachem, A.; Canha-Gouveia, A.; Hanbashi, A.; Garrappa, G.; Lopes, J.S.; Paris-Oller, E.; Bermejo-Álvarez, P.; Matas, C.; Romar, R.; Parrington, J.; Gadea, J.; Generation of Nonmosaic, Two-Pore Channel 2 Biallelic Knockout Pigs in One Generation by CRISPR-Cas9 Microinjection Before Oocyte Insemination. Cris. J. 2021, 4, 1–16, doi:10.1089/crispr.2020.0078.*

Abstract

Studies of knockout (KO) mice with defects in the endolysosomal two-pore channels (TPCs) have shown TPCs to be involved in pathophysiological processes, including heart and muscle function, metabolism, immunity, cancer, and viral infection. With the objective of studying TPC2's pathophysiological roles for the first time in a large, more humanlike animal model, TPC2 KO pigs were produced using CRISPR-Cas9. A major problem using CRISPR-Cas9 to edit embryos is mosaicism; thus, we studied for the first time the effect of microinjection timing on mosaicism. Mosaicism was greatly reduced when in vitro produced embryos were microinjected before insemination, and surgical embryo transfer (ET) was performed using such embryos. All TPC2 KO fetuses and piglets born following ET (i.e., F0 generation) were nonmosaic biallelic KOs. The generation of nonmosaic animals greatly facilitates germ line transmission of the mutation, thereby aiding the rapid and efficient generation of KO animal lines for medical research and agriculture.

CHAPTER 3

Generation of Calpain-3 knockout porcine embryos by CRISPR-Cas9 electroporation and intracytoplasmic microinjection of oocyte before insemination.

***Navarro-Serna, S**.; Dehesa-Etxebeste, M.; Piñeiro-Silva, C.; Romar, R.; Lopes, J.S.; López de Munaín, A.; Gadea, J. Generation of Calpian-3 knock-out porcine embryos by CRISPR-Cas9 electroporation and intracytoplasmic microinjection of oocytes before insemination. Theriogenology. 2022;186:175-184. https://doi.org10.1016/j.theriogenology.2022.04.012.*

Abstract

Limb girdle muscular dystrophy type R1 (LGMDR1) is an autosomal recessive myopathy described in humans resulting from a deficiency of calpain-3 protein (CAPN3). This disease lacks effective treatment and an appropriate model, so the generation of KO pigs by CRISPR-Cas9 offers a way to better understand disease ethology and to develop novel therapies. Microinjection is the main method described for gene editing by CRISPR-Cas9 in porcine embryo, but electroporation, which allows handling more embryos faster and easier, has also recently been reported. The objective of the current study was to optimize porcine oocyte electroporation to maximize embryo quality and mutation rate in order to efficiently generate LGMDR1 porcine models. We found that the efficiency of generating CAPN3 KO embryos was highest with 4 electroporation pulses and double sgRNA concentration than microinjection. Direct comparison between microinjection and electroporation demonstrated similar rates of embryo development and mutation parameters. The results of our study demonstrate that oocyte electroporation, an easier and faster method than microinjection, is comparable to standard approaches, paving the way for democratization of transgenesis in pigs.

CHAPTER 4

Effect of aphidicolin, a reversible inhibitor of eukaryotic nuclear DNA replication, on the production of genetically modified porcine embryos by CRISPR-Cas9.

***Navarro-Serna, S**.; Piñeiro-Silva, C.; Luongo, C.; Parrington, J.; Romar, R.; Gadea, J. 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. 2022, 23, 2135, doi:10.3390/ijms23042135.*

Abstract

Mosaicism is the most important limitation for one-step gene editing in embryos by CRISPR/Cas9 because cuts and repairs sometimes take place after the first DNA replication of the zygote. To try to minimize the risk of mosaicism, in this study a reversible DNA replication inhibitor was used after the release of CRISPR/Cas9 in the cell. There is no previous information on the use of aphidicolin in porcine embryos, so the reversible inhibition of DNA replication and the effect on embryo development of different concentrations of this drug was first evaluated. The effect of incubation with aphidicolin was tested with CRISPR/Cas9 at different concentrations and different delivery methodologies. As a result, the reversible inhibition of DNA replication was observed, and it was concentration dependent. An optimal concentration of 0.5 μM was established and used for subsequent experiments. Following the use of this drug with CRISPR/Cas9, a halving of mosaicism was observed together with a detrimental effect on embryo development. In conclusion, the use of reversible inhibition of DNA replication offers a way to reduce mosaicism. Nevertheless, due to the reduction in embryo development, it would be necessary to reach a balance for its use to be feasible.

CONCLUSIONS

1. Porcine oocyte microinjection before *in vitro* fertilization with Cas9 as ribonucleoprotein is a valid strategy to generate KO embryos and piglets in the first generation.

2. For the first time it has been generated nonmosaic, two-pore channel 2 biallelic knockout pigs in one generation by CRISPR-Cas9 microinjection before oocyte insemination.

3. The use of a reversible inhibitor of DNA replication, such as aphidicolin, can reduce mosaicism. However, under our experimental conditions, aphidicolin showed a toxic effect, so its regular use is not yet feasible.

4. Electroporation of porcine oocytes before *in vitro* fertilization achieves embryo development rate and mutation incidence similar to microinjection.

5. It has been established an efficient and optimized system for generating gene-edited embryos and pigs that might be used for models of human diseases.