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Molecular determinants involved in conceptus elongation in ungulates

Determinantes moleculares de la elongación del concepto de ungulados

D.ª Alba Pérez Gómez

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Summary

Embryo development in ungulates, the group of mammals including the most common farm animals, is characterized by a long preimplantation period that can be divided into two phases. The first preimplantation phase spans from fertilization to blastocyst formation and is common to all mammals. Following fertilization, totipotent cell termed zygote is formed and successive mitosis lead to the formation of a morula. At this stage of development, the blastomeres undergo polarization, triggering the first lineage differentiation. This initial differentiation event results in the formation of the two distinct cell lineages that compose the blastocyst: the inner cell mass (ICM) and the trophectoderm (TE). As a consequence of blastocyst growth and expansion, the embryo hatches from a glycoprotein layer termed zona pellucida, and implants into the uterus in some species such as rodents or primates, thereby ending preimplantation development. However, preimplantation embryo development in ungulates is prolonged in a second phase termed conceptus elongation. At the beginning of conceptus elongation, i.e., around blastocyst hatching, a second cell differentiation event differentiate the ICM cells into epiblast and hypoblast. Following the second cell lineage differentiation, hypoblast cells proliferate and migrate covering the entire inner surface of the trophectoderm. Hypoblast and trophectoderm constitute the extraembryonic membranes and proliferate extensively, transforming a spherical embryo of 150 μm into a filamentous structure of several decimeters by the time of implantation. Concomitantly, epiblast cells organize in a compact, bilaminar and flat structure termed embryonic disc, and that is similar to that observed in human embryos. The embryonic disc undergoes gastrulation during conceptus elongation, a complex process that results in the formation of the three germ layers -endoderm, mesoderm and ectoderm- that will give rise to all cell types of the individual, and in the establishment of the body axes. Due to the numerous cell proliferation, differentiation and migration events required for successful conceptus development during elongation, this period is the most susceptible period to embryonic loss. In the case of cattle, it has been observed that about one third of embryos arrest their development in the early stages of elongation. A better understanding of the factors governing conceptus elongation is required to develop strategies to reduce embryonic losses in cattle farming and to understand early embryonic losses in humans, given the similarities in developmental timing and gastrulation between ungulates and humans.

This thesis, divided into five independent chapters, analyzes the molecular aspects of bovine preimplantation development, focusing on the conceptus elongation period. Chapter 1 describes the main differences in preimplantation embryo development between mammals identified by means of transcriptomics analysis and genome edited models. The advances in genome editing have allowed the study the molecular basis of mammalian embryo development in other species different than mice and these experiments have served to refute dogmas of Developmental Biology established by means of knock-out (KO) mice models. The chapter also describes the identification of different lineage-specific markers that can be used to assess embryo quality in ungulates and summarizes recent advances in *in vitro* culture systems to recapitulate embryo development in ungulates beyond blastocyst hatching. The remaining chapters employ CRISPR/Cas9 gene editing technology to perform loss-of-function studies that provide new insights into the function of particular genes during preimplantation development. The emergence of CRISPR technology has been a breakthrough in the field of Developmental Biology. This technique can be applied to a wide variety of species to generate precise mutations in a wild-type (WT) embryo, eliminating the need for progenitors carrying the desired mutation. The experiments in these chapters primarily evaluate the developmental ability of bovine KO embryos for a specific gene during preimplantation embryo development. This was achieved using *in vitro* approaches -including a novel post-hatching culture system that extends traditional embryo culture up to Day (D) 12- and *in vivo* approaches, consisting of embryo transfers and recovery of elongated conceptus at a developmental stage equivalent to embryonic day (E) 14. KO embryos analyzed in this thesis have been generated using two different strategies. The first relies on the generation of frameshift insertions or deletions (indels) by conventional CRISPR-Cas9 system. Such frame disrupting indels at the open reading frame of the gene lead to the translation of a non-functional truncated peptide. The other strategy consists of introducing a stop codon in the open reading frame of the gene, preventing the complete synthesis of the protein. This is achieved by the technology of cytosine base editors, which constitutes a modified version of the CRISPR system, and act by modifying a cytosine at a specific locus to generate a stop codon.

Chapters 2 and 3 analyze the developmental ability of double-carrier bovine embryos of deleterious haplotypes. Deleterious haplotypes are alleles that cause

embryonic or fetal mortality when found in homozygosis. Determining the precise time at which such developmental arrest is produced is important to evaluate the economic impact associated to inadvertent crosses in cattle farming. Chapter 2 studies the developmental ability of double-carrier embryos of the Holstein Haplotype 3 (HH3), which consists of a T/C substitution that prevents the formation of a functional SMC2 (Structural Maintenance of Chromosomes 2) protein. SMC2 protein is an essential component of condensins I and II, protein complexes required for chromosome assembly and segregation during mitosis and meiosis. A preliminary study observed that double-carrier embryos resulting from crosses between carrier individuals were not able to develop to E14, as they showed neither embryonic disc nor proliferation of the extraembryonic membranes. To assess the effect of the ablation of condensins I and II at earlier developmental stages, *SMC2* KO embryos (functionally equivalent to HH3 double carriers) were generated using CRISPR technology. *SMC2* KO embryos were able to develop to the blastocyst stage, but showed a clear impairment in cell proliferation, evidenced by a significantly lower number of total, trophectoderm and inner cell mass cells than unmodified WT embryos. *SMC2* KO embryos were unable to survive to D12 *in vitro*. To determine if these developmental defects could be related to changes in telomere length, telomere length was analyzed at D8, D12 and E14, but no significant differences were found between embryos lacking condensins and WT embryos. In conclusion, condensins I and II are necessary for mitotic division of the blastomeres during early embryo development, and embryos lacking these complexes arrest their development right after blastocyst hatching, failing to reach elongated stages and to trigger maternal recognition of pregnancy.

Chapter 3 evaluates the developmental ability of double-carrier embryos of the Holstein haplotype 5 (HH5), which consists of a 138 kb deletion that prevents the formation of the TFB1M (Transcription Factor B1 Mitochondrial) protein, involved in mitochondrial translation. Initially, it was observed that E14 double-carrier embryos derived from crossing carrier cows with a carrier bull (heterozygous cross) showed a severe defect in the proliferation of extraembryonic membranes, necessary for maternal recognition of pregnancy. Subsequently, KO embryos for *TFB1M* (functionally equivalent to double HH5 carriers) were generated using CRISPR technology to assess their developmental ability before E14. *TFB1M* ablation did not alter embryo development to blastocyst *in vitro.* Total, ICM and TE cell numbers were similar

between blastocysts lacking *TFB1M* and WT blastocysts. Post-hatching embryo development up to D12 *in vitro* was also comparable between genotypes, with no significant differences found in embryo diameter, epiblast cell numbers or embryonic disc formation and complete hypoblast migration rates. The relative amount of mitochondrial DNA was also unaffected at D12. These results indicate that the development of HH5 double-carrier embryos is arrested prior to maternal recognition of pregnancy, at the transition between the spherical to ovoid/tubular conceptus.

Chapter 4 discusses the role of embryonic *PPARG* during early embryo development. PPARG is a receptor expressed in conceptus and endometrial cells and is involved in signaling mediated by lipid compounds such as prostaglandins. Previous loss-of-function studies based on intrauterine injection of morpholinos that prevent *PPARG* mRNA translation, demonstrated a critical role of PPARG in conceptus elongation in sheep, but this method does not specify whether PPARG is essential in the embryo or in the uterus. To gain further insight into the function of PPARG in the embryo, we analyzed the development of *PPARG* KO bovine embryos at D8 and D12 *in vitro* and also performed heterologous embryo transfers into recipient ewes to assess their developmental ability to reach early stages of elongation *in vivo*. Embryos without PPARG showed no developmental abnormalities until the most advanced stage analyzed. *PPARG* KO blastocyst showed similar total, ICM and TE cell numbers than WT embryos at D8. *PPARG* depletion did not affect cell differentiation and proliferation at D12 *in vitro.* Lastly, *PPARG* KO embryos were able to develop to elongated stages equivalent to E14, showing a similar degree of development of extraembryonic membranes and epiblast than WT embryos. These results evidence that PPARG-mediated lipid signaling in the embryo is not required for early elongation of the bovine conceptus.

Finally, Chapter 5 explores a dogma of Developmental Biology established from experiments in the mouse model: the essential role of the transcription factor TEAD4 (TEA Domain Transcription Factor 4) in trophectoderm differentiation. TEAD4 is essential for trophectoderm differentiation and, therefore, for blastocyst formation in mice, where it regulates the expression of other trophectoderm-specific factors such as *Cdx2*, *Eomes* or *Gata3*. This chapter analyzes the consequences of *TEAD4* depletion in two phylogenetically distant species (bovine and rabbit) and concludes that TEAD4 is not required for trophectoderm establishment and blastocyst formation in either species.

First, an experimental design similar to that of Chapter 4 was followed to determine the effect of *TEAD4* ablation in bovine embryos up to early elongated stages. *TEAD4* KO embryos reached the blastocyst stage and showed a normal expression pattern of the TE specific markers *GATA3* and *CDX2* and an overly similar transcriptome than WT embryos. *TEAD4* KO embryos were able to develop up to D12 *in vitro*, exhibiting a normal development of the epiblast, hypoblast and trophectoderm. At later developmental stages (E14, tubular conceptuses), no obvious defects in the proliferation of extra-embryonic membranes (trophectoderm and hypoblast) were observed in KO conceptuses and their development was comparable to that of WT embryos. In an additional experiment using rabbit embryos, a species that shares the same clade (Glires) as rodents, it was observed that TEAD4 was not required for trophectoderm differentiation and blastocyst formation. These results demonstrate that the crucial role of TEAD4 in trophectoderm differentiation is unique to rodents.

Resumen

El desarrollo embrionario de los ungulados, el grupo de mamíferos que incluye a los principales animales de granja, se caracteriza por un periodo preimplantacional largo que puede dividirse en dos fases. La primera fase engloba todos los eventos que ocurren desde la fecundación hasta la formación del blastocisto y es común a todos los mamíferos. Tras la fecundación se forma la célula totipotente denominada cigoto y se inician divisiones mitóticas sucesivas que resultan en la formación de una mórula. En este estadio de desarrollo, las blastómeras se polarizan iniciando la primera diferenciación celular. Este primer evento de diferenciación celular da lugar a los dos linajes celulares que forman el blastocisto: la masa celular interna (MCI) y el trofoectodermo (TE). Como consecuencia del crecimiento y expansión del blastocisto, el embrión eclosiona de su envuelta glicoproteica denominada zona pelúcida e implanta en el útero en algunas especies como los primates o los roedores, finalizando de este modo el desarrollo embrionario preimplantacional. Sin embargo, en ungulados el desarrollo embrionario preimplantacional se prolonga en una segunda fase conocida como elongación del concepto. Al inicio de la elongación, en torno a la eclosión del blastocisto, tiene lugar una segunda diferenciación celular en la MCI que resulta en la formación del epiblasto y del hipoblasto. Tras la segunda diferenciación de linajes, las células del hipoblasto proliferan y migran para cubrir toda la superficie interna del trofoectodermo. El hipoblasto y el trofoectodermo constituyen las membranas extraembrionarias del concepto y durante la elongación proliferan masivamente transformando a un embrión esférico de 150 µm en una estructura filamentosa de varios decímetros de longitud en el momento de la implantación. De forma concomitante al desarrollo de las membranas extraembrionarias, las células del epiblasto se organizan en una estructura bilaminar y plana denominada disco embrionario, que es similar a la que se observa en los embriones humanos. El disco embrionario gastrula durante la elongación del concepto, mediante un proceso complejo que da lugar a la formación de las tres capas germinales -endodermo, mesodermo y ectodermo- a partir de las cuales se forman todos los tipos celulares del individuo, y al establecimiento de los ejes corporales. Los numerosos eventos de proliferación, diferenciación y migración celular necesarios para la elongación del concepto hacen de este periodo de desarrollo el más susceptible a pérdidas embrionarias. En el caso de la especie bovina, se ha observado que alrededor un tercio de los embriones detienen su desarrollo en las primeras fases de la elongación. Un mayor entendimiento de los factores involucrados en la elongación del concepto es clave para desarrollar estrategias para reducir pérdidas embrionarias en

el ganado bovino y para entender las pérdidas embrionarias humanas, dada la similitud en el tiempo de desarrollo y gastrulación entre ungulados y humanos.

Esta tesis, dividida en cinco capítulos independientes, analiza diferentes aspectos moleculares del desarrollo preimplantacional bovino, centrándose en la fase de la elongación del concepto. El Capítulo 1, describe importantes diferencias en el desarrollo embrionario preimplantacional entre diferentes especies de mamíferos que han sido identificadas gracias a análisis transcriptómicos y modelos editados genéticamente. Los avances en la edición génica han permitido explorar la base molecular del desarrollo embrionario de los mamíferos en otras especies distintas al ratón y estos experimentos han servido para refutar dogmas de la Biología del Desarrollo establecidos en base a modelos *knock-out* (KO) en ratones. El capítulo también describe distintos marcadores específicos de linaje celular que pueden ser usados para evaluar la calidad embrionaria en embriones y revisa los últimos avances en sistemas de cultivo *in vitro* para desarrollar embriones de ungulados más allá de la eclosión del blastocisto. En el resto de los capítulos se emplea la tecnología de edición genómica CRISPR/Cas9 para realizar estudios de pérdida de función que sirven para conocer la función de un determinado gen durante el desarrollo preimplantacional. El surgimiento de la tecnología CRISPR ha supuesto un gran avance para el estudio de la Biología del Desarrollo. Esta técnica se puede aplicar a una gran variedad de especies para generaren mutaciones precisas en un embrión *wild-type* (WT), evitando la necesidad de tener progenitores portadores de la mutación deseada. Los experimentos de estos capítulos se basan principalmente en evaluar la capacidad de desarrollo de embriones bovinos KO para un determinado gen de estudio durante el desarrollo embrionario preimplantacional. Para ello, se emplean aproximaciones *in vitro* -incluyendo un novedoso sistema de cultivo post-eclosión que permite extender el cultivo embrionario tradicional hasta Día (D) 12– e *in vivo*, llevando a cabo transferencias embrionarias y recuperando conceptos elongados en un estadio equivalente a día embrionario (E) 14. Los embriones KO analizados en esta tesis se han generado mediante dos estrategias diferentes. La primera se basa en la generación de inserciones y deleciones (*indels*) que alteran el marco abierto de lectura mediante el sistema CRISPR-Cas9 convencional. Estos *indels* que alteran el marco de lectura del gen dan lugar a la traducción de péptidos truncados no funcionales. La otra estrategia consiste en introducir un codón de parada en el marco de lectura del gen que impide la síntesis completa de la proteína.

Esto es posible gracias a la tecnología de editores de bases de citosina, que constituye una versión modificada del sistema CRISPR y actúa modificando una citosina en una posición específica para generar un codón de parada.

Los capítulos 2 y 3 analizan la capacidad de desarrollo de embriones bovinos dobles portadores de haplotipos deletéreos. Los haplotipos deletéreos son alelos que causan mortalidad embrionaria o fetal cuando se encuentran en homocigosis. Conocer el momento en el que se produce el arresto en el desarrollo es clave para evaluar el coste económico asociado a los cruces involuntarios entre individuos portadores en las granjas vacunas. El capítulo 2 estudia la capacidad de desarrollo de embriones dobles portadores del Haplotipo Holstein 3 (HH3), que consiste en una sustitución T/C que impide la formación de una proteína SMC2 (*Structural Maintenance of Chromosomes 2*) funcional. La proteína SMC2 es un componente esencial de las condensinas I y II, complejos proteicos necesarios para el ensamblaje y segregación de los cromosomas durante la mitosis y meiosis. Un estudio preliminar observó que los embriones dobles portadores del haplotipo, procedentes de cruces entre individuos portadores, no eran capaces de desarrollarse hasta E14, no presentando ni disco embrionario ni proliferación de las membranas extraembrionarias. Para evaluar el efecto de la eliminación de las condensinas I y II en estadios previos del desarrollo embrionario, se generaron embriones KO para *SMC2* (funcionalmente equivalentes a los dobles portadores HH3) mediante la tecnología CRISPR. Los embriones KO para *SMC2* fueron capaces de desarrollarse hasta el estadio de blastocisto, pero mostraron un claro defecto en la proliferación celular, evidenciado por un número significativamente menor de células totales, de células del trofoectodermo y de la masa celular interna que los no modificados (WT). Los embriones KO para *SMC2* resultaron incapaces de sobrevivir hasta D12 *in vitro*. Para ver si los defectos en el desarrollo podían estar relacionados con cambios en la longitud de los telómeros se analizó la longitud de los telómeros a D8, D12 y a E14, pero no se encontraron diferencias significativas entre los embriones sin condensinas y los WT. En conclusión, las condensinas I y II son necesarias para la división mitótica de las blastómeras durante el desarrollo embrionario temprano y los embriones que carecen de estos complejos detienen su desarrollo justo después de la eclosión del blastocisto, sin poder llegar alcanzar estadios elongados y a desencadenar el reconocimiento materno de la gestación.

El Capítulo 3 evalúa la capacidad de desarrollo de embriones dobles portadores del Haplotipo Holstein 5 (HH5), que consiste en una deleción de 138 kb que impide la formación de la proteína TFB1M (*Transcription Factor B1 Mitochondrial*), que participa en la traducción mitocondrial. Inicialmente, se observó que los embriones dobles portadores en E14, derivados del cruce de vacas portadoras con un toro portador (cruce de heterocigotos), mostraban un defecto severo en la proliferación de las membranas extraembrionarias, necesarias para el reconocimiento materno de la gestación. Posteriormente, se generaron embriones KO para *TFB1M* (funcionalmente equivalentes a los dobles portadores HH5) mediante la tecnología CRISPR para evaluar la capacidad de desarrollo hasta estadios anteriores a E14. El desarrollo embrionario *in vitro* hasta blastocisto no se vio afectado por la eliminación de TFB1M, ya que el número de células totales, de trofectodermo y de la masa celular interna fueron similares entre los blastocistos con y sin *TFB1M*. El desarrollo post-eclosión hasta D12 también fue equiparable entre los distintos genotipos, no se encontraron diferencias significativas en el diámetro embrionario, número de células del epiblasto, o en tasas de formación de disco embrionario y migración completa del hipoblasto. La cantidad relativa de ADN mitocondrial tampoco se vio afectada a D12 *in vitro.* Estos resultados indican que el desarrollo de los embriones doble portadores de HH5 se detiene antes del reconocimiento materno de la gestación, en la transición entre concepto esférico y ovoide/tubular.

El Capítulo 4 analiza la función durante el desarrollo embrionario del receptor embrionario PPARG, que interviene en la señalización mediada por compuestos lipídicos como las prostaglandinas. *PPARG* se expresa en las células del concepto y en las células del endometrio. Estudios previos de pérdida de función de PPARG basados en la inyección intrauterina de morfolinos que impiden la traducción de ARN mensajero de *PPARG*, habían observado un papel fundamental de PPARG en la elongación del concepto ovino, pero dicho método no permite conocer si PPARG es esencial en el embrión o en el útero. Para conocer la función de PPARG en el embrión, se analizó el desarrollo de embriones bovinos KO para *PPARG* a D8 y D12 *in vitro* y se realizaron transferencias embrionarias heterólogas en ovejas receptoras para evaluar su capacidad de desarrollo *in vivo* durante las primeras etapas de la elongación. Los embriones sin PPARG no mostraron anomalías en el desarrollo hasta la fase más avanzada de desarrollo analizada. Los blastocistos KO para *PPARG* presentaron un número de

células totales, de trofoectodermo y de la masa celular interna similar al de los embriones WT en D8. Tampoco se observó ningún efecto de la eliminación del gen sobre la diferenciación y proliferación de los linajes embrionarios en embriones de D12 *in vitro.* Por último, los embriones sin PPARG fueron capaces de desarrollarse hasta estadios elongados equivalentes a E14, mostrando un desarrollo de las membranas extraembrionarias y del epiblasto similar al de los embriones WT. Estas evidencias indican que la señalización lipídica mediada por PPARG en el embrión no es necesaria para la elongación temprana del concepto bovino.

Finalmente, el capítulo 5 explora un dogma de la Biología del Desarrollo establecido en base a experimentos en el modelo de ratón: el papel esencial del factor de transcripción TEAD4 (*TEA Domain Transcription Factor 4*) en la diferenciación del trofoectodermo. TEAD4 es esencial para la diferenciación del trofoectodermo y, por tanto, para la formación del blastocisto en ratones, donde actúa regulando la expresión de otros factores específicos de trofoectodermo como *Cdx2*, *Eomes* o *Gata3*. En este capítulo se han analizado los efectos de la eliminación de *TEAD4* en dos especies filogenéticamente distantes (bovino y conejo) y se ha descubierto que *TEAD4* no es necesario para la diferenciación del trofoectodermo y formación del blastocisto en estas especies. Primero, se siguió un diseño experimental similar al del capítulo 4 para determinar el efecto de la eliminación de TEAD4 en embriones bovinos hasta estadios tempranos de la elongación. Los embriones KO para *TEAD4* alcanzaron el estadio blastocisto con un patrón de expresión normal de los marcadores específicos de trofoectodermo *GATA3* y *CDX2* y sin presentar cambios relevantes en su transcriptoma. Los embriones KO para *TEAD4* consiguieron desarrollarse hasta D12 *in vitro*, mostrando un desarrollo normal del epiblasto, hipoblasto y trofoectodermo. En estadios posteriores del desarrollo (concepto tubular de E14) no se observaron defectos evidentes en la proliferación de las membranas extraembrionarias (trofectodermo e hipoblasto) en los conceptos KO y su desarrollo fue equiparable al de los embriones WT. En un experimento adicional empleando embriones de conejo, una especie que comparte el mismo clado (Glires) que los roedores, se observó que TEAD4 no era necesario para la diferenciación del trofoectodermo y la formación del blastocisto. Estos resultados prueban que el papel crucial de TEAD4 en la diferenciación del trofoectodermo es exclusivo de roedores.

Introduction

Successful sexual reproduction relies on the formation of a novel, unique individual through the fusion of the female gamete, the oocyte, and the male gamete, the spermatozoon. In mammals, gamete fusion takes place in the oviductal ampulla, where a competent ovulated oocyte is fertilized by one of the few sperm that have reached that anatomical region (Wassarman, 1999). Following gamete fusion, a totipotent cell called zygote is formed, marking the onset of the embryonic preimplantation period. During the preimplantation period (i.e., the period compromised between fertilization and implantation), complex cell proliferation differentiation and migration processes occur as the embryo transits from the oviduct to the uterus, where implantation takes place. Following implantation, direct communication between the embryo and the mother is established, facilitating the evolution of the embryo into a fetus, which will ultimately give rise to a newborn.

In ungulates, a group that encompasses the most relevant mammalian livestock animals, including cattle, pigs, sheep and goats, preimplantation period is significantly prolonged and emerges as the most susceptible period to embryonic loss. Ungulate embryos continue their free-floating development in the uterus until their trophoblast cells attach to the uterine epithelium, which starts by Day (D) 19 in bovine (Guillomot & Guay, 1982), D15 in sheep (Boshier, 1969), or D13 in pigs (Geisert & Yelich, 1997). Conceptus elongations is the most susceptible period to embryonic loss (M. Diskin & Morris, 2008; Wiltbank et al., 2016). In cattle farms, about one-third of viable D6 blastocysts fail to elongate and maintain pregnancy by D28 (Santos, Thatcher, Chebel, Cerri, & Galvão, 2004), and embryo mortality during early conceptus elongation (D7-D14) ranges from 26% and 34% (Berg et al., 2010). Similarly, it has been estimated that in pigs, around 20-40 % of the embryonic losses occur from D10 to D20 of pregnancy (Bazer & First, 1983). From this perspective, understanding the mechanisms that drive preimplantation embryo development in ungulates is essential for developing interventions to improve the reproductive efficiency in these economically and ecologically valuable species.

1.1. Preimplantation bovine embryo development

Bovine embryo development, like that of other ungulates, is characterized by a long preimplantation period that can be divided in two distinct phases. The initial phase, universally shared among mammals, encompasses all events from fertilization to

blastocyst hatching, i.e. the release of the embryo from the surrounding glycoprotein layer known as the zona pellucida (ZP), which occurs in bovine by Day 8. The subsequent phase, termed conceptus elongation, is unique to ungulates, and expands from blastocyst hatching to implantation, which occurs around Day 21 in cattle (**Fig. 1,** Guillomot & Guay, 1982; Spencer & Hansen, 2015).

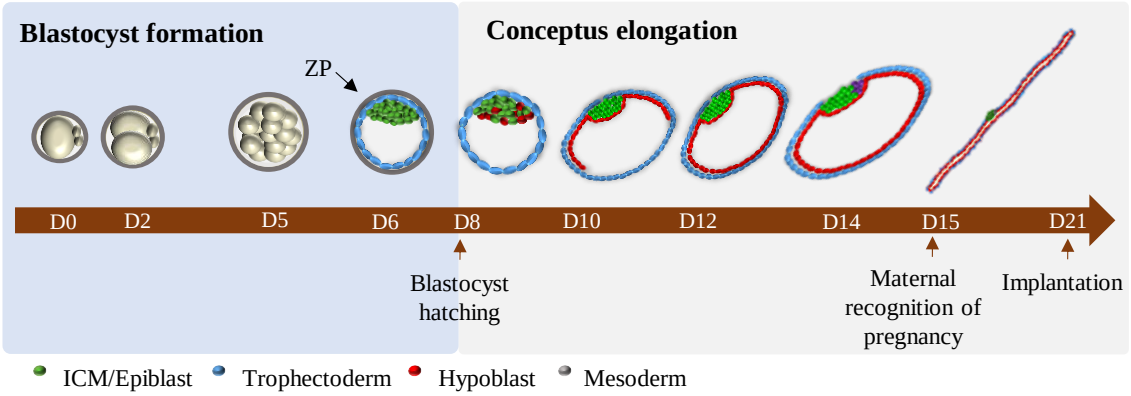

Fig. 1. Representation of bovine preimplantation development. Schematic images of bovine embryos at different time points, from the zygote to elongated stages.

1.1.1. Blastocyst formation

Following fertilization, the mature oocyte, which was arrested in metaphase II, resumes meiosis, and extrudes the second polar body. Male and female pronuclei are formed and there is an epigenetic reprogramming that gives rise to the totipotent cell termed zygote (Morgan, et al., 2005). The zygote undergoes a series of mitotic divisions, and the resulting daughter cells, the blastomeres, preserve their totipotency up to the eight-cell stage, meaning that they have the potential to give rise to all cell types, including both embryonic and extraembryonic tissues (Willadsen & Polge, 1981). During these first divisions, the embryo remains transcriptionally inactive, and it relies on maternally inherited mRNA and proteins present in the oocyte. In bovine, major embryonic genome activation (EGA) occurs at the eight-cell stage as evidenced by the detection of (i) embryonic transcripts, which are not present in oocytes; (ii) transcripts from the paternal allele; and (iii) primary transcripts with intronic sequences (Graf et al., 2014; Wei et al., 2017).

Then, by the 32-cell stage, cells start to undergo a process termed compaction, wherein they adhere tightly to each other, forming a compacted morula. At this point, first signs of cell differentiation become apparent as the morula is composed by apolar

and polar blastomeres allocated to inner and outer positions, respectively (van Soom et al., 1997). Consequently, the outer cells will differentiate into trophectoderm (TE) and the inner cells maintain their pluripotency forming the inner cell mass (ICM) at the blastocyst stage.

First lineage determination is partially understood across mammals and it may differ among species (Frankenberg, de Barros, Rossant, & Renfree, 2016). In mice, the most studied species, this process is regulated by the Hippo signaling pathway (Nishioka et al., 2009; Wicklow et al., 2014). Cell-cell interactions within the inner apolar cells trigger a phosphorylation cascade of Hippo components which prevents the activation of the transcription factor TEAD4. On the other hand, inactive Hippo pathway in the outer cells permits the translocation of non-phosphorylated Hippo components into the nucleus which bind TEAD4 (Hirate et al., 2013; Nishioka et al., 2009). *Tead4* activation in outer cells leads to the downregulation of the pluripotency factor *Sox2* and the concomitant upregulation of *Cdx2* from the morula stage (Ralston et al., 2010; Strumpf et al., 2005; Wicklow et al., 2014). *Cdx2* activation, in turn, downregulates *Oct4* (also known as *Pou5f1*, Niwa et al., 2005), and orchestrates the induction of other key markers associated with the TE lineage (Ng et al., 2008). The role and regulation of the Hippo signaling pathway components during bovine embryo development are not completely understood: the distinct localization patterns of Hippo components (Sharma & Madan, 2022) and the absence of effects on lineage segregation following Hippo pathway inhibition in bovine embryos (Sharma & Madan, 2020), suggest that the regulatory processes governing the first cell determination diverge among species. Interestingly, there are also some differences between mice and cattle regarding the pluripotency marker OCT4. In cattle, CDX2 does not repress *OCT4* expression as OCT4 is observed in all blastomeres at the blastocyst stage and is not restricted to the epiblast until later stages (Berg et al., 2011).

First cell lineage differentiation is evident at the blastocyst stage, when the embryo is composed by an outer layer of cells, the TE, which contributes to extraembryonic tissues essential for embryo implantation; and an inner cluster of pluripotent cells, the ICM, which will form the embryo proper (Marikawa & Alarcón, 2009; van Soom et al., 1997). The distinction of the first cell lineages is evidenced by the expression of lineage-specific transcription factors that regulate gene transcription differently for each lineage **(Fig. 2)**. Some specific lineage markers are shared among

mammals. For example, in mice, cattle, humans and pigs, TE cells specifically express CDX2 and GATA3 while SOX2 and NANOG expression is restricted to the ICM (Negrón-Pérez, Zhang, & Hansen, 2017; Ozawa et al., 2012; Pérez-Gómez et al., 2021). Apart from the establishment of the two first lineages, the blastocyst is characterized by a central fluid-filled cavity known as blastocele. This cavity is created by the entry of water into the embryo to balance the osmotic pressure generated by the active transport of ions by TE cells (Watson, 1992).

By the late blastocyst stage, second cell fate determination takes place in the ICM and implies the emergence of the epiblast and the extraembryonic hypoblast (**Fig. 2**). Specification of hypoblast in the mouse embryo depends on signaling by fibroblast growth factor (FGF). In mice, NANOG, OCT4, and SOX2 upregulate *Fgf4* and suppress *Gata6* expression in epiblast cells, while also inhibiting FGF signaling pathway. In contrast, in hypoblast precursors, FGF4 secreted from epiblast cells binds FGF receptor 2 (FGFR2) and activates FGF pathway. The higher FGF signaling in hypoblast cells enhances *Gata6* while supressing *Nanog* expression (Bessonnard et al., 2014; Chazaud & Yamanaka, 2016; Nichols et al., 2009; Plusa et al., 2008; Yamanaka, Lanner, & Rossant, 2010). FGF signaling and GATA6 cooperatively activate downstream genes, such as *Sox17* and *Gata4*, which are also hypoblast specific (Artus, Piliszek, & Hadjantonakis, 2011; Bessonnard et al., 2014; Chazaud & Yamanaka, 2016; Schrode et al., 2014). Consistent with these observations in mice, FGF4-treated bovine embryos developed ICMs composed entirely of GATA6-positive cells (Kuijk et al., 2012), and MEK signaling inhibition (downstream of FGF) blocked ICM differentiation towards hypoblast in cattle (Canizo et al., 2019). Nevertheless, GATA6 and NANOG mutually exclusive expression pattern is not conserved among mammals, as GATA6 is expressed in all cell lineages of the blastocysts in primates, pig, sheep and cattle, only becoming restricted to the hypoblast at later developmental stages (Barrangou et al., 2007; Blakeley et al., 2015; Ramos-Ibeas et al., 2019; Stirparo et al., 2018; Wei et al., 2017). Once specified, bovine hypoblast cells specifically express *GATA6, SOX17, FOXA2* and *PDGFR2α* (Pérez-Gómez et al., 2021) and reorganize to form an epithelium lying in contact with the blastocoel cavity that eventually migrates covering the inner surface of the embryo (Maddox-Hyttel et al., 2003).

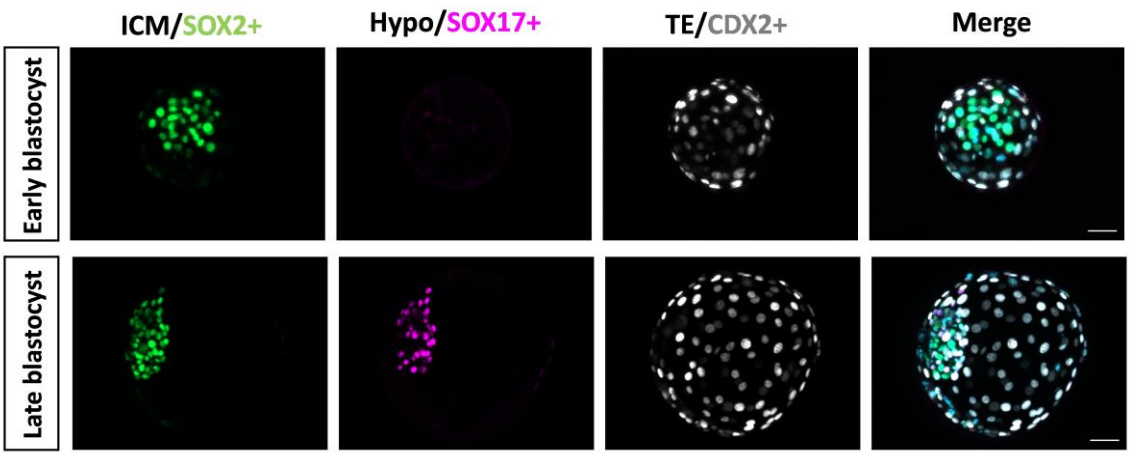

Fig. 2. Bovine blastocysts subjected to immunohistochemistry to identify cell linages. Early blastocysts (upper images) are composed by two cell lineages, ICM and TE, that specifically express SOX2 or CDX2, respectively. Hypoblast specific marker SOX17 begins to be expressed in the ICM of late blastocysts (lower images). Nuclei counterstained with DAPI. ICM, inner cell mass; Hypo, hypoblast; TE, trophectoderm. Scale bar 50 µm.

Up to the blastocyst stage, the embryo is surrounded by a protective glycoprotein layer termed zona pellucida (ZP). The release of the embryo from the ZP, i.e., blastocyst hatching, implies the end of the preimplantation development in many mammalian species, including human and mice. However, ungulate embryos undergo a prolonged period of preimplantation development termed conceptus elongation.

1.1.2. Conceptus elongation

Conceptus elongation, i.e., the embryonic period exclusive to ungulates that spans from blastocyst hatching up to implantation, is characterized by a massive proliferation of the extraembryonic membranes (EEMs) and the formation of a flat embryonic disc (ED), similar to that observed in humans, which ultimately undergoes gastrulation.

Following blastocyst hatching, the TE can be classified into mural TE, which covers the blastocoel cavity, and polar TE, covering the ICM. While in rodents and primates, polar TE derivatives contribute to implantation and become part of the placenta (Christodoulou et al., 2019; Weberling & Zernicka-Goetz, 2021), in ungulates, this function is assumed by the mural TE (Maddox-Hyttel et al., 2003). The polar TE,

also known as Rauber's layer (RL), undergoes apoptosis and is entirely removed around D9-D11 in pigs (Sun et al., 2015), D11-12 in sheep (Pérez-Gómez et al., 2021) and by D14 in cattle (van Leeuwen, Rawson, Berg, Wells, & Pfeffer, 2020), leaving the epiblast directly exposed to the uterine lumen. By the time the RL disappears, hypoblast cells have already spread to cover the entire inner surface of the mural TE (**Fig. 3,** Ramos-Ibeas et al., 2020). Then, both EEMs, including mural TE and hypoblast, undergo massive proliferation and the embryo becomes referred as conceptus (EEMs + embryo proper). During conceptus elongation, the embryo evolves from a spherical blastocyst of ~150 µM, progressing through ovoid and tubular stages, to a filamentous structure of approximately 30 cm in cattle by the time of implantation (Berg et al. 2010; Maddox-Hyttel et al., 2003). Proliferation of EEMs is crucial for maternal recognition of pregnancy (Spencer & Bazer, 2004), which occurs around D15-16 in cattle (Forde et al., 2011). It is established that production of interferon Tau (IFNT) by the proliferating trophoblast cells is required for endometrial cells to sense the embryo, thereby preventing luteolysis (Bazer, Ott, & Spencer, 1994; Roberts et al., 2008; Spencer, Ott, & Bazer, 1996).

Concomitant to the proliferation of the EEMs, epiblast cells develop tight junctions, form a basal lamina in contact to the hypoblast and finally give rise to a bilaminar epithelial structure termed embryonic disc (Artus, Hue, & Acloque, 2020; Betteridge & Fléchon, 1988). When the ED is fully formed, epiblast cells specifically express the core pluripotency transcription factors *OCT4*, *NANOG* and *SOX2* (**Fig. 3,** Berg et al., 2011; Negrón-Pérez et al., 2017; Pfeffer, Smith, Maclean, & Berg, 2017; Vejlsted et al., 2005; Wei et al., 2017). In the subsequent days (D14-15), the ED acquires an oval shape with increased density at the posterior edge, linked to the ingression of the first cells into the primitive streak and the onset of gastrulation (van Leeuwen, Berg, & Pfeffer, 2015). Understanding of cell fate decisions during gastrulation in non-rodent mammals remains incomplete, mainly due to the difficulties associated with obtaining embryos *in vivo*, the absence of an *in vitro* system that fully recapitulates these events and the historical lack of tools to perform targeted genome modifications in other mammals different than mice (described on the next section). Thereby, our knowledge of the genes and signaling pathways orchestrating mammalian gastrulation is mainly derived from research on mouse embryos, where gastrulation occurs in a three-dimensional structure called the egg cylinder and is governed by

signaling gradients which are not geometrically possible in a flat embryonic disc. Luckily, recent studies have shed light on the mechanisms that control this process in ungulates. NODAL/WNT signaling pathway, involved in the initiation of gastrulation in mice, similarly governs gene expression patterns at the onset of gastrulation in ungulates (Arnold & Robertson, 2009; Chhabra et al., 2019; Robertson, 2014; Simpson et al., 2023; van Leeuwen et al., 2015). Notably, WNT and NODAL induce epithelial-to-mesenchymal transition (EMT) in the posterior epiblast cells, which begin to express the mesoderm marker *BRACHYURY (T)* and to downregulate *SOX2* (**Fig. 3,** Blomberg et al., 2006; Guillomot, et al., 2004; Hue, Renard, & Viebahn, 2001; Pérez-Gómez et al., 2021; Pfeffer et al., 2017). *EOMES* and *MIXL1*, key genes involved in murine mesoderm formation (Arnold & Robertson, 2009; Hart et al., 2002), have also been identified in bovine mesoderm precursors (Pfeffer et al., 2017).

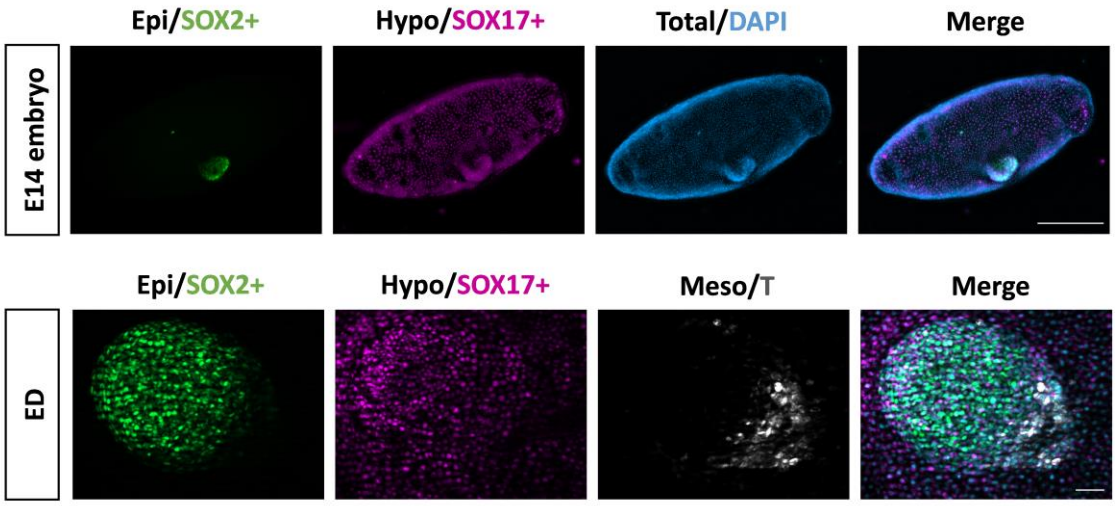

Fig. 3. E14 bovine conceptuses subjected to immunohistochemistry to identify cell lineages. The images of the upper low show a tubular conceptus with an embryonic disc (ED) and complete hypoblast migration. ED can be identified as a compact structure of epiblast cells (SOX2+). Hypoblast cells (SOX17+) extend throughout the entire inner surface of the TE. The images of the lower row show a higher magnification of an ED undergoing gastrulation. BRACHYURY (T) is specifically express by mesoderm cells at the posterior pole of the ED. Nuclei counterstained with DAPI. E, embryonic day; Epi, epiblast; Hypo, hypoblast, Meso, mesoderm. Scale bar: 500 µm for conceptus; 50 µm for ED.

These mesoderm precursors migrate into the space between the epiblast and hypoblast, constituting the nascent mesoderm. The mesoderm rapidly extends to cover the lateral surface of the ED (embryonic mesoderm) and migrates beyond its borders, lining the mural trophoblast and hypoblast (extraembryonic mesoderm) (Pfeffer et al., 2017). Simultaneously, more epiblast cells, expressing *FOXA2* and *CER1*, continue egressing through the primitive streak and intercalate to the dorsal hypoblast to form the endoderm (Simpson et al., 2023; van Leeuwen et al., 2015). Epiblast cells that do not pass through the primitive streak contribute to the ectoderm (Pfeffer et al., 2017; van Leeuwen et al., 2015). Hence, gastrulation concludes with the establishment of the three germ layers that will give rise to all embryonic tissues: ectoderm (outer layer), mesoderm (middle layer) and endoderm (inner layer).

Once gastrulation has been completed and the filamentous conceptus has occupied both uterine horns, implantation takes place around D21 in cattle, as a result of complex crosstalk between the endometrium and the elongating conceptus (Guillomot & Guay, 1982; Spencer & Hansen, 2015). The implantation process in ungulates is multicotyledonary and epitheliochorial (non-invasive) as the trophectoderm of the conceptus only adheres to the uterine luminal epithelial, without penetrating the basal laminal underneath the epithelia (Bazer et al., 2009; Davenport et al., 2023). Implantation in ungulates requires the differentiation of trophectoderm cells into binucleate cells (BNCs) (Bazer et al., 2010) and the loss of anti-adherent molecules by the endometrial cells (Johnson et al., 2001). Later, during placentation, BNCs form the placental cotyledons. These cotyledons attach to endometrial specialized aglandular structures termed caruncles, and together constitute the placentomes. Subsequently, placentomes undergo vascularization to provide essential nutrients for fetal development (Davenport et al., 2023).

Chapter 1 of this thesis covers the fundamental aspects of preimplantation embryonic development in ungulates, delving into the cell differentiation events that take place during this process and comparing these events across mammalian species. In addition, this chapter details the lineage-specific markers that can be used to assess proper embryo development in ungulates and discusses a novel *in vitro* culture system that allows extending traditional embryo culture to the beginning of conceptus elongation.

1.1.3. Post-hatching bovine embryo development *in vitro*

Conceptus elongation is the developmental period most susceptible to embryonic loss in ungulates (Berg et al., 2010; Diskin & Sreenan, 1980). However, research into the factors contributing to embryonic mortality during this period is limited by the absence of *in vitro* culture systems that recapitulate conceptus elongation. Conventional *in vitro* culture systems support embryo development until blastocyst hatching (Holm et al., 1999) but thereafter, embryos arrest their development and die. This is presumably due to the requirement for substances present in the uterine lumen, collectively termed histotroph (Forde et al., 2014; Gray et al., 2001; Lonergan, 2011; Simintiras et al., 2019).

In recent years, attempts have been made to extend embryo culture beyond the blastocyst stage in ungulates. Pioneer studies established an *in vitro* post-hatching development (PHD) system in agarose gel tunnels to physically induce conceptus elongation. These tunnels were filled with PHD medium that consisted of synthetic oviductal fluid (SOF) medium, a widely used medium for bovine embryo culture up to blastocyst hatching (Holm et al., 1999), supplemented with fetal bovine serum (FBS) and high glucose concentration (Brandão et al., 2004; Vajta, Alexopoulos, & Callesen, 2004). Embryos in this system showed high proliferation of the extraembryonic membranes up to D15, acquiring a tubular shape due to physical constricts. However, it remained unclear if hypoblast cells migrated covering the entirely inner surface of the embryo, and epiblast cells, the most sensitive lineage to develop, failed to form embryonic disc structures (Brandão et al., 2004; Machado et al., 2013; Vajta et al., 2004; Vejlsted et al., 2006).

Fortunately, a post-hatching embryo culture system has recently been developed that maintains epiblast survival and allows complete hypoblast migration and embryonic disc development in cattle (Ramos-Ibeas et al., 2020) and sheep (Ramos-Ibeas et al., 2022). For the development of this culture system, D9 bovine blastocysts were cultured following conventional *in vitro* culture (Holm et al., 1999) and then they were transferred to different culture conditions to promote post-hatching development. Culture in agarose tunnels was shown to restrict embryo growth, and thereby embryos were cultured free-floating in this system. Blastocysts were transferred to two different media: PHD (previously described) or N2B27, a rich medium commonly used for

embryonic stem cell culture (Kinoshita et al., 2021). PHD medium did not support epiblast survival and complete hypoblast migration along the entire inner surface of the embryo. In contrast, when N2B27 medium was used, all embryos showed complete hypoblast migration, around 60 % maintained epiblast survival, and epiblast cells managed to organize into a compact ED-like structure in about 20% of the embryos (**Fig. 4,** Ramos-Ibeas et al., 2020). N2B27 medium allows embryo development up to Day 12, when embryos reach a developmental stage equivalent to embryonic day 10-11 (own data). It is important to note that N2B27 medium does not allow embryo development from the zygote, and recent improvement of the system have identified that transferring bovine embryos from SOF to N2B27 medium in Day 7 instead of Day 9 enhances post-hatching development, particularly epiblast survival (Ramos-Ibeas et al., 2023).

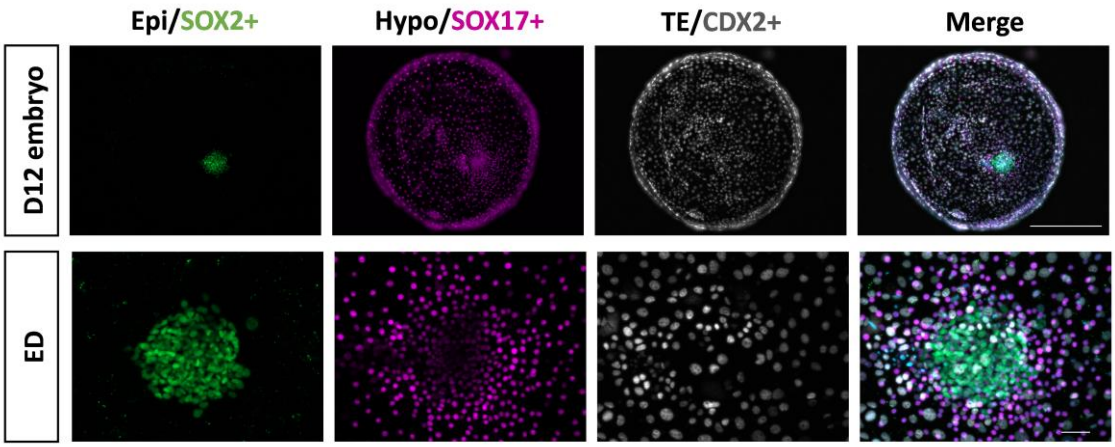

Fig. 4. D12 embryo produced *in vitro* using a post-hatching culture system based on N2B27 medium and subjected to immunohistochemistry for SOX2 (epiblast), SOX17 (hypoblast) and CDX2 (TE). Pictures of the whole spheric D12 embryo showing complete hypoblast migration (above) and its ED-like structure at a higher magnification (bellow). Complete hypoblast migration is evidenced by the presence of SOX17+ cells throughout the entire inner surface of the TE. ED-like structure consists of a compact mass of SOX2+ cells. Nuclei counterstained with DAPI. D, day; ED, embryonic disc, Epi, epiblast; Hypo, hypoblast; TE, trophectoderm. Scale bar: 500 µm for embryo; 50 µm for ED.

This culture system can be applied to study embryo development for different aims, bypassing the need for experimental animals. For instance, it can be used to test

the effect of *in vitro* embryo production systems or other assisted reproductive techniques on post-hatching development, serving as a platform to try future improvements without the need of time and resource consuming experiments involving embryo transfer. The system also provides a platform to directly test the role of specific metabolites, hormones, and growth factors beyond blastocyst hatching by simple compound addition/removal experiments. Last, and particularly relevant for this thesis, the system provides an extension of the period that can be observed *in vitro* allowing to check the development of genome edited embryos to post-hatching stages. These loss-of-function studies to investigate the role of a gene in embryo development of ungulates have been made possible by the advent of the CRISPR/Cas9 technology tool and its optimization for generating genetically modified embryos in livestock species.

1.2. CRISPR/Cas9 gene editing tool

The lack of tools to perform targeted mutagenesis (i.e., genome modifications at specific loci) in ungulate embryos has been another important limitation for the study of conceptus elongation (reviewed by Lamas-Toranzo et al., 2017). As it will be explained in more detail below, before the advent of site-specific endonucleases, such as CRISPR, site-specific genetic modification could only be achieved by homologous recombination (HR), which was extremely inefficient when applied to zygotes (~0.1 % efficiency; (Brinster et al., 1989). The low efficiency was circumvented by applying HR to embryonic stem cells (ESC), which once modified and selected were injected into an embryo to generate chimeric individuals harboring the intended mutation. Unfortunately, such method was only available for mice, as truly pluripotent stem cells were not available in any other mammal, and this technical limitation has been the responsible for the widespread use of mice as the main mammalian model in detriment to other previously well-appreciated models in the pre-genomics era, such as guinea pigs, rabbits or ungulates. In any other mammal different than mice, Somatic Cell Nuclear Transfer (SCNT also known as embryo cloning) constituted the only means to achieve targeted mutagenesis by applying HR in fibroblasts which –upon genome modification and selection- were used to generate a genetically modified embryo. Unfortunately, this method holds major limitations, as SCNT is a difficult to master and inefficient technique, resulting in the best case scenario in a 5 % of live calves (Kato, Tani, & Tsunoda, 2000). These problems have been surpassed by the eruption of gene editing by site-specific endonuclease, such as CRISPR, which currently allow the

generation of genome edited ungulate embryos to understand their own development and that of humans (Ismael Lamas-Toranzo et al., 2018).

1.2.1. Evolution of CRISPR/Cas9: from an adaptative immune system of prokaryotes to a genomic engineering tool

CRISPR/Cas9 editing technology arises from a natural system that provides prokaryotes with an adaptive defense mechanism against foreign DNA invasion. CRISPR stands for "Clustered Regularly Interspaced Short Palindromic Repeats" and are repetitive motifs found in many prokaryotic genomes, which prompted research into their biological function (Ishinoet al., 1987; Mojica et al., 2000; Mojica et al., 2005). The CRISPR locus is composed of repetitive sequences interspersed with small non-repetitive fragments, known as spacers, and encompasses a set of CRISPR-associated (Cas) genes. During the immunization process, Cas effector endonucleases cleave the genome of the invading virus, and the resulting DNA fragments are integrated as spacers into the CRISPR locus, forming a genetic record of the infection, which enables a rapid response to future invasions (Mojica et al., 2005). Upon a reinfection event, these spacers are transcribed into CRISPR RNA (crRNA) and guide Cas endonuclease to the foreign DNA (protospacer) containing a sequence complementary to the spacer (Deltcheva et al., 2011). In the type II CRISPR system, the better characterized, (two more types are currently known; type I and III), the crRNA binds to a trans-activating CRISPR RNA (tracrRNA), facilitating its maturation and association with Cas9 endonuclease (Deltcheva et al., 2011). Once assembled, the crRNA-tracrRNA-Cas9 complex, guided by the crRNA, binds the target DNA through base pairing. The protospacer must be immediately followed by a Protospacer-Adjacent Motif (PAM) sequence. Depending on the bacterial or archaeal species, Cas9 will recognize specific PAM sites and induce a double-strand break (DSB) about three base pairs (bp) upstream of these motifs. PAM sequences provide a mechanism to distinguish between self and foreign genomes (Barrangou et al., 2007; Jinek et al., 2012).

Based on this mechanism of adaptive immunity of prokaryotic cells, a very efficient gene editing technique has been designed. The type II CRISPR system derived from *Streptococcus pyogenes* has been most widely applied in genomic engineering. The Cas9 endonuclease from *S. pyogenes* (SpCas9) recognizes a PAM sequence NGG, where N represents any nucleotide, located at the 3' end downstream of the protospacer

(Mojica et al., 2009). To simplify the system, the endonuclease is directed to the target site to introduce a DSB via a single-guide RNA (sgRNA), which results from the fusion of crRNA and tracrRNA and consists of 20 nucleotides (Jinek et al., 2012). It is important to clarify that a DSB by itself does not generate mutations; mutations arise during the repair process when cell's DNA repair mechanisms attempt to fix the break. All these components of the CRISPR/Cas9 system can be introduced into cells in several forms: (i) DNA plasmid encoding Cas9 protein and sgRNA, (ii) Cas9 mRNA together with an independent sgRNA or (iii) Cas9 protein associated with sgRNA, forming a ribonucleoprotein complex.

1.2.2. Consolidation of CRISPR/Cas9 as the predominant genome editing tool

The emergence of the CRISPR/Cas9 genome editing system has been a breakthrough in the field of genome engineering. CRISPR/Cas9 was first used as a genome editing tool in scientific research in the early 2010s (Cong et al., 2013; Mali et al., 2013) and has become the predominant tool, largely mitigating the drawbacks of previous genome editing techniques.

Primarily, CRISPR/Cas9 is a targeted mutagenesis that enables the introduction of mutations at a precise locus within the genome. This represents a significant advance over earlier non-targeted mutagenesis techniques, which resulted in unpredictable and undesired mutations These non-targeted techniques involved the introduction of a foreign DNA sequence (i.e. transgene) into the genome through different methods such as transduction (Jaenisch, 1976), pronuclear injection (Brinster et al., 1981; Costantini & Lacy, 1981; Gordon & Ruddle, 1981), or intracytoplasmic sperm injection (García-Vázquez et al., 2009; Perry et al., 1999) but neither the insertion site nor the copy number could be controlled.

In addition to allowing targeted mutagenesis, CRISPR/Cas9 technology is highly efficient and can be applied at the zygote stage, enabling direct generation of genetically modified individuals across a wide range of organisms. The pioneer method to achieve targeted mutagenesis relied on homologous recombination (HR), a biological process wherein two similar or identical DNA molecules exchange or recombine to create new DNA sequences. HR-based mutagenesis involved introducing a DNA molecule flanked by sequences complementary to the genome region of interest into cells. This facilitates the integration of the transgene into a specific genomic region via

DNA repair mechanisms, primarily in response to a DSB (Smithies et al., 1985; Thomas, Folger, & Capecchi, 1986; K. Thomas & Capecchi, 1986). The main drawback of HR-based methods was their low efficiency (<1 %), limiting its direct application to zygotes (Brinster et al., 1989). To bypass the low efficiency issue, HR was successfully applied in ESCs, as millions of cells are available in culture, and the very few ones introducing the mutation can be selected by introducing –together with the transgene– a selection cassette which confers to those cells resistance to a cytotoxic agent (Doetschman et al., 1987; Thomas & Capecchi, 1987). Mouse ESCs maintain their pluripotency and when introduced into a preimplantation embryo, they integrate into the developing embryo ultimately forming a chimeric mouse composed of cells derived from both, the initial embryo and the mutated ESCs (Robertson, 1986). ESCs can contribute to the germline and after two crosses of these mosaic animals, homozygous offspring for the desired mutation can be obtained (**Fig. 5,** Evans et al., 1985). Nevertheless, truly pluripotent ESCs able to form chimeras are not available in any other mammalian species. The only solution available to circumvent the need of ESC involved combining HR with SCNT, which had already been implemented in livestock species (Baguisi et al., 1999; Campbell et al., 1996; Cibelli et al., 1998; Polejaeva et al., 2000). SCNT consists of transferring the nucleus of a somatic cell into an enucleated oocyte, ultimately generating an organism genetically identical to the somatic cell. From this perspective, ESCs can be replaced by somatic cells (usually fibroblasts) that are genetically modified by HR and similarly selected to be used as nuclear donors (**Fig. 5)**. This approach resulted in the first gene-targeted livestock animals (Denning et al., 2001; McCreath et al., 2000) and offered a substantial advantage over the production of chimeric animals using gene-targeted ESCs: it allows the direct generation of bi-allelically modified animals, without the need for chimeric progenitors. However, this technique proves ineffective, as only 5 % of the transferred blastocysts results in live births (Kato et al., 2000; Wilmut et al., 1997). Furthermore, the costly offspring generated by SCNT often results in neonatal death and abnormal phenotypes due to epigenetic aberrations. Luckily, all these critical limitations have been surpassed by site specific endonucleases, whose high efficiency allows direct genome modification in embryos, bypassing the need of intermediary cells such as ESCs or fibroblasts.

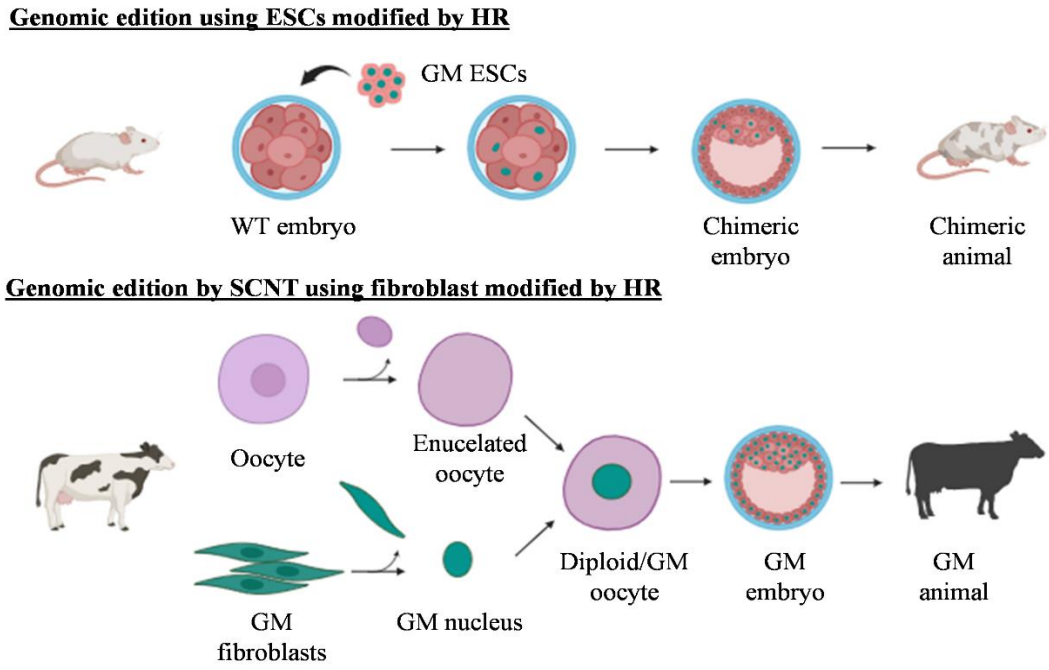

Fig. 5. Generation of genetically modified animals using homologous recombination (HR). Chimeric mouse can be produced by introducing genetically modified ESCs in an early embryo. To produce genetically modified animals in any other mammalian species, SCNT is performed, which consists of transferring the nucleus of a genome edited fibroblast into an enucleated oocyte. GM, genetically modified; ESCs, embryonic stem cells; SCNT, somatic cell nuclear transfer.

Between the different site-specific endonucleases available, CRISPR/Cas9 stand out from other previously developed methods due to its simplicity of design and high efficiency. Zinc-Finger Nucleases (ZFN; Geurts et al., 2009) and Transcription Activator-Like Effector Nucleases (TALEN; Tesson et al., 2011) are other site-specific endonucleases that share the same mechanism of action as CRISPR but differ in their targeting methods and their endonuclease domains. Both ZFN and TALEN are guided by artificial proteins fused to the DNA-cutting domain of the Fok1 restriction endonuclease. Since Fok1 domains only cut DNA when they are present as dimers, ZFN and TALEN require heterodimers that specifically bind each DNA strand and generate DSB (Christian et al., 2010; Porteus & Carroll, 2005). The major advantage of CRISPR over ZFN and TALEN is the use of a guiding RNA molecule, which is highly specific and considerably simpler to design and produce than the artificial proteins that drive sequence specificity in the other two systems. In the same line of simplicity, CRISPR

system is associated to Cas9 endonuclease domain that can introduce directly DSBs, eliminating the need to design an additional guide for the complementary DNA strand (Jinek et al., 2012).

1.2.3. Genomic mutations generated by CRISPR/Cas9 technology

As previously mentioned, DSBs generated by CRISPR/Cas9, do not induce mutations directly; mutations arise when cell's DNA repair mechanisms resolve the break. Eukaryote cells mainly repair DSB by two different mechanisms: Non-Homologous End Joining (NHEJ) or HR. NHEJ is an error-prone repair pathway that causes mutations due to random insertion or deletion of bases (indels). The generation of indels is very useful for the establishment of knock-out (KO) models, particularly when CRISPR is targeted to the coding sequence of a specific gene. Indels that are not multiple of three disrupt the open-reading frame (ORF), resulting in a frame-shift mutation that generates a non-functional truncated peptide. Alternatively, DSB may be repaired by HR, which can restore the original sequence using the sister chromatid as template or, if an exogenous DNA is provided, insert the given sequence, resulting in sequence insertion (knock-in, KI), **(Fig. 6)**. CRISPR is designed against a specific DNA sequence, so the system remains functional until a mutation in the target sequence prevents the sgRNA from recognizing it. In the context of KI generation, it is crucial that the insertion disrupts the target sequence; otherwise, CRISPR continues inducing a DSB, resulting in either an indel (NHEJ) or multiple insertions at the target site (Lamas-Toranzo et al., 2017).

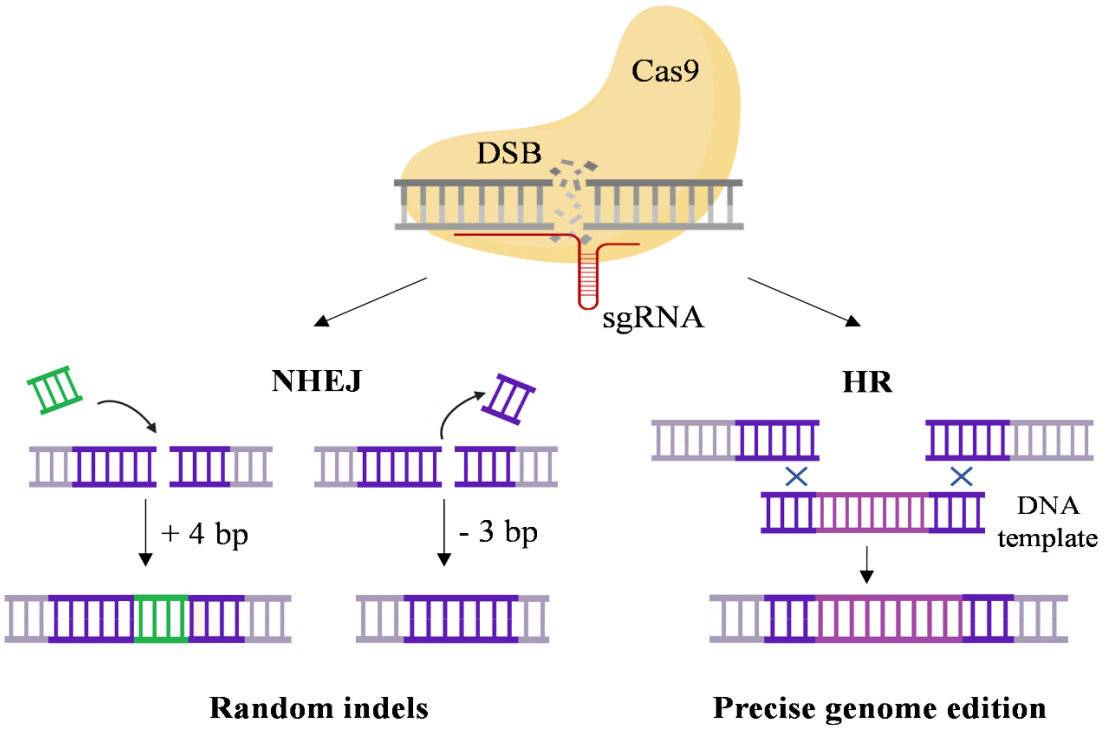

Fig. 6. Gene editing mechanisms of CRISPR/Cas9 tool. Cas9, targeted by a sgRNA, induces a DSB in the DNA that can be repaired by NHEJ (left) or HR (right). NHEJ results in random insertions and deletions (indels) while HR enables precise genome edition if an exogenous DNA template is provided. DSB, double-strand break; HR, homologous recombination; NHEJ, non-homologous end joining; sgRNA, single-guide RNA.

CRISPR can be directly applied to zygotes, allowing the generation of KI and KO individuals in a single step. CRISPR can be used to generate KIs by HR, as HR efficiency improves by >1,000-fold when a DSB is induced at the target *locus* (Smih, Rouet, Romanienko, & Jasin, 1995). KO embryos are typically produced by NHEJ, with the requirement that all alleles within the embryo carry frameshift mutations (i.e. KO alleles). Nevertheless, since indels are generated randomly, they can also generate mutations multiple of three that result in the insertion or deletion of a few amino acids, with the rest of the protein remaining unaltered and probably functional (known as in frame –IF- alleles). This inherent randomness in indel generation means that, despite a 100 % genome edition rate (edited embryos/injected embryos) is technically possible, achieving a 100% KO generation rate (KO embryos/injected embryos) is statistically improbable, since some edited embryos will carry IF alleles that do not disrupt the ORF (Lamas-Toranzo et al., 2017).

In the context of the random generation of indels by NHEJ, the presence of more than one cell type in the same individual (termed mosaicism), with each cell type composed by different alleles decreases significantly KO generation rate (Bevacqua et al., 2016; Crispo et al., 2015; Sato et al., 2015). The logical behind that decrease is simple: the more cell types within an individual, the more alleles it will have and the less likely will be that all of them will be KO alleles (i.e., composed by frame-disrupting indels). Mosaicism following CRISPR-mediated genome edition is the consequence of more than two recombination events in the same target. At the early zygote stage, the embryo contains two copies from each target, each inherited from each parent (2n2c), but shortly after the DNA replicates doubling the genomic content to 2n4c. When CRISPR edition takes place after zygote DNA replication (2n4c), 4 independent genome editing events will take place, resulting in the generation more than two alleles. In this scenario, two cell types will emerge following the first embryo cleavage, each harboring different alleles and leading to genetic mosaicism (Lamas-Toranzo et al., 2017). To reduce mosaicism, CRISPR-induced genome modifications should be generated before the first DNA replication, which is known to occur in bovine embryos between 8-18 h post-insemination (Eid, Lorton, & Parrish, 1994). During conventional IVF, gametes are co-incubated ~20 h (Parrish et al., 1986), and it was observed that the introduction of CRISPR components just prior fertilization significantly reduced mosaicism (Lamas-Toranzo et al., 2019). Following this approach, KO generation rates achieved in bovine embryos vary between 25-60%, depending on the sgRNA used and the genomic region targeted (own data). To identify an embryo as KO, it is imperative to use an accurate genotyping system that enables the identification of all alleles harbored by the embryo and to confirm the presence of a frameshift mutation in all of them. The advent of next-generation sequencing techniques (Illumina), which generate thousands of reads of a PCR product encompassing the target sequence, offers a highly precise method for genotyping CRISPR-edited embryos (Lamas-Toranzo et al., 2019).

1.2.4. Base editors, a technology derived from CRISPR/Cas9 system

Circumventing the difficulties in genotyping inherent to the presence of multiple alleles composed by indels, KO alleles can also be generated by introducing a premature stop codon into the ORF, which prevents the synthesis of the complete protein. This is possible thanks to an adaptation of the CRISPR system, termed Base Editors (BEs, particularly Cytosine Base Editor, CBE), which instead of generating DSBs that will normally result in an indel, change a specific DNA base into another. CBE resulted from the fusion of a catalytically dead Cas9 (dCas9, unable to induce a DSB, but able to carry a fused protein to a genomic target), with a cytidine deaminase enzyme that mediates the conversion of cytosine (C) to uracil (U), which has the base-pairing properties of thymine (T), thereby mediating a C→T substitution (Komor et al., 2016). These cytosine BEs are valuable for creating specific mutations and inducing a premature stop codon by converting the four codons CAA, CAG, CGA and TGG into stop codons TAA, TAG or TGA (Billon et al., 2017; Kuscu et al., 2017). The alleles generated by CBE are considerably easier to genotype than those generated by conventional CRISPR/Cas9 and based on indels. As indels are random and alter the PCR product length, Sanger sequencing of a PCR product of an embryo containing several alleles cannot faithfully identify each of the alleles, as they lead to mixed peaks in the Sanger chromatogram. For this reason, embryo genotyping following this strategy requires allele individualization either by clonal sequencing or by high throughput methods (Lamas-Toranzo et al., 2019). In contrast, CBE-generated alleles do not alter the PCR product length and thereby multiple alleles can be easily identified in the Sanger sequencing of a single PCR product (**Fig. 7)**. Beyond this relevant advantage, CBEs effectively reduce mosaicism and notably increase the KO generation rate, as the system is constantly seeking to introduce the same mutation into all available targets, often resulting in the generation of the same allele in all of them. In this way, KO generation rates close to 100 % can be achieved (own unpublished data).

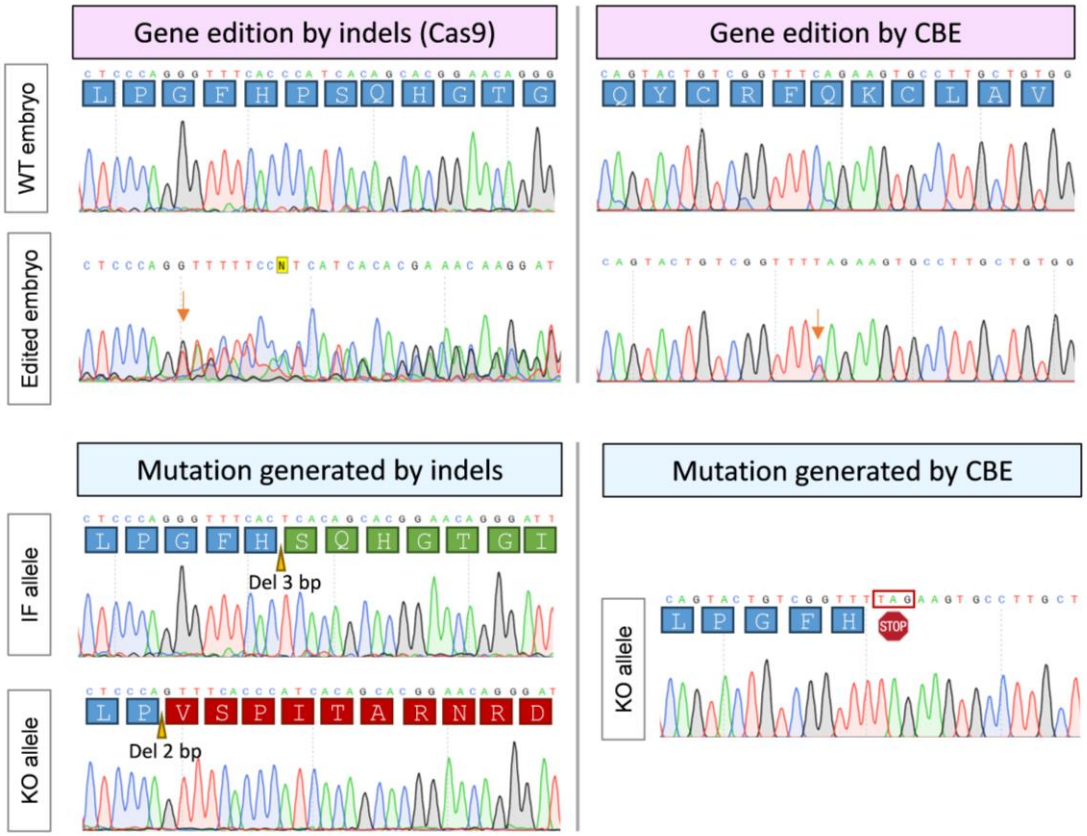

Fig. 7. Sanger sequencing chromatograms of wild-type (WT) and genetically modified embryos for *PPARG* following two gene ablation strategies. Images on the left show Sanger sequencing chromatograms of genome edited embryos by indel generation at the target site. The upper sequence represents a WT embryo without any mutations, and the sequence bellow corresponds to an edited embryo harboring several alleles. From the orange arrow the peaks resulting from each allele overlap, making it impossible to determine the sequence of each allele. The lower rows show two examples of individual mutated alleles generated by indels; these alleles have been individualized by clonal sequencing. In frame (IF) alleles are composed by indels multiple of three (3 bp deletion in the example) that do not disrupt the open reading frame (ORF). In contrast, knock-out (KO) alleles carry frameshift mutations (2 bp deletion in the example) that alters the ORF, impeding protein translation. Images on the right show Sanger sequencing reactions of PCR products amplified from embryos generated using the cytosine base editor (CBE) strategy, which generates a stop codon at the target site. The edited embryo in the example is a heterozygous (WT/KO) embryo that shows two nucleotides at the same position, indicating the presence of two alleles (marked with orange arrow: C in the WT allele and the intended T generating a TAG

stop codon in the KO allele). Only one type of mutation can be generated by CBE (C→T conversion), which always results in a KO allele (lower row).

In this thesis, both indel and base editor-based strategies have been used to generate KO embryos. KO embryos are composed only by KO alleles, which are frame-disrupting alleles when using the indel-based strategy or alleles with a premature stop codon when using CBE. Edited non-KO embryos are referred to as in frame (IF) or heterozygous (HZ) embryos, depending on whether indel or base editor KO generation strategy was used, respectively. IF embryos harbor at least one allele with a mutation that is multiple of three that do not alter the ORF and HZ embryos are composed by two different alleles: edited with a stop codon and WT.

1.2.5. CRISPR/Cas9 technology in livestock species

The development of CRISPR/Cas9 system has simplified the generation of genetically modified mice, but the greatest achievement of this technology is that it has opened the possibility of extending gene edition to other species, including livestock animals. While genetically modified farm animals have not yet been approved for human consumption, CRISPR technology has been used to design animals with specific traits, such as disease resistance (Gao et al., 2017; Whitworth et al., 2016) or enhanced meat production (Crispo et al., 2015; Wang et al., 2015), primarily for research purposes. Besides, genetically modified animals (GMA) can serve as bioreactors to produce therapeutic proteins (Whitelaw et al., 2016) and could be a source of humanized organs with reduced immune rejection (Wolf et al., 2019).

The generation of GMAs other than mice has also important applications in biomedical research. Although genetically modified mice have been an invaluable asset to understand several aspects of human physiology and to model certain diseases, many other human diseases and physiological processes cannot be recapitulated in this species. Farm animals, which are closer to humans regarding size and anatomy, become a feasible alternative to carry on more accurate studies. Accordingly, human diseases such as Huntington's disease (Yan et al., 2018), Parkinson's disease (Wang et al., 2016), atherosclerosis (Huang et al., 2017), adenocarcinoma (Wang et al., 2017), cystic fibrosis (Fan et al., 2018), hypophosphatasia (Williams et al., 2018), Duchenne muscular dystrophy (Yu et al., 2016) or congenital cataract (Yuan et al., 2016) have

been modelled in genetically modified livestock animals generated by CRISPR, enabling the study of potential therapies for patients.

Research on Developmental Biology has been also benefitted from the use of the CRISPR/Cas9 system to ablate genes in ungulate embryos. Beyond constituting the best model to understand their own economically relevant reproductive failures, ungulate embryos arguably constitute a closer model to human embryology than murine embryos. Morphologically, eutherian mammals develop to the blastocyst stage in a very similar manner, albeit timing of development is more similar in ungulates and humans compared to mice. However, despite the morphological similarities, key transcriptional regulators of lineage differentiation differ in their localization patterns and roles (reviewed in **Chapter 1**). In the absence of KO models in other mammalian species, first lineage differentiation, was largely believed to be regulated by the mutually exclusive expression patterns of the transcription factors *Cdx2* (expressed in TE) and *Oct4* (expressed in ICM) observed in mice (Niwa et al., 2005; Strumpf et al., 2005). However, human and ungulate embryos display different OCT4 expression dynamics than mice (Berg et al., 2011; Cauffman et al., 2004; Kirchhof et al., 2000; Kuijk et al., 2008), and whereas *Oct4* KO mouse embryos develop to blastocysts unable to develop hypoblast, *OCT4* KO bovine (Simmet et al., 2018) and human embryos (Fogarty et al., 2017) show impaired blastocyst formation and, surprisingly, a reduced expression of *CDX2*. In the same line of divergence, whereas the ablation of *Cdx2* impairs blastocoel maintenance in murine embryos (Strumpf et al., 2005), *CDX2* downregulation (Goissis & Cibelli, 2014) or ablation ((Shi et al., 2023) and own unpublished observation) in bovine embryos does not disrupt blastocyst formation.

1.3. General overview of the Chapters 2-5

In **Chapters 2, 3, 4 and 5**, KO bovine embryos were generated by CRISPR to determine the effect of the ablation of specific genes on preimplantation embryo development for different purposes: **Chapters 2 and 3** aim to characterize the developmental arrest induced by naturally occurring mutations in the Holstein population, **Chapter 4** assesses the role of PPARG (a major effector of lipid signaling) on embryo development, and **Chapter 5** deepens into the known molecular differences in the regulation of first lineage differentiation by assessing the role of TEAD4, the major regulator of TE differentiation in mice.

Chapters 2 and 3 focus on two deleterious haplotypes present in Holstein population: HH3 and HH5, respectively. Deleterious haplotypes are alleles that are never found at homozygosity at birth in genotyping programs, meaning that they cause pre-term mortality (VanRaden et al., 2011). The developmental stage at which double-carrier embryos (DC, i.e., homozygous for the deleterious allele) arrest their development is economically relevant. Embryonic losses occurring before maternal recognition of pregnancy derive in economic losses similar to a non-fertilizing insemination, whereas later embryonic losses increase both the non-productive periods (open days) and the risk of uterine pathologies associated to abortions if the DC embryo achieves implantation. HH3 haplotype consists in a T by C substitution within exon 24 of the Structural Maintenance of Chromosomes 2 (*SMC2*) gene, which encodes for a core subunit of condensins I and II (Stray et al., 2005), large protein complexes required for chromosome assembly and segregation during mitosis and meiosis (Hirano, 2016). The single nucleotide substitution inactivates the protein by substituting amino acid 1135 from phenylalanine (TTC) to serine (TTC) within its NTPase domain (Daetwyler et al., 2014; McClure et al., 2014). HH5 haplotype is the result of a large 138 kb deletion on chromosome 9 that ablates the Transcription Factor B1 Mitochondrial (*TFB1M*) gene (Schütz et al., 2016), which encodes for a protein involved in mitochondrial translation (McCulloch, Seidel-Rogol, & Shadel, 2002). In both cases, *in vivo* experiments aimed to evaluate the developmental ability of double carrier embryos generated by the cross of heterozygous (carrier) individuals were complemented with *in vitro* experiments where the causative mutation was mimicked by KO alleles generated by CRISPR.

Chapter 4 addresses the role of Peroxisome proliferator-activated receptor gamma (PPARG), a receptor expressed by the ungulate embryo and endometrium (Cammas et al., 2006) and is involved in lipid signaling (Lehrke & Lazar, 2005). During conceptus elongation, there is an increase in the amount of lipids in the uterine fluid, and higher *PPARG* expression has been reported in bovine and ovine conceptuses (Cammas et al., 2006; Ribeiro et al., 2016), suggesting an important role for lipid signaling through PPARG during this developmental period. Furthermore, *PPARG* knock-down induced by intrauterine infusion of Morpholino Antisense Oligonucleotides (MAO) in ewes drastically impaired conceptus elongation (Brooks, Burns, & Spencer, 2015). However, by this approach both endometrial and embryonic

PPARG were depleted, and thereby the specific role of *PPARG* in the conceptus remained unclear. To solve that question, the developmental ability of bovine *PPARG* KO embryos generated by CRISPR was assessed under *in vitro* and *in vivo* approaches.

Finally, **Chapter 5** tests a dogma of mammalian Developmental Biology established by experimentation in the mouse model: the key role of TEAD4 for trophectoderm differentiation. In mice, TEAD4 governs TE differentiation by regulating the expression of other TE specific genes such as *Cdx2*, *Eomes* and *Gata3* (Nishioka et al., 2008; Ralston et al., 2010; Yagi et al., 2007). *Tead4* ablation blocks the expression of these genes preventing blastocyst formation (Nishioka et al., 2009; Yagi et al., 2007). However, as mentioned above, major differences in the roles of OCT4 and CDX2 –essential for ICM and TE differentiation in mice– have been observed between mice and ungulates or humans (Berg et al., 2011; Cauffman et al., 2004; Fogarty et al., 2017; Goissis & Cibelli, 2014; Kirchhof et al., 2000; Kuijk et al., 2008; Shi et al., 2023; Simmet et al., 2018), questioning the essential role of TEAD4 –the upstream regulator of CDX2- on TE commitment or development in non-rodent mammals. To solve that question, the developmental ability of *TEAD4* KO embryos was assessed in cattle and rabbits, which share the same clade (Glires) than the order Rodentia (Álvarez-Carretero et al., 2022) while being closer to humans or ungulates in terms of OCT4 expression pattern (Kobolak et al., 2009).

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Objectives

General objective:

The main objective of this thesis has been to deepen in the molecular knowledge of bovine preimplantation development by performing loss-of-function studies using CRISPR/Cas9 technology.

Specific objectives

1) To critically revise current knowledge on the molecular regulation of early embryo development in mammals, highlighting the differences between mice – the best studied mammalian model– and ungulates.

2) To characterize the developmental arrest induced by the deleterious haplotype HH3, which prevents condensins I and II formation by impairing the translation of functional SMC2 protein.

3) To identify the developmental stage at which double-carrier bovine embryos for the lethal haplotype HH5, which lack *TFB1M* gene, arrest their development.

4) To determine if embryonic PPARG, a major receptor for lipid signaling, is required for bovine embryo development up to maternal recognition of pregnancy.

5) To assess if the essential role of TEAD4 in trophectoderm differentiation in mice is conserved in cattle and rabbits.

Chapter 1

Lineage Differentiation Markers as a Proxy for Embryo Viability in Farm Ungulates

Lineage Differentiation Markers as a Proxy for Embryo Viability in Farm Ungulates

Pérez-Gómez, A., González-Brusi, L., Bermejo-Álvarez, P., & Ramos-Ibeas, P. (2021). Lineage Differentiation Markers as a Proxy for Embryo Viability in Farm Ungulates. *Frontiers in veterinary science, 8,* 680539. https://doi.org/10.3389/fvets.2021.680539

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PhD student's contribution to the work

Analysis of interspecific differences on lineage-specific markers during early embryo development. Elaboration of all the figures of the paper. Drafting and revision of the manuscript.

Lineage Differentiation Markers as a Proxy for Embryo Viability in Farm Ungulates

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Abstract

Embryonic losses constitute a major burden for reproductive efficiency of farm animals. Pregnancy losses in ungulate species, which include cattle, pigs, sheep and goats, majorly occur during the second week of gestation, when the embryo experiences a series of cell differentiation, proliferation, and migration processes encompassed under the term conceptus elongation. Conceptus elongation takes place following blastocyst hatching and involves a massive proliferation of the extraembryonic membranes trophoblast and hypoblast, and the formation of flat embryonic disc derived from the epiblast, which ultimately gastrulates generating the three germ layers. This process occurs prior to implantation and it is exclusive from ungulates, as embryos from other mammalian species such as rodents or humans implant right after hatching. The critical differences in embryo development between ungulates and mice, the most studied mammalian model, have precluded the identification of the genes governing lineage differentiation in livestock species. Furthermore, conceptus elongation has not been recapitulated *in vitro*, hindering the study of these cellular events. Luckily, recent advances on transcriptomics, genome modification and post-hatching *in vitro* culture are shedding light into this largely unknown developmental window, uncovering possible molecular markers to determine embryo quality. In this review, we summarize the events occurring during ungulate pre-implantation development, highlighting recent findings which reveal that several dogmas in Developmental Biology established by knock-out murine models do not hold true for other mammals, including humans and farm animals. The developmental failures associated to *in vitro* produced embryos in farm animals are also discussed together with Developmental Biology tools to assess embryo quality, including molecular markers to assess proper lineage commitment and a post-hatching *in vitro* culture system able to directly determine developmental potential circumventing the need of experimental animals.

Chapter 2

SMC2 ablation impairs bovine embryo development shortly after blastocyst hatching

SMC2 ablation impairs bovine embryo development shortly after blastocyst hatching

Pérez-Gómez, A., Flores-Borobia I., Hamze J.G., Galiano-Cogolludo B., Lamas-Toranzo I., González-Brusi L., Ramos-Ibeas P., & Bermejo-Álvarez P. (2024). SMC2 ablation impairs bovine embryo development shortly after blastocyst hatching. *Reproduction (Cambridge, England).* Advance online publication. https://doi.org/10.1530/REP-24-0211.

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PhD student's contribution to the work

In vitro production of bovine embryos. This process involves collection of ovaries from the slaughterhouse, *in vitro* maturation of cumulus-oocyte complexes, *in vitro* fertilization and *in vitro* culture up to day 8 or 12. For *SMC2* ablation, microinjection of CRISPR/Cas9 components into matured oocytes was performed. Immunohistochemistry of *in vivo* and *in vitro* produced embryos and assessment of embryo development at each developmental stage. Embryo genotyping by Sanger and miSeq sequencing. Besides, statistical analysis of the results and generation of figures. Lastly, participation in manuscript drafting and revision.

SMC2 ablation impairs bovine embryo development shortly after blastocyst hatching

Pérez-Gómez A[1], Flores-Borobia I[1], Hamze JG[1,2], Galiano-Cogolludo B[1], Lamas-Toranzo I[1,2], González-Brusi L[1], Ramos-Ibeas P[1], Bermejo-Álvarez P[1].

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Abstract

Condensins are large protein complexes required for chromosome assembly and segregation during mitosis and meiosis. Mouse or bovine embryos lacking SMC2 (a core component of condensins I and II) do not complete development to term, but it is unknown when they arrest their development. Herein, we have assessed the developmental ability of bovine embryos lacking SMC2 due to a naturally occurring mutation termed HH3 (Holstein Haplotype 3) or by CRISPR-mediated gene ablation. To determine if embryos homozygous for HH3 allele survive to maternal recognition of pregnancy, embryonic day (E)14 embryos were flushed from superovulated carrier cows inseminated with a carrier bull. Mendelian inheritance of HH3 allele was observed at E14 conceptuses but conceptuses homozygous for HH3 failed to achieve elongation and lack embryonic disc. To assess the consequence of the ablation of condensins I and II at earlier developmental stages, SMC2 KO bovine embryos were generated *in vitro* using CRISPR technology. SMC2 KO embryos were able to form blastocysts but exhibited reduced cell proliferation as evidenced by a significantly lower number of total, trophectoderm (CDX2+) and inner cell mass (SOX2+) cells at Day (D) 8 post-fertilization compared to their WT counterparts and were unable to survive to D12 *in vitro*. SMC2 ablation did not alter relative telomere length at D8, D12 or E14. In conclusions, condensins I and II are required for blastomere mitosis during early development and embryos lacking those complexes arrest their development shortly after blastocyst hatching.

Chapter 3

HH5 Double-Carrier Embryos Fail to Progress through Early Conceptus Elongation

HH5 Double-Carrier Embryos Fail to Progress through Early Conceptus Elongation

Pérez-Gómez, A., Hamze, J. G., Flores-Borobia, I., Galiano-Cogolludo, B., Lamas-Toranzo, I., González-Brusi, L., Ramos-Ibeas, P., & Bermejo-Álvarez, P. (2024). HH5 Double-Carrier Embryos Fail to Progress through Early Conceptus Elongation. *Journal of dairy science*, S0022-0302(24)00760-4. Advance online publication. https://doi.org/10.3168/jds.2023-24482

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PhD student's contribution to the work

In vitro production of wild-type and genetically modified bovine embryos. This process includes collection of ovaries from the slaughterhouse, *in vitro* maturation of cumulus-oocyte complexes, *in vitro* fertilization and *in vitro* culture until day 8 or day 12. For *TFB1M* KO embryo generation, microinjection of CRISPR/Cas9 components into mature oocytes was performed. Immunohistochemistry of embryos obtained *in vivo* and *in vitro* and embryo development assessment at each developmental stage. Embryo genotyping by Sanger and miSeq sequencing. Statistical analysis of the results and elaboration of figures. Finally, participation in the drafting of the article and revision.

HH5 Double-Carrier Embryos Fail to Progress through Early Conceptus Elongation

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Abstract

Massive genotyping in cattle has uncovered several deleterious haplotypes that cause pre-term mortality. Holstein Haplotype 5 (HH5) is a deleterious haplotype present in the Holstein Friesian population that involves the ablation of the Transcription Factor B1 mitochondrial (TFB1M) gene. The developmental stage at which HH5 double-carrier (DC, homozygous) embryos or fetuses die remains unknown and this is a relevant information to estimate the economic losses associated to the inadvertent cross between carriers. To determine if HH5 DC survive to maternal recognition of pregnancy, embryonic day (E)14 embryos were flushed from superovulated carrier cows inseminated with a carrier bull. DC E14 conceptuses were recovered at Mendelian rates but they failed to achieve early elongation, as evidenced by a drastic (>26-fold) reduction in the proliferation of extraembryonic membranes compared to carrier or non-carrier embryos. To assess development at earlier stages, *TFB1M* knock-out (KO) embryos -functionally equivalent to DC embryos- were generated by CRISPR technology and cultured to the blastocyst stage -Day (D)8- and to the early embryonic disc stage -D12-. No significant effect of *TFB1M* ablation was observed on the differentiation and proliferation of embryonic lineages up to D12. In conclusion, HH5 DC embryos are able to develop to early embryonic disc stage but fail to undergo early conceptus elongation, required for pregnancy recognition.

Chapter 4

PPARG is dispensable for bovine embryo development up to tubular stages

PPARG is dispensable for bovine embryo development up to tubular stages

Pérez-Gómez, A., González-Brusi, L., Flores-Borobia, I., Martínez de Los Reyes, N., Toledano-Díaz, A., López-Sebastián, A., Santiago Moreno, J., Ramos-Ibeas, P., & Bermejo-Álvarez, P. (2024). PPARG is dispensable for bovine embryo development up to tubular stages. *Biology of reproduction*, ioae083. Advance online publication. https://doi.org/10.1093/biolre/ioae083

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PhD student's contribution to the work

In vitro production of bovine embryos. This process includes collection of ovaries from the slaughterhouse, *in vitro* maturation of cumulus-oocyte complexes, *in vitro* fertilization and *in vitro* culture until day 8 or day 12. For *PPARG* disruption, microinjection of CRISPR/Cas9 components into mature oocytes. Immunohistochemistry of embryos obtained *in vivo* and *in vitro* and embryo development assessment at each developmental stage. Embryo genotyping by Sanger and miSeq sequencing. Statistical analysis of the results and elaboration of figures. Finally, participation in the drafting of the article and revision.

PPARG is dispensable for bovine embryo development up to tubular stages

Pérez-Gómez A[1], González-Brusi L[1], Flores-Borobia I[1], Martínez de los Reyes N[1], Toledano-Díaz A[1], López-Sebastián A[1], Santiago Moreno J[1], Ramos-Ibeas P[1], Bermejo-Álvarez P[1*]

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Abstract

Following blastocyst hatching, ungulate embryos undergo a prolonged preimplantation period termed conceptus elongation. Conceptus elongation constitutes a highly susceptible period for embryonic loss and the embryonic requirements during this process are largely unknown, but multiple lipid compounds have been identified in the fluid nourishing the elongating conceptuses. Peroxisome proliferator-activated receptors (PPARs) mediate the signaling actions of prostaglandins and other lipids and, between them, PPARG has been pointed out to play a relevant role on conceptus elongation by a functional study that depleted PPARG in both uterus and conceptus. The objective of this study has been to determine if embryonic PPARG is required for bovine embryo development. To that aim, we have generated bovine *PPARG* KO embryos *in vitro* by two independent gene ablation strategies and assess their developmental ability to the blastocyst stage (day –D- 8), early post-hatching stage (D12) and tubular stage (E14). *In vitro* development to blastocyst was unaffected by PPARG ablation, as total, inner cell mass and trophectoderm cell numbers were similar between WT and KO D8 embryos. Post-hatching development to D12 *in vitro* was also comparable between different genotypes, as embryo diameter, epiblast cell number and embryonic disc formation and hypoblast migration rates were unaffected by the ablation. The development to tubular stages equivalent to E14 was assessed following a heterologous transfer experiment, observing that the development of extra-embryonic membranes and of the embryonic disc was not altered by *PPARG* ablation. In conclusion, PPARG ablation did not impaired bovine embryo development up to tubular stages.

Chapter 5

The role of TEAD4 in trophectoderm commitment and development is not conserved in non-rodent mammals

The role of TEAD4 in trophectoderm commitment and development is not conserved in non-rodent mammals

Pérez-Gómez, A., González-Brusi, L., Flores-Borobia, I., Galiano-Cogolludo, B., Lamas-Toranzo, I., Hamze, J. G., Toledano-Díaz, A., Santiago-Moreno, J., Ramos-Ibeas, P., & Bermejo-Álvarez, P. (2024). The role of TEAD4 in trophectoderm commitment and development is not conserved in non-rodent mammals. Development (Cambridge, England), dev.202993. Advance online publication. https://doi.org/10.1242/dev.202993

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PhD student's contribution to the work

In vitro production of bovine embryos (collection of ovaries, *in vitro* maturation, fertilization and culture until day 8 or day 12). For *TEAD4* ablation, microinjection of CRISPR/Cas9 components into mature oocytes. Immunohistochemistry of bovine and rabbit embryos and developmental assessment. Embryo genotyping by Sanger and miSeq sequencing. RNA-seq library preparation. Statistical analysis of the results and elaboration of figures. Finally, participation in the drafting of the article and revision.

The role of TEAD4 in trophectoderm commitment and development is not conserved in non-rodent mammals

Pérez-Gómez A[1], González-Brusi L[1], Flores-Borobia I[1], Galiano-Cogolludo B[1], Lamas-Toranzo I[1], Hamze JG[1], Toledano-Díaz A[1], Santiago-Moreno J[1], Ramos-Ibeas P[1], Bermejo-Álvarez P[1]

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Abstract

The first lineage differentiation in mammals gives rise to the inner cell mass (ICM) and the trophectoderm (TE). In mice, TEAD4 is a master regulation of TE commitment, as it regulates the expression of other TE-specific genes and its ablation prevents blastocyst formation, but its role in other mammals remains unclear. Herein, we have observed that TEAD4 ablation in two phylogenetically distant species (bovine and rabbits) does not impede TE differentiation, blastocyst formation and the expression of TE markers such as GATA3 and CDX2, although a reduced ICM cell number was observed in bovine TEAD4 KO blastocysts. Transcriptional analysis in bovine blastocysts revealed no major transcriptional effect of the ablation, although the expression of hypoblast and Hippo signaling-related genes tended to be decreased in KO embryos. Experiments were conducted in the bovine model to determine if TEAD4 was required for post-hatching development. TEAD4 KO spherical conceptuses showed normal development of the embryonic disc and TE, but hypoblast migration rate was reduced. At later stages of development (tubular conceptuses) no differences were observed between KO and WT conceptuses.

Conclusions

1) The molecular regulation of the cell differentiation events occurring during preimplantation embryo development is remarkably different between mice and other mammals, precluding the direct extrapolation of the knowledge gathered from murine KO models to other mammals, including ungulates.

2) Embryos lacking condensins I and II due to SMC2 ablation (i.e., HH3 double-carriers) are able to reach the blastocyst stage but exhibit cell proliferation defects and are unable to survive to Day 12 *in vitro*, thereby being unable to trigger maternal recognition of pregnancy.

3) Embryos lacking *TFB1M* (i.e. HH5 double-carriers) develop normally to pre-elongation stages but arrest their development during early conceptus elongation, before maternal recognition of pregnancy.

4) *PPARG* is not required for bovine embryo development up to tubular stages, suggesting that lipid signaling is either dispensable up to E14 or is mediated by a PPARG-independent pathway.

5) TEAD4 is dispensable for trophectoderm differentiation in cattle and rabbits, in striking contrast to its essential role in mice. TEAD4 is not required either for bovine trophectoderm proliferation to tubular stages.

Appendix

Appendix I

List of abbreviations

A	Adenine
ANOVA	Analysis of variance
BE group	Control group, microinjected with BEs
BE+G group	Experimental group, microinjected with BEs and sgRNA
BEs	Base editors
BNCs	Binucleated cells
bp	Base pair
BSA	Bovine serum albumin
C	Cytosine
C group	Control group, microinjected with Cas9
C+G group	Experimental group, microinjected with Cas9 and sgRNA
Cas	CRISPR-associated
cDNA	Complementary deoxyribonucleic acid
CBE	Cytosine base editor
COC	Cumulus oocyte complex
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
D	Day
DAPI	4 ',6-diamidino-2-fenilindol

DC	Double-carrier
dCas9	Catalytically dead Cas9
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide tri-phosphate
DSB	Double-strand break
E	Embryonic day
e.g.	Exempli gratia (for example)
ED	Embryonic disc
EEMs	Extraembryonic membranes
EGA	Embryonic genome activation
EGF	Epidermal growth factor
EMT	Epithelial-to-mesenchymal transition
ESC	Embryonic stem cell
F	Forward
FBS	Fetal bovine serum
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FGFR2	Fibroblast growth factor receptor 2
G	Guanine
GMA	Genetically modified animal
HH3	Holstein haplotype 3

HH5	Holstein haplotype 5
hpi	Hours post-insemination
HR	Homologous recombination
HZ	Heterozygous
i.e.	Id est (that is)
ICM	Inner cell mass
IF	In-frame
IFNT	Interferon tau
IHC	Immunohistochemistry
Indel	Insertion/deletion
IVF	*In vitro* fertilisation
kb	Kilobase
KI	Knock-in
KO	Knock-out
MAO	Morpholino Antisense Oligonucleotide
mRNA	Messenger RNA
NC	Non-carrier
NHEJ	Non-homologous end joining
non-KO	Edited non-knock-out
NTPase	Nucleotide triphosphatase
ORF	Open reading frame

p (-value)	Probability value
PAM	Protospacer-adjacent motif
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PHD	Post-hatching development
PPARG	Peroxisome proliferator-activated receptor gamma
PPARs	Peroxisome proliferator-activated receptors
R	Reverse
RL	Rauber's layer
RNA	Ribonucleic acid
RT	Room temperature
s.e.m.	Standard error of the mean
SC	Single-carrier
SCNT	Somatic cell nuclear transfer
sgRNA	Single-guided RNA
SMC2	Structural Maintenance of Chromosomes 2
SNP	Single-nucleotide polymorphism
SOF	Synthetic oviductal fluid
SpCas9	Cas9 endonuclease from *S. pyogenes*
T	Brachyury

T	Thymine
TALEN	Transcription activator-like effector nucleases
TALP	Tyrode/Albumin/Lactate/Pyruvate medium
TE	Trophectoderm
TEAD4	TEA Domain Transcription Factor 4
TF	Transcription Factor
TFB1M	Transcription Factor B1 Mitochondrial
tracrRNA	Trans-activating crRNA
TXPBS	0.1 % Triton X-100 in PBS
U	Uracil
v	volume
WT	Wild-type
ZFN	Zinc-finger nuclease
ZP	Zona pellucida

Units (volume, weight, time, …) are abbreviated following the International System of Units.

Chemical substances are named following the IUPAC (International Union of Pure and Applied Chemistry) nomenclature.

Genes and proteins are named by their symbols following the nomenclature of the National Center for Biotechnology Information´s (https://www.ncbi.nlm.nih.gov/pubmed).

Appendix II

Curriculum Vitae

Education

- PhD in Biology and Reproductive Health Technology, University of Murcia, Murcia (Spain), 2019-2024.
- MSc Degree in Mammal Reproduction Biology and Technology, University of Murcia, Murcia (Spain), 2018-2019.
- BSc Degree in Biotechnology, University of Zaragoza, Zaragoza (Spain), 2014-2018.
- Professional Degree in Flute, Professional Conservatory of Music of Zaragoza, Zaragoza (Spain), 2010-2017

Work history

- Research associate. Aragon Institute for Health Research (IIS), Zaragoza (Spain), Jun 2024 – at present
- PhD candidate at Animal Reproduction Department, INIA, CSIC, Madrid (Spain), Oct 2020 – Oct 2023.

Research stays

- Developmental Biology Department at the University of Nottingham. Dr. Ramiro Alberio. May 2023- Aug 2023.
- Cell Biology and Histology Department of the Medicine Faculty at the University of Murcia. Dra. María Jiménez Movilla and Dra. Raquel Romar. Nov 2019 – Mar 2020

Publications in indexed journals

Pérez-Gómez, A., Flores-Borobia I., Hamze J.G., Galiano-Cogolludo B., Lamas-Toranzo I., González-Brusi L., Ramos-Ibeas P., & Bermejo-Álvarez P. (2024). SMC2 ablation impairs bovine embryo development shortly after blastocyst hatching.

Reproduction (Cambridge, England). Advance online publication. https://doi.org/10.1530/REP-24-0211.

Pérez-Gómez A, González-Brusi L, Flores-Borobia I., Galiano-Cogolludo B, Lamas-Toranzo I, Hamze JG, Toledano-Díaz A, Santiago-Moreno J, Ramos-Ibeas P, Bermejo-Álvarez P. (2024). The role of TEAD4 in trophectoderm commitment and development is not conserved in non-rodent mammals. *Development.*

Pérez-Gómez A, González-Brusi L, Flores-Borobia I., Martínez de Los Reyes N, Toledano-Díaz A, López-Sebastián A, Santiago Moreno J, Ramos-Ibeas P, Bermejo-Álvarez P. (2024). PPARG is dispensable for bovine embryo development up to tubular stages. *Biol Reproduction*, ioae083.

Pérez-Gómez A, Hamze, JG, Flores-Borobia I, Galiano-Cogolludo B, Lamas-Toranzo I, González-Brusi L, Ramos-Ibeas, P, Bermejo-Álvarez P. (2024). HH5 Double-Carrier Embryos Fail to Progress through Early Conceptus Elongation. *J Dairy Sci*, S0022-0302(24)00760-4.

González-Brusi L, **Pérez-Gómez A**, Quiroga AC, Tamargo C, Ramos-Ibeas P, Bermejo-Álvarez P. (2023). Effect of arachidonic acid on pre- and post-hatching in vitro bovine embryo development. *Reprod Fertil Dev*, Jul; 35(12):614-621.

Ramos-Ibeas P, **Pérez-Gómez A,** González-Brusi L, Quiroga AC, Bermejo-Álvarez P. **2023.** Pre-hatching exposure to N2B27 medium improves post-hatching development of bovine embryos in vitro. *Theriogenology*, 205:73-78.

Martínez-Moro Á, Lamas-Toranzo I, González-Brusi L**, Pérez-Gómez A**, Padilla-Ruiz E, Garcia-Blanco J, Bermejo-Álvarez P. **2022.** mtDNA content in cumulus cells does not predict development to blastocyst or implantation. *Hum Reprod Open*, 2022(3):hoac029.

Pérez-Gómez A, González-Brusi L, Bermejo-Álvarez P, Ramos-Ibeas P. **2021**. Lineage Differentiation Markers as a Proxy for Embryo Viability in Farm Ungulates. *Front Vet Sci*, 8:680539.

Presentations in international congresses as first author

Pérez-Gómez A, Galiano-Cogolludo B, Hamze JG, Ramos-Ibeas P, Bermejo-Álvarez P. (2023) Transforming growth factor beta (TGFβ) pathway is required for the proliferation of bovine extra-embryonic membranes following blastocyst hatching. 39th Association of Embryo Technology in Europe (AETE) Meeting. Heraklion, Greece. **Oral communication.**

Pérez-Gómez A, Álvarez-Sala M, Inglés Pedreño A, Lamas-Toranzo I, Galiano-Cogolludo B, Hamze JG, Sevim Tütem E, Ramos-Ibeas P, Bermejo-Álvarez P. (2023). 49th International Embryo Technology Society (IETS) Annual Meeting. Lima, Peru. **Poster.**

Pérez-Gómez A, Ramos-Ibeas P, Bermejo-Álvarez P. (2022). Homozygous bovine embryos for HH3 haplotype fail to survive through elongation. 16th Spanish Association of Animal Reproduction (AERA) Congress. Leon, Spain. **Poster**.

Pérez-Gómez A, Ramos-Ibeas P, Bermejo-Álvarez P. (2022) HH5 double-carrier bovine embryos show impaired development through elongation. 38th Association of Embryo Technology in Europe (AETE) Meeting. Utrecht, Netherlands. **Oral communication**.

Pérez-Gómez A, Hamze JG, Galiano-Cogolludo B, Lamas-Toranzo I, Ramos-Ibeas P, Bermejo-Álvarez P. (2022). Activin A enhances epiblast survival during bovine post-hatching development.55th Society for the Study of Reproduction (SSR) Conference. Spokane, Washington, USA. **Poster.**

Pérez-Gómez A, Sevim Tütem E, Lamas-Toranzo I, Hamze JG, Galiano-Cogolludo B, Ramos-Ibeas P, Bermejo-Álvarez P. (2022). TEAD4 is dispensable for bovine conceptus elongation. 19th International Congress on Animal Reproduction (ICAR). Bologna, Italy. **Poster.**

Pérez-Gómez A, González-Brusi L, Ramos-Ibeas P, Bermejo-Álvarez P. (2022). Transcription factor TEAD4 is not required for bovine blastocyst formation. 48th International Embryo Technology Society (IETS) Annual Meeting. Savannah, Georgia, USA. **Flash talk and Poster.**

Pérez-Gómez A, González-Brusi L, Toledano-Diaz A, López-Sebastián A, Ramos-Ibeas P, Bermejo-Álvarez P. (2021). Embryonic PPARG is dispensable for bovine

conceptus elongation. 54th Society for the Study of Reproduction (SSR) Conference. Saint-Louis, Missouri, USA. **Oral communication.**

Pérez-Gómez A, Muniesa-Martinez I, Garcia-Sacristán P, González-Brusi L, Ramos-Ibeas P, Bermejo-Álvarez P. (2021). Kinetics of bovine embryonic lineages development during post-hatching embryo culture. 37th Association of Embryo Technology in Europe (AETE) Meeting. Online. O**ral communication.**

Pérez-Gómez A, Ramos-Ibeas P, González-Brusi L, Quiroga A, Bermejo-Álvarez P. (2021).Pre-hatching exposure to N2B27 medium improves post-hatching development of bovine embryos in vitro. 37th Association of Embryo Technology in Europe (AETE) Meeting. Online. **Poster.**

Presentations in national congresses as first author

Pérez-Gómez A, Álvarez-Sala M, Inglés Pedreño A, Lamas-Toranzo I, Galiano-Cogolludo B, Hamze JG, Sevim Tütem E, Ramos-Ibeas P, Bermejo-Álvarez P. (2022). La eliminación del gen SMC2 disminuye la proliferación celular del embrión antes del estadio de blastocisto. VI Jornadas Doctorales UM. Murcia, Spain. **Oral communication.**

Pérez-Gómez A, González-Brusi L, Ramos-Ibeas P, Bermejo-Álvarez P. (2021) Developmental effects of arachidonic acid supplementation during posthatching bovine embryo development. VI Jornadas Doctorales UM. Murcia, Spain. **Oral communication.**

Awards

Winner of the 38th Association of Embryo Technology in Europe (AETE)'s Student Competition. Utrecht (Netherlands), September 2022.

Peter Farin Travel Award at the 48th International Embryo Technology Society (IETS) Annual Meeting. Savannah, Georgia (USA). January 2022.

Best oral communication at the 37th Association of Embryo Technology in Europe (AETE) Meeting. Online. September 2021.

Courses

ABC Certificate in Practical Small Animal Care Skills (Animal Training)

Third Cell Fit Training School, 2020, Ponte di Legno, Milan, Italy