EXPLORING METHODS TO UNDERSTAND BOVINE EMBRYO COMPETENCY *IN VITRO*

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ABSTRACT

The development of a preimplantation embryo is a stepwise process consisting of morphological, biochemical, and genomic changes. Much remains unknown about the attainment of embryo competency to develop and establish pregnancy. To investigate this, we compared methods of selection at the oocyte or embryo level for improved blastocyst production. Brilliant cresyl blue staining was used to sort oocytes by their growth status (not fully grown vs. fully grown) and the timing of the first embryonic cell division to sort embryos. We found that an embryo's cleavage kinetics are more indicative of their competency than the growth status of the oocyte that gave rise to that embryo. We further investigated the cryopreservation survival of embryos with fast or slow cleavage kinetics and found no significant differences in their ability to hatch post-thawing. Next, we used the complete sequence of the cattle Y chromosome to identify oligonucleotides for efficient sexing of samples. These materials may be used to understand sexual dimorphism as a biological factor in future experiments. Finally, we designed a new method to induce targeted DNA sequence deletions and mRNA cleavage in zygotes using CRISPR-Cas. We targeted the gene *OCT4,* since the literature shows variable knockout outcomes. Our method improved deletion efficiency while accounting for preexisting or maternally inherited mRNA of the target gene. Our findings can be used to better understand early embryo development and biological drivers of quality, which can be leveraged to improve embryo production and transfer outcomes.

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GENERAL AUDIENCE ABSTRACT

The development of an early embryo involves many biological and structural changes. Much remains unknown about the influences on embryo quality and ability to successfully develop. To investigate this, we compared methods for selecting the highest quality cattle eggs or embryos. We found that the observation of an embryo's development speed is better for selecting high quality embryos than egg quality. We further investigated the freezing survival of embryos with fast or slow growth. We found that the freezing survival of fast and slow growing embryos is not different. Next, we used the complete sequence of the cattle Y chromosome to identify PCR primers for determining sample sex. These resources can help us understand how an individual's sex can influence biological differences. Finally, we designed a new method for removing the total function of a gene in embryos. For this, we deleted segments of DNA and cut RNAs. Our findings can be used to better understand early embryo development and biological drivers of quality, which can be leveraged to improve embryo production and transfer outcomes.

DEDICATION

I dedicate this thesis to my grandparents, Brenda and Houston Lindsay.

Because of you, I will always choose to take ‘the road less traveled by’. Thank you for your unconditional support throughout this journey.

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I would like to thank my mother, Jennifer Gibson, and little sister, Julie Gibson, for always believing in me. You have been my biggest cheerleaders, and I will forever appreciate your support. I could not have conquered this without you both.

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LIST OF ABBREVIATIONS

AMOT	Angiomotin
BCB	Brilliant cresyl blue
CDK	Cyclin dependant kinase
ChrY	Y chromosome
COC	Cumulus oocyte complexes
CTCF	Corrected total cell fluorescence
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphate
G6PHD	Glucose-6-phosphate dehydrogenase
GFP	Green fluorescent protein
gRNA	Guide ribonucleic acid
HPF	Hours post-fertilization
HPT	Hours post-thawing
IVC	*in vitro* culture
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
IVP	*in vitro* embryo production
KO	Knock out
LH	Luteinizing hormone
MPF	Mitosis promoting factor
mRNA	Messenger ribonucleic acid
OMM	Oocyte maturation medium
PCR	Polymerase chain reaction
PZ	Putative zygotes
RFP	Red fluorescent protein

RNP	Ribonucleoprotein
sgRNA	Single guide ribonucleic acid
SOF-BE1	Synthetic oviductal fluid culture medium
SOF-FERT	Synthetic oviductal fluid fertilization medium
SOF-HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered synthetic oviductal fluid
TEAD	Transcriptionally enriched associated domain
YAP	Yes-associated protein

CHAPTER 1: LITERATURE REVIEW

Introduction

Producing embryos in the laboratory has become a popular and valuable tool for improving livestock, and the use of assisted reproduction technologies, like *in vitro* embryo production (IVP) and subsequent embryo transfer, supports improved genetic advancement in cattle[1-3]. The number of *in vitro* produced cattle embryos transferred to recipients has far surpassed those produced *in vivo* in the United States[4,5], showing that this technology is steadily being adopted by producers wishing to improve their her compositions. Cattle embryos produced *in vitro* are also valuable for translational and biomedical studies[6] due to their remarkable similarity to human biology. The early embryonic development schedule, morphology, and gene expression of cattle and human embryos show notable similarity[7,8], and it is suggested that cattle may soon replace the murine model for translational studies of early embryo development[9]. Cattle and women also show similarities in their reproductive physiology. For example, both are mono-ovulatory, with similar reproductive cycle and gestation lengths[10]. Apart from use as an infertility treatment, IVP is a valuable tool to further our understanding of early embryogenesis in livestock and humans, while providing a means for the improvement of livestock genetic merit and production potential.

Despite the numerous benefits and applications of producing embryos *in vitro*, several limitations exist that hinder this technology's effectiveness for research or livestock production. Additionally, biological differences that may impact developmental potential exist between IVP and *in vivo* derived embryos. These limitations translate to large losses throughout the duration of embryo production and culture. This literature review will discuss the biological factors supporting early embryonic development, the limitations of cattle IVP, and methods to predict embryo development *in vitro,* with a focus on cattle. Early embryogenesis encompasses a vast assortment of cooperative biological processes resulting in varying developmental competencies, and therefore, this literature review is only able to capture a small portion of this complexity.

Biological factors contributing to preimplantation development in cattle

The development of an embryo is an exceedingly complex process involving tightly coordinated temporal genomic, metabolic, and morphological changes. For successful embryonic development to occur, specific developmental milestones must be completed within a defined timeframe. Deviations away from the characteristic execution of these milestones are expected to negatively impact early embryonic development, resulting in poor quality or degenerate embryos. There are countless factors spanning the parental and embryonic lifetime that may influence early development, therefore, only a portion of the influences that are important for the successful completion of early embryogenesis will be discussed in the following section of this literature review. Additionally, much of the research done on these processes have been completed in organisms other than cattle. Therefore, though most examples will be of studies completed in cattle embryos, some examples from other organisms will be included.

Oocyte competency

The oocyte (female gamete) is of principal importance to preimplantation development, as it provides the early embryo with its valuable cytoplasm, stockpiled with RNAs[11], proteins[12,13], and other cellular machinery[14]. Attainment of oocyte competence to produce a viable embryo is a continuous and stepwise progression completed throughout oogenesis and prior to ovulation[15]. Groups of primary oocytes, enclosed in tertiary follicles, are recruited into the growth pool under the control of follicular stimulating hormone and luteinizing hormone from the female's anterior pituitary. Most of the recruited follicles and oocytes will undergo atresia or cell death, which is coordinated through bidirectional and paracrine communication between the gamete and follicle cells[16]. Atresia occurs at all stages of oogenesis across all mammalian species and is an important biological feature that aims to best support female fertility through stringent quality control of the oocyte selected for ovulation, especially in those species that are mono-ovulatory, like cattle or women. In fact, it has been suggested that in cows, 80-90% of ovarian follicles will fail to be selected for ovulation and instead undergo atresia[17], with as little as 1% of follicles ovulating an oocyte[18]. The oocytes that are selected to continue growth and maturation, bypassing atresia, must next gain the ability to complete meiosis and prepare the resources needed for early embryonic development.

As the oocyte grows, its volume and diameter increase, with fully grown oocytes reaching 110-130 µm in diameter[19]. Aguila and colleagues suggest that cattle oocytes reach peak developmental competence at a diameter of 120 µm[20]. Another study confirmed this theory by showing that oocytes with large cytoplasmic volumes, or 120-130 µm in diameter, have a greater capacity for completing the initial cell division and producing a normal or hatched blastocyst than oocytes with small volumes, or less than 120 µm in diameter[19]. The increase in ooplasm volume and oocyte competence is likely due to the improved stockpiles of molecules and cellular machinery present in fully grown oocytes. During its growth phase, the oocyte's chromatin is decondensed and actively transcribing the maternal stockpiles of mRNAs, but as growth persists, the chromatin becomes increasingly condensed[21,22]. The mRNAs transcribed during the growth phase, before global chromatin condensation, reside within the oocyte cytoplasm in mitochondria-associated compartments until their translation[23]. It is also theorized that the granulosa cells of the ovarian follicle, in direct contact with the oocyte, may aid in establishing the maternal stockpiles by transporting additional mRNAs and proteins to the oocyte's cytoplasm through transzonal projections[24]. These mRNAs have short poly-A tails that allow them to remain stable and dormant until their recruitment for translation, which occurs by poly-A lengthening and transport to the ribosome[25]. The global shortening of mRNA poly-A tails occurs throughout oocyte maturation, in preparation for embryogenesis, as germinal vesicle stage oocytes contain higher levels of polyadenylated mRNAs than those at metaphase II[26].

In conjunction with increased oocyte diameter, the mitochondria located in the oocyte cytoplasm undergo multiplication during the growth period, with fully grown oocytes containing the transcripts of nearly 10,000 protein coding genes[11] and more than 1,000,000 copies of mitochondrial DNA[27,28]. These mRNAs and organelles will provide the basis for cellular survival to the early embryo. Therefore, the proper stockpiling of the ooplasm with resources is vital for successful preimplantation development. Expectedly, oocytes that have not completed growth are less competent to produce a blastocyst than their fully grown counterparts[28-34]. Both the mitochondrial DNA[28,35,36] and maternal mRNA[28,37,38] content of oocytes has been correlated with their developmental capability to produce a blastocyst, and decreases in these stockpiles result in poor embryo competency, possibly due to their lack of the necessary cellular contents for successful development.

Interestingly, recent literature has shown that not fully grown oocytes contain more copies of mitochondrial DNA, and likely more mitochondria[28]. It is unclear why immature oocytes would contain more mitochondria than those that are fully grown. These findings are incongruent with other reports showing greater mitochondrial DNA copy number in germinal vesicle, or immature, oocytes versus metaphase II, or fully grown, oocytes[27]. This may be due to the increased energetic needs of the transcriptionally active immature oocyte. It is possible that increased numbers of mitochondria present in immature oocytes may negatively impact embryonic development if selected for culture *in vitro*. This could occur through the overproduction of reactive oxygen species, a byproduct of the mitochondria's energetic processes[39], which cause cellular damage and embryo arrest when in excess[40,41].

Fertilization

Following the luteinizing hormone (LH) surge, but prior to ovulation, the oocyte begins the process of maturation, where it obtains its competence to resume meiosis, arrest at metaphase II, undergo fertilization, and begin a mitotic cell cycle. The process of maturation includes many molecular and structural alterations, including changes in organelle localization[42,43], cytoskeleton organization[44], and gene expression[11] of the oocyte. Additionally, following fertilization and resumption of meiosis, the oocyte must complete the unsymmetric division from one diploid cell to two haploid cells, or the large mature oocyte and the small discarded polar body. If the meiotic spindle is improperly placed away from the periphery of the ooplasm, the polar body will contain more cytoplasmic volume than necessary. Consequently, the early embryo may not have the materials needed to persist until genomic activation when it no longer relies on the maternal storages. Interestingly, polar body size may be correlated with oocyte developmental competence[45,46], but the rarity of large polar body extrusion makes this abnormality difficult to study[47]. Failure of an oocyte to equally segregate its chromosomes between the polar body and mature oocyte results in non-mosaic aneuploidies[48] in the conceived offspring which may lead to embryonic arrest[49] or birth defects, should the pregnancy proceed to term.

Fertilization of the mature oocyte by a capacitated spermatozoa involves many processes crucial for the commencement of early embryo development. As the spermatozoa contacts the oocyte's zona pellucida, hydrolytic enzymes are released and the spermatozoa bores into the perivitelline space and through the plasma membrane[50]. After the spermatozoa fuses with the oocyte, it releases its nucleus and spermatozoa-specific factors into the cytoplasm, including its centriole, which will serve as the mitotic center for the initial zygotic cleavage divisions[51]. After entry into the cytoplasm, the spermatozoa factor, Izumo, binds to the oocyte factor, Juno. Additionally, phospholipase C zeta is released. Together, these factors trigger cytoplasmic calcium oscillations and the calcium-dependent exocytosis of the oocyte's cortical granules, also known as the cortical reaction[52]. This reaction is characterized by structural and chemical modifications to the oolemma which render it unenterable by additional spermatozoa[53]. Calcium oscillations also trigger the activation of the oocyte[54]. Interestingly, the profile of an activated oocyte's calcium oscillations may influence many aspects of embryonic development far past the polyspermy block, including the regulation of mitochondrial activity[55] and maternal mRNA recruitment[56]. Failure of the oocyte to initiate and successfully block additional spermatozoa from depositing their nucleus in the cytoplasm results in aneuploidies, multipolar mitotic spindle formation, and might lead to morphological errors, like direct zygotic division to more than two cells. Additionally, the entry of the spermatozoa activates the oocyte to complete metaphase II of meiosis[57]. Development will cease to occur if spermatozoa do not activate the oocyte. Total fertilization failure occurs in approximately 20% of cattle oocytes fertilized *in vitro*[58]. These failures may be directly caused by lack of oocyte activation resulting from a lack of, or abnormally functioning, phospholipase C zeta[59], Juno[60], or Izumo[61].

Early embryonic development

Following spermatozoa and oocyte fusion and activation, the gamete genomes migrate toward each other and unify to establish the embryonic genome. The genomic content of the oocyte, inherited by the early embryo, contains a disproportionate amount of methylation when compared to other cell types. Additionally, gamete and embryonic genome methylation is highly dynamic in comparison to the established and stable methylation of mature somatic cell genomes[62]. The methylation of gamete genomes is sex-specific and functionally non-equivalent, with differentially methylated regions. Both maternal and paternal epigenetic imprinting are required for successful embryonic and fetal development. Particularly, gynogenetic or androgenetic embryos show placenta or fetal development errors, resulting from a lack of maternal or paternal methylation[63]. Shortly after its establishment, the embryonic genome undergoes intense epigenetic reprogramming, or the removal and addition of methylation until approximately the late blastocyst stage in mice[64], when the epigenome becomes stable. The reestablishment of methylation imprinting does not occur until the formation of primordial germ cells in the fetal gonads[65]. The epigenetic remodeling of the early embryo occurs through a combination of active demethylation, or the cleavage of methyl groups by enzymes, and passive demethylation, or dilution, achieved by replication of DNA without maintenance of the methyl tags on the newly synthesized sequences[66,67]. Irregular epigenetic remodeling following successful fertilization, commonly seen in cloned embryos[68,69], results in decreased developmental capacity. Interestingly, many studies involving embryos produced, or children conceived, by IVP are affected by irregular epigenetic profiles that persist into adulthood and may cause disease or reproductive complications[70,71].

The zygote must next complete a series of cell divisions, using a modified cell cycle characterized by an extraordinarily fast S-phase and the absence growth phases, resulting in each division producing two sister blastomeres containing only half of the founding cytoplasmic volume[72,73]. This process, and particularly the first mitotic division, is highly error prone and lends to chromosome segregation errors[74,75]. These errors result in mosaic aneuploid embryos, and depending on the severity of the aneuploidy, embryos may arrest their development. The transition of the zygote from a meiotic to a modified mitotic cell cycle involves the cyclic activation and deactivation of mitosis promoting factor (MPF), which consists of two subunits, cyclin B and cyclin dependent kinase (CDK)[76]. This continues until the embryo has completed three cleavage divisions to become an eight-cell embryo, or around 48 hours post-fertilization in cattle[77]. Many morphological errors have been observed during these cleavage divisions, such as direct divisions to greater than two cells, reverse or incomplete divisions, and asymmetric cell division, which have negative implications for early development and may be hallmarks of aneuploidies within the embryonic cells, likely caused by multipolar mitotic spindle formation, poor spindle placement, or cell cycle checkpoint errors[78-80]. Additionally, cleavage divisions are typically symmetrical and result in two sister blastomeres of equal size. When a zygote divides asymmetrically, its development potential is reduced [81], likely from the unequal sharing of cytoplasmic contents between sister blastomeres.

As the maternal stores are depleted through their transcription and the dilution of cytoplasm in each additional cleavage division, the early embryo must gradually activate its own genome over time and transition from the highly differentiated genome of the sperm and oocyte to a totipotent cell type[82,83]. This includes the further clearance of maternal products, chromatin remodeling, and accompanying epigenetic reprogramming of the transcriptionally repressed embryonic genome. The exact trigger of the transition from oogenetic to embryonic supported development is unknown, though theorized mechanisms include egg activation serving as a biological timer through expression transcriptional activators and the increase of nucleus to cytoplasm ratio in the dividing blastomeres[73,84]. Genomic activation entails both major and minor waves, with the minor wave occurring around the 4-cell stage, and the major wave occurring between the 8-cell and 16-cell stages in cattle[85], though exact characterization is difficult due to the presence of maternal mRNAs within the cytoplasm that are further expressed by the embryonic genome. The minor genome activation wave involves the epigenetic clearance and transcriptional availability of select genes, while the major wave is characterized by global epigenetic reprogramming and amplified transcriptional activity. Failure to activate the embryonic genome in a timely manner will always result in death of the early embryo, as it would be unable to support its growth, or cell plasticity, without transcription and translation of gene products important for its baseline biological processes.

The maternal to zygotic transition is followed by the genetic establishment of blastomere totipotency, then the specification of two pluripotent cell lineages by the blastocyst stage. Inner cell mass versus trophectoderm specification occurs in the morula stage when the embryonic cells are tightly gathered, and apical and basolateral domains are established[86], though some blastomeres may have developmental bias toward one lineage as early as the 4-cell stage[87]. This decision may be controlled by the sensing of cell position by bound or unbound cell-cell junctions, followed by recruitment of F-actin to form the apical actin network and mechanotransducers for the position-dependent activation of the HIPPO signaling pathway[86,88,89]. In the HIPPO pathway, the apical actin network in the apical cells sequesters angiomotin (AMOT), while it remains active in the basolateral cell cytoplasm. In the basolateral cells, AMOT becomes phosphorylated to activate large tumor suppressor kinase 1 and 2, which phosphorylates the yes-associated protein (YAP) and its transcriptional co-activator TAZ. The YAP/TAZ complex then loses the ability to traverse the nuclear membrane and the expression of inner cell mass specific genes persists. Conversely, in the apical cells, AMOT is unable to become phosphorylated to activate LATS 1 and 2, subsequently blocking the phosphorylation of YAP/TAZ. The unphosphorylated YAP/TAZ then cross the apical cell's nuclear membrane and act as a transcriptional activator with the transcriptionally enhanced associated domain (TEAD) for trophectoderm lineage specific gene expression[90-92]. Aberrant gene expression during the first lineage specification event in the preimplantation embryo results in poor formation or a lack of the inner cell mass or trophectoderm populations. For example, knockout of TEAD, the major controller of trophectoderm specific gene expression, halts cell lineage specification and embryonic viability in mice[93].

The lineage-specific expression of developmentally important genes is vital for the survival and continual development of the preimplantation embryo, as these cell populations establish the placenta and fetal tissues. The coordinated expression of the genes *CDX2, NANOG, and OCT4,* directed by the hippo signaling pathway, are required for effective lineage segregation and maintenance of inner cell mass and trophectoderm lineages in the murine[94] and cattle models[95]. In the cattle trophectoderm cells, *CDX2* regulates a variety of other trophectoderm, and pluripotency associated genes, such as *NANOG* and *GATA3*[96-98]. Similarly, in the cattle inner cell mass cells, *OCT4* participates in the maintenance of cell pluripotency through their individual and dual action as transcription factors[99] promoting the expression of additional genes, such as NANOG[100] and other pluripotency factors[101]. It is not until the late blastocyst stage that the lineage specific expression and localization of CDX2 in the trophectoderm and OCT4 in the inner cell mass cells occur in mice[102], with *CDX2* acting to repress *OCT4* expression in the trophectoderm[103]. Conversely, in cattle, *OCT4* and *CDX2* are co-expressed in the inner cell mass and trophectoderm, without repression of *OCT4* by *CDX2*[104]. In the murine model, *OCT4* null embryos fail to establish the inner cell mass lineage and *CDX* null embryos fail to establish trophectoderm[105]. Similarly, this was recently shown by *OCT4* null cattle embryos, which failed to produce a visually observable or dense inner cell mass[101]. The study of key developmental genes such as *OCT4*, *NANOG*, and *CDX2* may provide greater insight to the coordinated gene expression necessary for preimplantation development, and how deviations in the expression of these genes may impact embryo competency to form a blastocyst, establish and carry out pregnancy, or produce healthy offspring.

As the first cell lineage differentiation is underway, a coupled series of morphological changes occur to establish the first embryonic cavitation. This involves the maturation of cell-cell junctions between neighboring cells and the expanding of apical actin networks which zipper together with that of neighboring cells to establish tight adherens junctions[106-108]. Next, water is passively transported across the trophectoderm cells by aquaporins and into the intracellular spaces through the active influx of sodium through sodium-potassium pumps[109,110]. The water collects in the center of the embryo and physically pushes the cells outward into a spherical organization, while applying hydrostatic pressure on the zona pellucida. This outward force, along with the release of digestive enzymes by the embryo[111-113] and uterine environment[114,115] allow the embryo to escape from its zona pellucida and eventually contact the maternal endometrium. Incomplete or abnormal formation of the blastocoel cavity is shown to result in abnormal development or embryonic lethality, possibly by failure to hatch or poor cell to cell communication. It has been suggested that filopodia-like projections of the trophectoderm cells' cytoplasm may aid in breeching of the zona pellucida through additional mechanical force and delivery of proteolytic factors[116,117]. Any deviation of an embryo from the standard path of development, from formation to hatching, is expected to be detrimental to its viability.

The limitations of cattle *in vitro* embryo production

There are many obstacles limiting the success of IVP in a production or research setting, including poor blastocyst development and post-transfer pregnancy rates of IVP embryos when compared to embryos conceived and developed in the uterus (termed *in* *vivo* derived embryos). More specifically, there are large losses seen *in vitro*, with only 80-90% of collected oocytes progressing to metaphase II following *in vitro* maturation[118,119], 80% successfully completing fertilization and the first cleavage event, and 30-40% producing a blastocyst[58]. This equates to an average of 60-70% losses across a typical IVP program. Furthermore, of the few embryos that develop to the blastocyst stage, only a small proportion will be candidates for transfer into a recipient. Following transfer, only ~29% of IVP cattle embryos will establish pregnancy and give rise to live offspring, compared to ~45% of *in vivo* derived and transferred embryos[120,121] or ~55% of pregnancies resulting from one round of estrus synchronization and artificial insemination service[122]. The large deviation of embryo development toward early demise seen *in vitro* may be explained by inadequate culture conditions or poor gamete quality that result in unhealthy or unviable growth. Despite improvements in current practices, embryos produced *in vitro* are known to be of lesser quality than those produced *in vivo.* This is likely because gametes and embryos are removed from the reproductive tract, which causes abnormal divergences in biological processes. These abnormalities may be triggered by cellular stress, stimulated through unavoidable light exposure, temperature, and even gas concentration fluctuations during embryo handling. Additionally, *in vitro* culture conditions cannot perfectly mimic the dynamic uterine environment, which may impact embryo growth and development[123]. Examples of abnormal developmental processes seen in IVP embryos include aberrant gene expression[124], metabolic differences,[125] genomic instability[126], reduced intercellular communication[127], and even ultrastructural variations[128,129]. It is undoubtable that these abnormalities are a direct cause of the widespread pregnancy failure seen following transfer of an IVP embryo to a recipient.

Molecular deviations of *in vitro* produced embryos

Genomic integrity is essential for the function of all cells, especially those comprising the early embryo, as the these will give rise to all other cell types in the organism. Poor genome integrity, or increased genome instability, is characterized by any genomic sequence changes or chromosomal aberrations, including insertions, deletions, translocations, and even whole chromosome duplications or losses[130]. The change of a nucleotide sequence may impact its transcription, translation, and protein characteristics, which may impact downstream biological functions. Of all chromosomal aberration types, aneuploidy is the leading category observed in preimplantation embryos[131]. Using single blastomere sequencing of cattle embryos, one group indicated that approximately 85% of embryos produced from *in vitro* maturation and culture are aneuploid, compared to only 19% of *in vivo* derived embryos[132]. Other groups have found that approximately 60% of embryos produced from *in vitro* matured oocytes contain the improper number of chromosomes, compared to 33% of embryos produced from *in vivo* matured oocytes, though this study reported a small sample size and may not be representative[133]. This suggests that *in vitro* maturation may lead to chromosome segregation errors in the zygote and early embryo, leading to aneuploidies. The mechanism in which *in vitro* maturation may lead to chromosomal aberrations is unclear, and further investigation is needed to understand these observations. Though some aneuploidies are not lethal, others result in early embryonic demise. It has been estimated that only 5.8% of transferred aneuploid cattle blastocysts will establish pregnancy and 5% will produce live offspring, compared to 59.6% and 46.7% for euploid embryos, respectively[134]. The increased rate of chromosomal aberrations seen *in vitro* likely contribute to early embryonic losses seen during culture or post-transfer to a female.

Tightly coordinated, dynamic, and stage-specific gene expression following genome activation is required for successful early embryo development in all cases. Interestingly, *in vitro* and *in vivo* derived embryos have different transcript[135-139] and protein[140] expression profiles, even across various stages of development. This deviation from normal embryonic gene expression occurs as soon as 10 hours following placement into *in vitro* culture[141]. Distinct embryonic gene expression profiles are also seen when comparing embryos produced across different culture systems[142], and interestingly, these changes are greater in male embryos[143]. These findings point to the removal of gametes and embryos from the reproductive tract, in addition to our current inability to perfectly mimic the maternal environment, as the culprit of abnormal embryonic gene expression seen in IVP embryos. Furthermore, proper methylation and demethylation of the embryonic genome is vital for the stage specific and dynamic control of gene expression required for the progression of development by the early embryo[144]. Methylation is a biochemical alteration to the structure of the DNA sequence, typically in the form of a methyl group added to a nucleotide. Most methylation occurs on CpG islands, or on a cytosine neighboring a guanine, and functions in the control of sequence availability or recruitment of anti-transcription factors[145]. Interestingly, when *in vivo* produced embryos are placed into *in vitro* culture around the anticipated time of their embryonic genome activation, changes in the methylation profile observed in their blastocysts differed from those that were retained *in vivo*[146]. This group also indicated a decrease in global methylation resulting from *in vitro* culture, which aligns with previous reports that *in vitro* cattle embryos contain more total transcripts, pointing to increased transcriptional activity, than those produced *in vivo*[147]. Data also indicates that these variations in genome methylation affect developmentally important genes, which may influence embryo viability and offspring health[148-150].

Developmental deviations of *in vitro* produced embryos

In addition to precise genomic control and proper chromosomal segregation, the early embryo must successfully complete a series of developmental events in a timely manner. Many groups have indicated that an optimal developmental schedule does exist in embryos across species, with prolonged development resulting in decreased viability[151-153]. Historically, faster cleaving embryos were seen as more competent than their slowly cleaving counterparts[153-155]. Further investigation has revealed that a moderate developmental schedule, versus one that is delayed or precocious, best supports optimal preimplantation development[152,156]. Embryos of varying developmental speeds also show different gene expression profiles[157]. Interestingly, *in vitro* culture delays preimplantation development[158]. In fact, this delay can persist for as long as 18-24 hours[159,160], and likely impairs embryo viability. Interestingly, IVP embryos also show increased metabolic activity when compared to their *in vivo* counterparts[161]. These findings agree with the "quiet embryo" and "goldilocks zone" hypotheses[162], that state the optimal range of embryonic metabolism is neither high nor low, but intermediate, and the observation that embryos produced *in vitro* are less competent than those produced *in vivo*[163]. The increase in embryo metabolism and slowing of developmental kinetics may be a consequence of utilizing a static culture environment that does not adequately support the dynamic energetic needs of everchanging cell types comprising the embryo.

Blastocyst hatching is a vital prerequisite for uterine attachment and pregnancy establishment. Of those embryos cultured *in* vitro that produce a blastocyst and are selected for embryo transfer, only ~29% will produce a live calf[120], which may be a result of the poor hatching competence of *in vitro* produced embryos. Following transfer, the embryo must quickly escape its zona pellucida and establish maternal contact. This involves mechanical and chemical forces exerted by the embryo and uterine environment that weaken the embryo's glycoprotein coating until its rupture and escape[164]. Low rates of cattle blastocyst hatching are reported *in vitro*, with nearly 60% of zygotes failing to produce a hatched blastocyst by day 8 to 12 across various culture systems[165-170]. Remarkably, almost 70% of murine blastocysts produced *in vivo* failed to complete hatching by day 5[171], comparable to day 9 in cattle[172]. Additionally, assisted hatching significantly improves pregnancy rate following transfer of IVP cattle embryos[173]. These data point to a reduction in blastocyst hatching potential when embryos are produced *in vitro.* It is likely that the absence of maternal enzymes, and erroneous embryonic gene expression and developmental schedules seen in IVP embryos, described previously, directly impacts hatching ability and pregnancy establishment following transfer to a recipient. Overall, there are many factors influencing the growth of an embryo *in vitro* or *in vivo*. The stage specific completion of developmental events is vital for successful production of live offspring, and abnormalities caused by *in vitro* culture are a major limitation for the effective production of embryos for production or research purposes.

Understanding and predicting embryo development in vitro

An important aspect of effective IVP is the selection of the most competent embryos to maintain in culture or utilize for cryopreservation and transfer to a surrogate. When embryos are cultured in cohorts, there is a sharing of autocrine and paracrine factors throughout the medium[174,175]. Degenerate oocytes and embryos secrete molecules that negatively impact the development of their neighbors, while healthy embryos support their cohort's development[176]. In fact, high quality cattle embryos have even been used as companions for single embryos of difficult-to-culture species, such as felines, and this co-culture supports embryonic development through sharing of growth factors[177]. The efficient selection and removal of degenerate or lesser competent embryos from the growth pool may improve cohort development[178,179]. Likewise, the enhanced selection of competent blastocysts for cryopreservation and transfer to a surrogate improve subsequent pregnancy rates[180]. This emphasizes the necessity of embryo selection techniques in clinical embryology and for livestock production purposes.

Developmental kinetics

In hopes to improve the selection of competent embryos for culture and transfer, many groups have characterized approaches for predicting and understanding embryo health[181]. The most common method for determining embryo quality is through morphological observations. It is well documented that the incidence of slow cleavage[182], direct cell divisions[183], reverse cell divisions[184], fragmentation[185], and apoptosis[186] are correlated with poor embryonic development and pregnancy outcome. Some early studies focused on the timing of the first embryonic cell division and found that slower cleaving embryos are less competent for producing a blastocyst[29,154,187] or establishing pregnancy following transfer[188]. Other groups have investigated the components of the spent culture medium as a non-invasive screening of development potential[189,190]. Of particular use in clinical embryology is preimplantation genetic testing for chromosomal aberrations, which may be used for selecting against aneuploid embryos for transfer. Interestingly, retrospective analysis of clinical embryology data suggests that excluding embryos presenting these abnormalities does not significantly improve pregnancy outcome following embryo transfer, unless the mother is of advanced age[191]. Therefore, this method may not be applicable for selection of embryos with the greatest developmental competence to maintain in culture or for transfer. The implementation and constant evolution of embryo selection is paramount for the success of IVP and transfer programs.

Early studies of developmental kinetics were limited in the number of observations made, as each one would require the exposure of embryos to suboptimal culture conditions, like light, temperature, and gas fluctuations, that trigger embryonic stress responses[192]. Recent technological advancements have produced incubators equipped with microscopes and cameras. Time-lapse incubators provide extremely detailed observations of embryo development without removal from the culture environment, which has improved our understanding of embryo development and may provide enriched selection for embryo transfer in clinical settings[193,194]. The popular use of time-lapse incubators in clinical embryology or research studies, coupled with recording of genetic testing or pregnancy outcome, has provided scientists opportunities to identify morphological and kinetic parameters predicative of an embryo's competence to produce a blastocyst[195-197], its ploidy[198-200], and even its ability to establish a successful pregnancy[201-203], though there is much improvement to be made in commercial algorithm accuracy[204]. Unfortunately, reproducibility of results between clinics has been challenging[205], and therefore selection of embryos for transfer by time-lapse data and prediction algorithms has not been widely implemented in IVP programs. The advancement of these tools may someday result in automated embryo selection algorithms, which could improve the outcome of IVP for transfer and provide new outlooks on preimplantation embryogenesis.

RNA Sequencing

Gene expression analysis plays a pivotal role in understanding the intricacies of embryo competency, providing valuable insights into the molecular mechanisms underlying early developmental processes. By examining the dynamic patterns of gene activation and suppression, researchers can identify key genetic signatures associated with embryonic competence. This information is crucial for understanding the factors that contribute to successful embryo development, implantation, and ultimately, a healthy pregnancy. Through technologies like RNA sequencing[206] and microarray analysis[207], scientists can assess the transcriptomic profile of embryos, allowing them to pinpoint specific genes and pathways that correlate with developmental competence[208]. While microarrays were a revolutionary technology for transcriptome analysis, RNA-Seq offers several advantages that have made it a preferred choice in many studies, such as the interrogation of global gene expression and improved sensitivity[209]. Through RNA sequencing, it has been shown that the first cell fate inclination occurs at the 2-cell stage in mice[210], the embryo and endometrium form co-expression networks at the time of implantation[211], and *in vitro* embryo production causes abnormal expression patterns in the embryo and endometrium across various days of pregnancy[212]. This knowledge not only enhances our understanding of the molecular events governing embryogenesis but also holds great promise for predicting embryo viability in assisted reproductive procedures. By identifying biomarkers indicative of optimal embryo development, we may have the ability to make more informed decisions during IVP that improve developmental outcomes.

Removal of gene function

The removal of gene function during preimplantation embryogenesis is a popular method for understanding the mechanistic role and interaction of the target gene in biological pathways. Through targeted genetic manipulations, researchers can selectively disable specific genes to observe the resultant effects on the developing embryo. This approach allows scientists to delineate the functions of individual genes, uncovering their roles in key developmental processes. By systematically deleting or knocking out genes associated with various embryonic stages, researchers can identify critical regulators, signaling pathways, and molecular cascades that orchestrate early development. The insights gained contribute to our comprehensive understanding of normal embryonic development and the consequences of genetic aberrations. Many methods exist for this purpose, but the most popular method is by introduction of CRISPR-Cas DNA endonuclease mRNAs or ribonucleoproteins for the deletion of targeted sequence sections[213]. For removal of gene function without genome sequence modification, siRNAs[214] or CRISPR-Cas RNA endonucleases[215] have been utilized. Studies of gene function typically involve sequence deletions, gene and protein expression analyses, and phenotypic observations. The limitation of using CRISPR-Cas in early embryos is the poor efficiency of sequence deletions and the presence of maternally inherited mRNAs of the target gene, which may influence knockout phenotype. New methods have been suggested in attempts to overcome these challenges[216]. The technologies described above, coupled with molecular diagnostics, are a powerful tool for understanding embryogenesis and improving *in vitro* embryo production.

Concluding remarks

The development of a pluripotent and multicellular embryo from two highly differentiated gametes is a complex and stepwise process. Scientists have long sought to understand these processes, and though progress has been made, much is left to discover. By broadening our understanding of ‘normal’ embryo development, we may improve assisted reproduction technologies for couples, the production and sustainability of livestock production operations, and the study of preimplantation embryogenesis. The gaps in our knowledge of early development directly reflect the suboptimal culture conditions and developmental hinderance seen *in vitro*. This diminished competence is a profound obstruction to the widespread implementation of assisted reproductive technologies in the livestock industry. The differences between embryos produced naturally or *in vitro* have been well documented and range from variations in epigenetic profiles to disparities in cell structure. The biological differences seen between IVP and *in vivo* derived cattle embryos may be a limitation for the study of ‘normal’ preimplantation embryogenesis, as resulting data may be influenced by culture-driven divergences in their development. Application of embryo selection methods throughout IVP may provide advantages for commercial or research embryo production. As *in vitro* produced embryos face unique challenges when compared to their *in vivo* counterparts, only the most developmentally capable individuals will succeed in their development, and removal of subpar embryos from the growth or transfer pool results in improved outcomes. Researchers have long sought the best method for replication of *in vivo* development *in vitro*, and enhanced replication may allow for lesser competent embryos to succeed, but many improvements are necessary before this goal may be accomplished, as biological system are difficult to imitate within a laboratory setting.

There are many gaps in our understanding of early embryonic development and methods to select the most competent embryos for maintaining in culture or as candidates for transfer into a recipient. In the following chapters, I will discuss research that fills some of these gaps in our knowledge. In the second chapter, we compared two methods of selection for oocyte or embryo competency to produce a blastocyst. We hypothesized that the combined use of brilliant cresyl blue staining of oocytes and sorting of embryos by the timing of their cleavage would result in improved development to the blastocyst stage. Our objectives were to compare these methods alone and in conjunction, then compare developmental outcomes between selection methods. In chapter three, we identified oligonucleotides for efficient sexing of cattle embryonic tissues by PCR. Our objective was to design oligonucleotides of greater specificity using a complete version of the cattle Y chromosome, which was previously unavailable. These oligonucleotides provide researchers with a method to observe differences due to sexual dimorphism in their data with greater specificity. In the third chapter, we designed a new method for knocking out a gene and knocking down its transcripts with CRISPR-Cas in cattle zygotes. We targeted the gene *OCT4* and hypothesized that the ablation of its function would result in decreased development to the blastocyst stage. Our objectives were to design a method for highly efficient gene and transcript deletion in zygotes, knockout the gene *OCT4*, and observe its impact of embryonic development. This study provides researchers with a method for efficient knockouts and knockdown of genes and their products in zygotes, which can be used for studying the function of specific genes in preimplantation development. This study also provides information on the effect of *OCT4* deletion on the development of cattle embryos.

CHAPTER 2

Cleavage kinetics is a better indicator of embryonic developmental competency than brilliant cresyl blue staining of oocytes

Abstract

In vitro production of embryos (IVP) is a valuable technology to produce embryos of high genetic value. Despite advances in IVP, the efficiency of culture systems remains low. One method to increase IVP success is the early selection of oocytes or embryos that may have greater developmental potential. The objective of this study was to investigate two methods of selection, namely brilliant cresyl blue (BCB) staining and cleavage kinetics, both individually and in conjunction, for improved developmental outcomes in vitro. We hypothesized that a synergistic use of both BCB staining and cleavage kinetics would result in identification of embryos of greater developmental potential. The selection of oocytes by BCB staining does select for those oocytes with higher developmental potential, as noted by a greater blastocyst development between BCB positive (32.6%) and BCB negative (22.0%) on day 8 post-fertilization. However, the utilization of BCB staining and cleavage kinetics in tandem resulted in a complete masking of the effect observed when using BCB alone. We obtained the highest proportion of blastocyst development per selection group using cleavage kinetics alone, in which 53.1% of embryos grouped as Fast produced a blastocyst, which was significantly different from the three other groups (Fast+, Slow, not cleaved). We observed, however, that the separation of embryos by cleavage kinetics did not predict their survival to cryopreservation. In conclusion, in standard culture systems, cleavage kinetics is an effective method for the selection of embryos with increased developmental potential to develop blastocysts, however, it may not be effective to select healthy embryos for transfer following cryopreservation.

Introduction

Only a fraction of oocytes placed into IVP systems will develop into a viable blastocyst that can survive cryopreservation and result in a successful pregnancy. Though it has been shown that approximately 90% of bovine oocytes placed into IVP systems will complete fertilization and their first cleavage event to the two-cell stage, only 30-40% of zygotes will produce a blastocyst[38,58]. Even fewer of those blastocysts will be classified as grade one[217], which poses limitations to producers utilizing embryo transfer programs, as embryo quality is correlated to successful cryopreservation and post-transfer pregnancy rates[163]. Typically, embryo transfer programs select oocytes and their resultant embryos for transfer into a recipient based on morphology alone. It has been suggested that utilizing different methods of selection either at the oocyte or embryo stage could allow for an increase in development, cryopreservation survival, and pregnancy success[20,163,218].

Brilliant cresul blue staining is a method for the selection of oocytes in vitro which have completed their growth *in vivo*. BCB is a supravital stain with the unique ability to select for oocyte developmental competence and cytoplasmic maturation while posing no adverse effect on developmental outcome[33]. The completion of cytoplasmic maturation is an important factor for the acquisition of oocyte developmental competence and encompasses many processes which support successful nuclear maturation, fertilization, cleavage events, and blastocyst formation[219]. Additionally, the completion of cytoplasmic maturation is coupled with a decrease in glucose-6-phosphate dehydrogenase (G6PDH) enzymatic activity[31]. Reduced molecules of BCB stain are easily oxidized to their colorless form by the enzyme G6PDH, which is present in high concentrations in immature oocytes which have not yet completed growth and cytoplasmic maturation[31]. Those oocytes that have completed cytoplasmic maturation and are fully grown contain low levels of the enzyme G6PDH and retain the stain in its reduced, blue form[31]. While the assessment of oocyte developmental competence by BCB staining can improve blastocyst yields[31,32], these improvements are limited.

The cleavage kinetics of early embryos can serve as a non-invasive marker for embryonic development potential. The timing of the first cleavage event influences many factors of early embryonic development in many species, including cows, mice, and humans[220-222]. Length of time from insemination to first embryonic cleavage can affect embryonic cell number and morphology, both of which are crucial to the health and development of the embryo[223]. The timing of embryo cleavage in cattle has been further investigated and showed that early cleaving embryos presented improved blastocyst yield, cryotolerance, and pregnancy rates when compared to later cleaving embryos[154,220,224-226].

Strategies for the selection of putatively competent oocytes or embryos utilized in tandem with IVP have been studied independently; however, the consequence of multiple selection strategies on blastocyst yields remains unknown. Here, we hypothesized that the selection of oocytes by BCB staining, followed by a selection of embryos by cleavage kinetics, would increase blastocyst yield when compared to either selection approach employed individually. In this study, we aimed to test whether (i) selection of oocytes, (ii) selection of embryos or (iii) a synergistic use of both methods would produce different blastocyst yields in an in vitro production system.

Materials and methods

All reagents were purchased from Sigma-Aldrich unless otherwise specified.

Collection of cumulus-oocyte complexes (COCs)

Bovine ovaries were obtained from a slaughterhouse and transported to our laboratory in saline (0.9% NaCl) and 1x antibiotic antimycotic solution. Using a regulated vacuum system coupled to a collection tube containing BoviPlus oocyte washing medium with BSA (Minitube, Verona, WI) (OCM) and an 18g needle, we aspirated ovarian follicles 3 to 8mm in diameter to retrieve cumulus oocyte complexes (COCs). Cumulus oocyte complexes were then placed through two consecutive washes in OCM. If COCs were to undergo BCB staining, as described below, the staining process was completed at this timepoint. Only COCs with heterogenous cytoplasm and three or more layers of compact cumulus cells surrounding the zona pellucida were *in vitro* matured.

In vitro maturation (IVM)

All following procedures and media formulations were adapted from a previously identified standard *in vitro* production of embryos protocol[227]. COCs were first placed through three washes of oocyte maturation medium (OMM) (TCM-199 + Earle’s Salts (Thermofisher Scientific, Waltham, MA) supplemented with 5 µg/ml Folltropin-V (Vetoquinol USA, Fortworth, TX), 0.05 µg/ml human recombinant epidermal growth factor (Thermofisher Scientific, Waltham, MA), 50 µg/ml gentamycin, 1 mM GlutaMAX

(Thermofisher Scientific, Waltham, MA), 22 µg/ml sodium pyruvate, and 10% HyClone Fetal Bovine Sera Defined (Hyclone Laboratories, Logan, UT)). We then placed COCs in 50µl droplets of OMM covered in mineral oil. All COCs were incubated at 38.5ºC in a humidified atmosphere at 5% CO_2 for 22-24 hours. The COCs separation into different groups is described below.

In vitro fertilization (IVF)

COCs were placed through three washes of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered synthetic oviductal fluid (SOF-HEPES) (10mM HEPES-TL (Caisson Laboratories, Smithville, UT) supplemented with 3 mg/ml fraction V BSA, 0.2 mM sodium pyruvate, 7.5 µg/ml gentamicin, 4.92 mM sodium DL-lactate, 100 mM sodium chloride, 23.27 mM sodium bicarbonate, 1.09 mM calcium chloride dihydrate, 1.1 mM potassium phosphate monobasic monohydrate, 6.64 mM potassium chloride, and 0.45 mM magnesium chloride hexahydrate) and two washes of synthetic oviductal fluid fertilization medium (SOF-FERT) (6 mg/ml essentially fatty acid free BSA (Millipore Sigma, Rockville, MD), 22 µg/ml sodium pyruvate, 5 µg/ml gentamicin, 20 µg/ml heparin, 1 mM caffeine (VWR, Philadelphia, PA), 5.3 mM sodium DL-lactate, 107.7 mM sodium chloride, 25.07 mM sodium bicarbonate, 1.17 mM calcium chloride dihydrate, 1.19 mM potassium phosphate monobasic monohydrate, 7.16 mM potassium chloride, and 0.49 mM magnesium chloride hexahydrate) before transfer into a final plate of SOF-FERT.

Straws containing frozen semen were prepared by following a previously described protocol [227] with a minor modification. We used BoviPure and BoviDilute (Nidacon International, Molndal, Sweden) to separate spermatozoa by a density isolation gradient. Spermatozoa were introduced to COCs in our final fertilization plate at a concentration of 1,000,000 spermatozoa/ml, as described elsewhere[228]. Plates containing COCs and spermatozoa were incubated for 16 to 18 hours under the same conditions described for IVM.

In vitro culture (IVC)

Presumptive zygotes were denuded of their cumulus cells by vortexing for 5-7 minutes, then transferred through three washes of SOF-HEPES. Presumptive zygotes were then placed through two washes of synthetic oviductal fluid culture medium (SOF-

BE1) (4mg/ml essentially fatty acid free BSA, 4.92 mM sodium DL-lactate, 100 mM sodium chloride, 23.27 mM sodium bicarbonate, 1.09 mM calcium chloride dihydrate, 1.1 mM potassium phosphate monobasic monohydrate, 6.64 mM potassium chloride, and 0.45 mM magnesium chloride hexahydrate, 1 mM GlutaMAX, 0.37 mM sodium pyruvate, 1x MEM-non-essential amino acid solution, 1x BME-essential amino acid solution, 23.3 µg/ml gentamicin, 0.46 mM sodium citrate, and 2.56 mM myo-inositol) and were then placed in groups of 20-30 in 50µl droplets of SOF-BE1 submerged in mineral oil. Plates containing presumptive zygotes in SOF-BE1 were incubated for 8 days at 38.5ºC in a humidified atmosphere at 5% CO_2 and 5% O_2. Sorting of embryos by cleavage kinetics took place at both 22-23hpf and 37-38hpf. Blastocyst development was recorded at 165-166- and 190-191-hours post-fertilization (hpf).

Brilliant cresyl blue staining and COC classification

We adapted previously specified BCB staining protocols[31,229]. Immediately after collection of COCs by aspiration of ovarian follicles, COCs were washed in OCM. This was followed by staining of COCs in 26µM BCB diluted in OCM for 20 minutes at 32.5ºC on a slide warmer. After staining was complete, COCs were placed through two washes of SOF-HEPES and grouped based on their cytoplasmic coloration (BCB positive, blue cytoplasm; BCB negative, lack of blue cytoplasm) (Figure 1.). Only those COCs which presented dark blue coloration of their cytoplasm with no lightly colored regions, which would indicate a reduction of the stain to its colorless form, were deemed BCB positive. In each replicate, a random group of COCs were placed in IVM without staining or withholding period. We carried out this procedure across nine replicates with a total of 1718 presumptive zygotes being placed into culture.

Sorting of embryos by cleavage kinetics in BCB separated COCs

We stained cumulus-oocyte complexes as described above and classified the oocytes as BCB positive or negative. A randomly selected group of COCs bypassed BCB staining and served as a control group for each replicate. All groups were *in vitro* matured, fertilized, and cultured separately. At 37-38 hpf, all groups underwent separation of embryos by classification of four or more cells (Fast group), two to three-cell embryos (Slow group), and putative zygotes not cleaved into fresh SOF culture medium (50µl) and immediately returned the culture plate to the incubator under the same conditions described for IVC. We employed a combination of BCB staining and sorting by cleavage divisions in five replicates with a total of 1354 presumptive zygotes placed into culture.

Sorting of embryos by cleavage kinetics

At 22-23 hpf, two-cell embryos (Fast+ group) were transferred into fresh SOF culture medium (50µl) and immediately returned to the incubator under the same conditions described for IVC (Figure 1.). At 37-38 hpf, those putative zygotes that had not cleaved at 22-23 hpf were classified as having reached or developed past the four-cell stage (Fast group), two to three-cell stage (Slow group), or not cleaved, and sorted into new 50µl droplets of SOF-BE1 (Figure 1.). The culture dishes were immediately returned to the incubator under the same conditions described for IVC. All SOF-BE1 droplets contained no more than 30 presumptive zygotes or embryos. We completed this method in seven replicates with a total of 2573 presumptive zygotes being placed into culture.

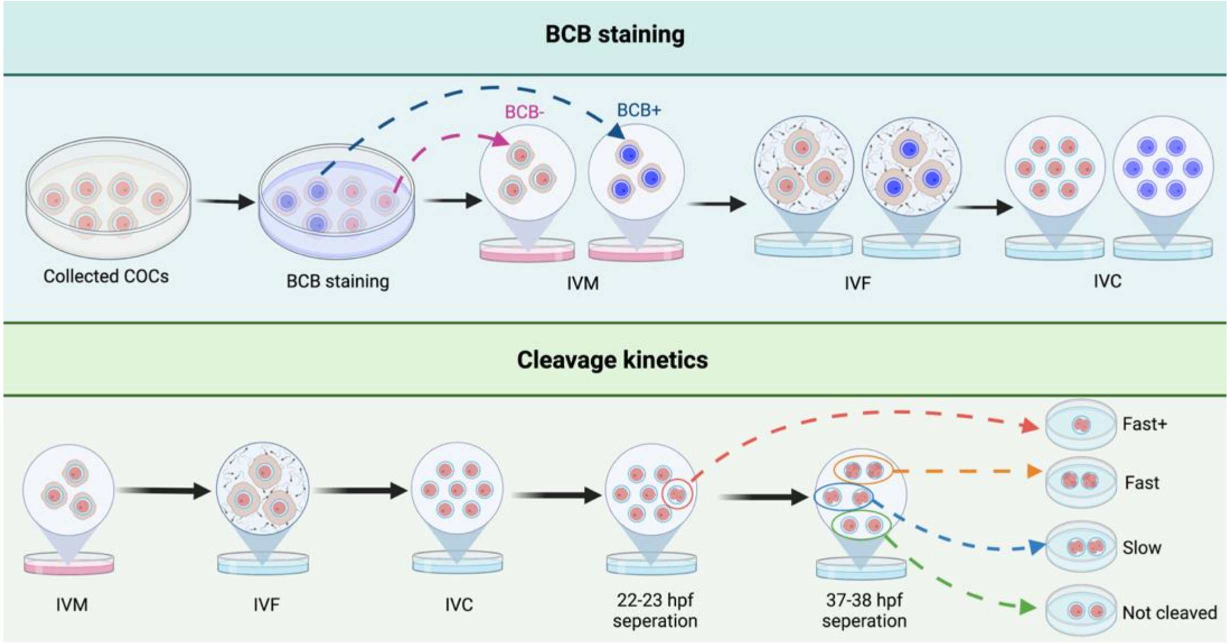


Figure 1. Schematics of the separation of embryos by BCB staining of cumulus-oocyte complexes (COCs) or cleavage kinetics. Created with BioRender.com.

Cryopreservation of embryos

Previously described slow freezing of embryos protocols were adapted for use in this experiment [230-232]. Blastocysts which were deemed grade one[217] and had not begun or completed hatching were selected to undergo cryopreservation. Embryos were removed from SOF culture medium droplets and transferred through three washes of SOF-HEPES. Those embryos selected for cryopreservation were placed into 500µl of ethylene glycol with sucrose (MWI Animal Health, Boise, ID) for 5-15 minutes, or until equilibration had been reached, and were then transferred into a new dish containing 500µl of ethylene glycol before loading into straws. Using an embryo transfer straw connected to a 1ml syringe, embryos were loaded into each straw as previously described [230-232], and straws were sealed with embryo transfer identification rods. Straws underwent slow freezing using a Crysalys Cryocontroller PTC 9500 (Biogenics, Harriman, TN). After two minutes at -6ºC, straws were seeded by evoking ice crystal formation on the topmost area of the straw by exposure to liquid nitrogen. Cycling conditions were as follows: -6ºC for 8 minutes, followed by a ramp of 0.6ºC/min until reaching -32ºC, and held at -32ºC until removal. After the slow freezing program was complete, straws were stored in liquid nitrogen.

Thawing and evaluation of cryopreserved embryos

Straws containing cryopreserved embryos were thawed in a Cito Thaw (CITO Products, Watertown, WI) at approximately 35.5ºC for 30 seconds. Straw contents were dispensed into SOF-HEPES and progressed through three total SOF-HEPES washes followed by two washes of SOF-BE1 with 10% FBS. Groups of 5 blastocysts were plated into 500µl of SOF-BE1 with 10% FBS covered in mineral oil and placed into a humidified tri-gas incubator at 38.5ºC. Blastocyst hatching was recorded at 24-, 48-, and 72-hours post-thawing (hpt).

Embryo sexing using a polymerase chain reaction (PCR)

We designed oligonucleotides (F: AGGGTGAAGCAAATGGTCGT, R: GGAGCAACAGTGTCCTGTGT) to target the male-specific region of the Y (MSY) chromosome (CM011803.1 [233]) using NCBI Primer BLAST[234]. In addition, we multiplexed the reaction with a pair of oligonucleotides (F: GCCCAGACCCAGCATCATT, R: GGGAGTGCCCAAAGCTCTAAA) that targeted a segment of the gene *CDK1* located on the chromosome 28.

A total of 60 hatched blastocysts, post-thawing, from Fast and Slow groups were used for sexing. Blastocysts individually collected in one μl underwent DNA exposure by addition of 5 μl of Lucigen QuickExtract DNA Extraction Solution (Lucigen, Middleton, WI) per embryo followed by incubation at 65ºC for 15 minutes followed by 98ºC for 2 minutes.

We used DNA obtained from a heifer and a steer, respectively, as positive controls. Blood samples were obtained post-mortem, and the buffy coat was separated by centrifugation as we described elsewhere[235,236]. The DNA was extracted from peripheral white blood cells using the Zymo Direct-zol DNA/RNA Miniprep Kit (Zymo Research, Irvine, CA).

We carried out PCR reactions using the total lysate solution added to a reaction mix composed of 0.2 IU/μL Phusion Hot Start II DNA Polymerase (Thermofisher Scientific, Waltham, MA), 1X Phusion HF Buffer, 3% DMSO, 200 μM dNTPs (Promega, Madison, WI), and forward and reverse oligonucleotides (IDT, Coralville, Iowa) for autosome and MSY at 0.12 μM each oligo, in a final volume of 20 μl. Reactions were carried out in 0.2 mL clear PCR tubes (VWR, Philadelphia, PA) using a thermocycler (Eppendorf, Enfield, CT). Cycling conditions were as follows: 98ºC for 30s, followed by 35 cycles of 98ºC for 10s, 60ºC for 30s, and 72ºC for 30s, followed by a final extension of 72ºC for 5 minutes.

We determined the sex of each embryo by agarose gel electrophoresis, using a 2% agarose gel (VWR, Philadelphia, PA) in 1x Tris-Acetate EDTE buffer (Thermofisher, Waltham, MA). The PCR products were loaded into wells at 10 μl each, and electrophoresis was conducted at 90v for 40 minutes. We stained the DNA by immersing the gels into a solution of Promega Diamond Nucleic Acid Dye (Promega, Madison, WI) and 1x Tris-Acetate EDTE buffer for 30 minutes. Gels were imaged using a BioRad Gel Doc XR and Image Lab program (BioRad, Hercules, CA). We determined females by the presence of one band resultant from the amplification of the autosome chromosome, and we determined males by the presence of bands resultant from the amplification of fragments from the autosome and Y chromosomes.

Statistical analysis

All analyses were carried out using R software[237]. Statistical significance was inferred if $P < 0.05$ and a statistical trend was assumed when $0.05 \leq P \leq 0.10$. For transparency and reproducibility of our work, the data collected on embryo *in vitro* production and the analytical work executed is available at the figshare [238] repository and at: https://biase-lab.github.io/BCB_Cleavage_Kinetics/index.html.

Effect of selection on blastocyst yield

We recorded the number of embryos that developed to the blastocyst stage and the number of putative zygotes with arrested development prior to blastocyst formation for each culture drop. We analyzed count data (success of blastocyst development or developmental arrest) using a general linear model with a binomial family, which results in logistic regression analysis[239], using the “glmer” function from the R package “lme4” [240]. We used the number of blastocysts and the number of putative zygotes that failed to develop into blastocysts as the dependent variable, and we added the blastocyst group as a fixed effect, whereas technical replicate was considered a random effect.

Groups were designated based on the selection approach used. For separation based on oocyte BCB staining, three groups were defined: BCB positive, BCB negative, and control. For separation based on cleavage kinetics, four groups were defined: Fast+, Fast, Slow, and not cleaved. For separation using both selection approaches, eight groups were defined: BCB positive Fast+, Fast, Slow, or none; BCB negative Fast+, Fast, Slow, or none.

We carried out a type III analysis of variance on blastocyst proportion with the Wald Chi-square test[241] using the “Anova” function in the R package “car”[242]. Hypothesis tests on linear contrasts were further assessed with odds ratio[243] and multiple comparisons of probabilities using the “emmeans” function in the R package “emmeans”.

Effect of embryo kinetics on survival to cryopreservation

We analyzed the survival post cryopreservation using a similar approach to the one described for blastocyst yield. We used the proportion of blastocysts that hatched as dependent variable, while group and time post-thaw were considered fixed effects and replicate was considered a random variable. We fitted the linear model using the “glmer” function from the R package “lme4” [240]. We tested the significance of effects using type III analysis of variance with the Wald Chi-square test[241]. We used the Fisher’s exact test to assess whether there was a significant difference in the proportion of males and female among the blastocysts that hatched in each of the two groups analyzed.

Results

Blastocyst yield following the separation of COCs by BCB staining

Overall, we classified 37.8% and 62.2% of the oocytes as BCB positive and BCB negative, respectively. There was a significant effect ($P<0.05$, Figure 2.) of oocyte separation by BCB staining on cleavage percentage, in which BCB positive oocytes had 10.9 percentage points greater cleavage relative to unstained oocytes. There was also a significant effect ($P<0.05$, Figure 2.) of oocyte separation by BCB staining on blastocyst yield at 191 hpf, in which BCB positive oocytes had 10.6 percentage points greater yield relative to BCB negative oocytes. This difference represents 1.7 likelihood of a putative zygote to develop a blastocyst in the BCB positive versus the BCB negative group ($P=0.0001$).

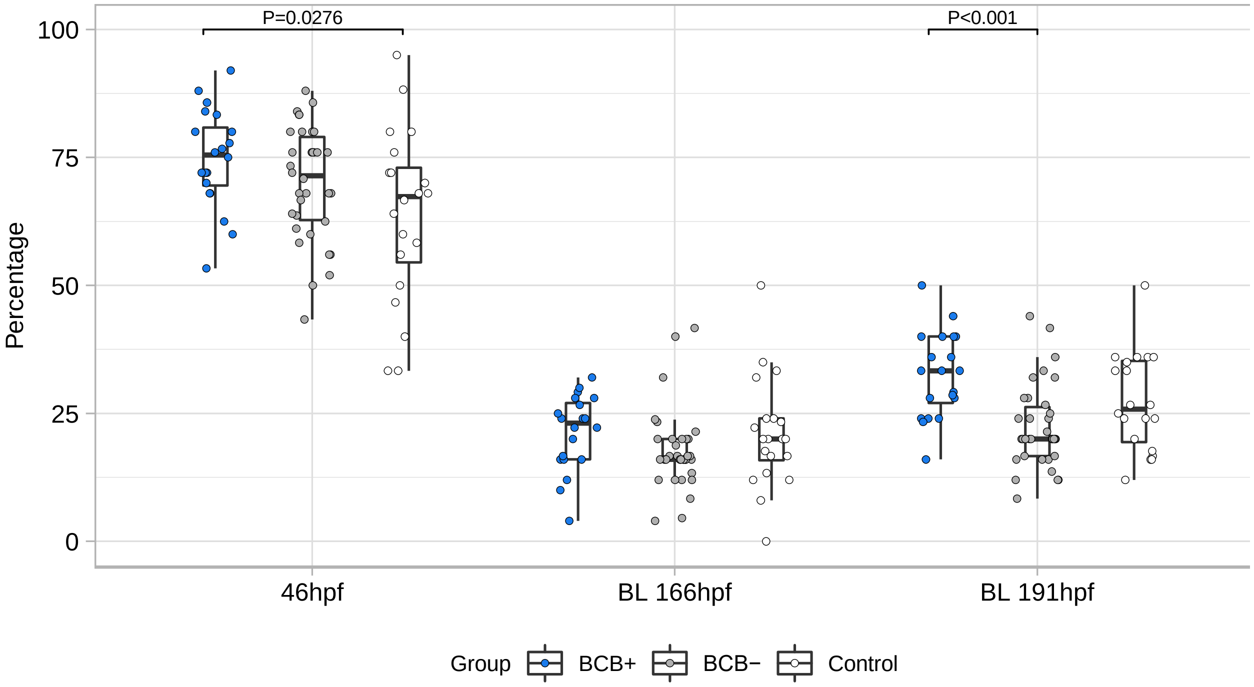


Figure 2. Embryo development for COCs subjected to BCB staining or controls. Each dot represents data from one culture drop. X-axis indicates developmental time points and Y-axis indicates percent developed (46hpf % values are: # embryo cleaved / # embryos placed into culture; BL 166 and 191hpf % values are: # blastocysts / # embryos placed into culture). Each circle within group indicates development data from one culture drop. The median is indicated by a horizontal line, with upper and lower quartiles shown by the boxed area for each group. Box whiskers show the maximum and minimum percentage for each group. Outliers are shown by solid grey circles.

Blastocyst yield following the separation COCs by BCB staining and early cleavage kinetics

Next, we tested whether separating embryos based on cleavage kinetics after the classification of oocytes by BCB staining would improve the separation of embryos with better developmental potential. Within each cleavage group (Fast, Slow, No cleavage) there was no statistical difference between the averages of blastocyst yield at either 166 hpf or 191 hpf across different BCB staining groups.

Within the BCB staining group (BCB positive, BCB negative, control), there was an effect of cleavage kinetics at 166 hpf ($P<0.05$, Figure 3.). In our *in vitro* embryo culture, putative zygotes that did not cleave by 37 hpf had significantly less likelihood of producing a blastocyst relative to those that cleaved by 37 hpf ($P<0.05$). Embryos that developed from BCB negative oocytes and were classified as Fast cleavage produced significantly more blastocysts at 166 hpf relative to their counterparts classified as Slow cleavage. ($P<0.05$, Figure 3.).

Within the BCB staining group (BCB positive, BCB negative, control), there was also an effect of cleavage kinetics at 191 hpf ($P<0.05$, Figure 3.). In BCB treated cumulus-oocyte complexes, embryos in the Fast group presented a trend of producing more blastocysts than the Slow group ($P=0.09$ in BCB positive and $P=0.10$ in BCB negative, Figure 3.). Both Fast and Slow groups produced more blastocysts compared to those presumptive zygotes that did not cleave by 37 hpf ($P<0.05$, Figure 3.) regardless of the BCB group.

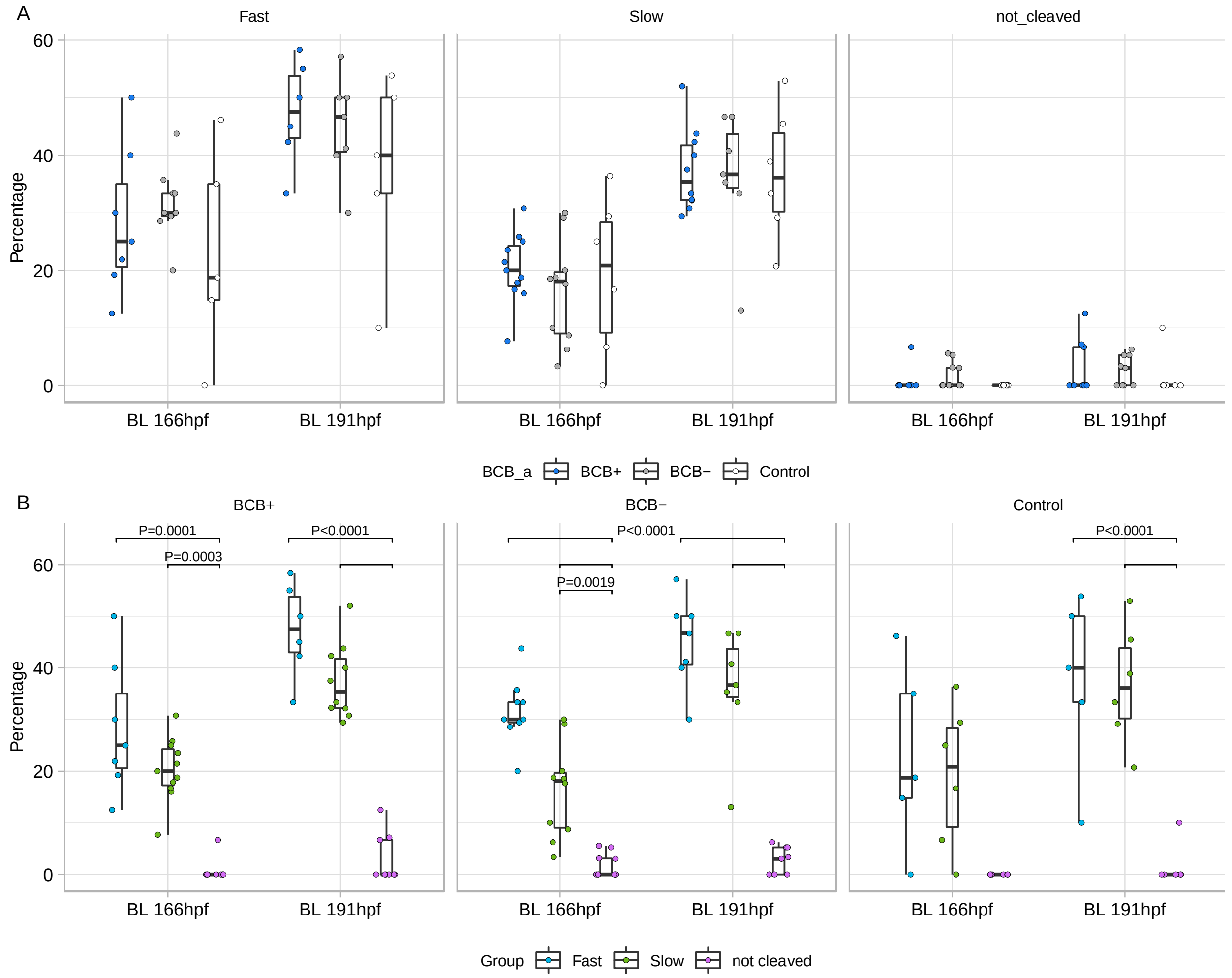


Figure 3. Embryo development following the separation of COCs based on BCB staining and cleavage kinetics. Each dot represents data from one culture drop. Data are arranged to emphasize the comparisons (A) of BCB-stained oocytes within groups of cleavage kinetics and (B) cleavage kinetics within groups of BCB-stained oocytes. X-axis indicates developmental time points and Y-axis indicates percent developed (BL 166 and 191hpf % = # blastocysts / # embryos placed into culture). The median is indicated by a horizontal line, with upper and lower quartiles shown by the boxed area for each group. Box whiskers show the maximum and minimum percentage for each group. Outliers are shown by solid grey circles.

Blastocyst yield following the separation of embryos by early cleavage kinetics

There was a significant effect of the cleavage kinetics on blastocyst yield at 166 hpf and 191 hpf (P<0.05, Figure 4.). Embryos in the Fast group produced more blastocysts than the Slow group at 166 hpf (P<0.05, Figure 4.). At 191 hpf there were more blastocysts produced in the Fast group compared to the Fast+ and Slow groups (P<0.05, Figure 4.). At both timepoints (166 hpf and 191 hpf), the group of putative zygotes that had not cleaved by 37 hpf produced fewer blastocysts then all other groups (P<0.05, Figure 4.).

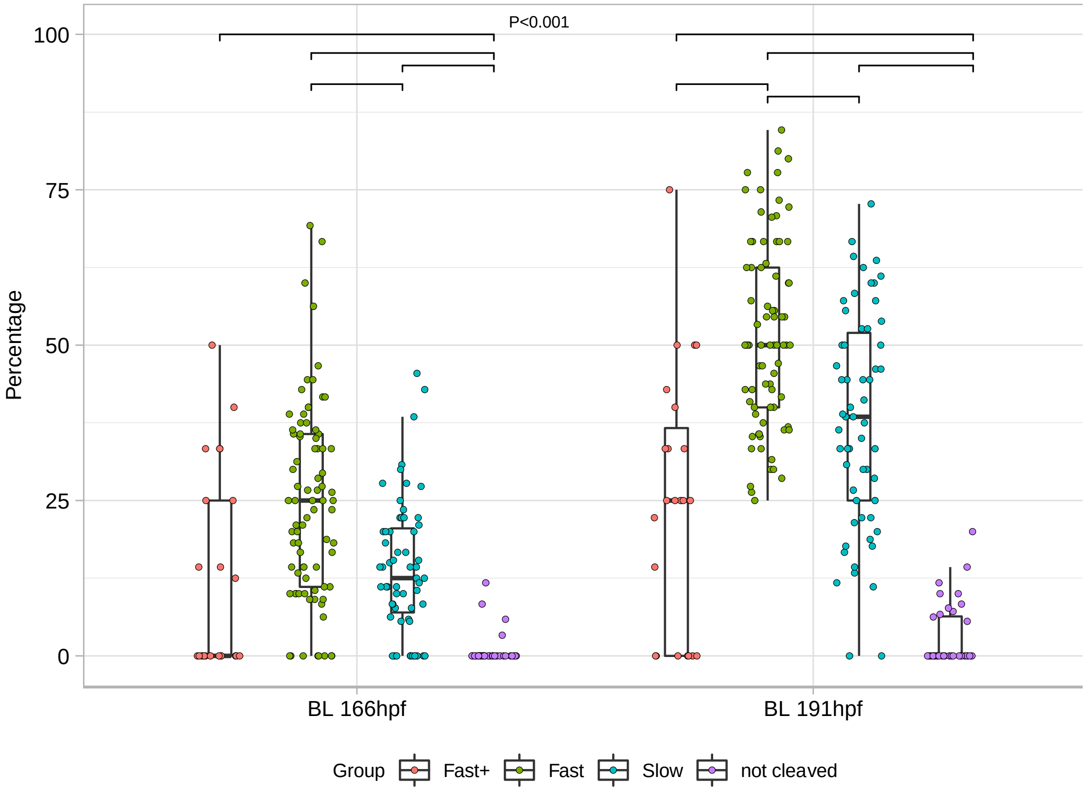


Figure 4. Embryo development following a separation based on cleavage kinetics. Each dot represents data from one culture drop. Fast+: 2 or more cells at 22hpf, Fast: 1-cell at 22hpf, 4 or more cells at 37hpf, Slow: 1-cell at 22hpf, 2 or 3-cells at 37hpf, not cleaved: 1-cell at 37hpf. X-axis indicates developmental time points and Y-axis indicates percent developed (BL 166 and 191hpf % values are: # blastocysts / # embryos placed into culture). Each circle within group indicates development data from one culture drop. The median is indicated by a horizontal line, with upper and lower quartiles shown by the boxed area for each group. Box whiskers show the maximum and minimum for each group. Outliers are shown by solid grey circles.

Assessment of blastocyst developmental potential using the freeze-thaw approach

We tested whether the groups of embryos cleaving between 22 and 37 hpf (Fast and Slow) differ in their potential to survive cryopreservation. As expected, there was an increase of embryos hatched as time progressed. Although there was a seven-point percentage difference between the Fast and Slow groups at 72 hpt, these estimates were not statistically different (P=0.14, Table 1). In addition, we observed no significant deviation in the proportion of male and female embryos that hatched in either group (Fast: 40% female and 60% male, Slow: 46% female and 54% male, P=0.76).

Table 1. Estimates of hatching after the cryopreservation of blastocysts.

Groups	*n**	24 hpt	48 hpt	72 hpt
Slow	44	15.4 ± 3.6	29.4 ± 5.1	41.5 ± 5.7
Fast	98	19.6 ± 3.6	35.8 ± 4.4	48.7 ± 4.6

* Number of blastocysts thawed and cultured in two replicates.

Discussion

The results of the present study confirm that staining of oocytes by BCB can select for oocytes with greater developmental potential, and that the cleavage kinetics of the initial cleavage event is an indicator of embryo developmental potential, as observed by the development of blastocysts in both cases. However, contrary to our expectation, both selection approaches are not synergistic in identifying embryos of greater developmental potential. Our results show that the separation of embryos based on their initial cell division masks the effect of selecting oocytes by BCB staining.

The results we obtained from the selection of COCs are well aligned with previous reports that those oocytes which are capable of degrading BCB dye produce fewer blastocysts compared to the oocytes that cannot break down BCB, and thus retain the stain in its blue form[31,32,34,229,238,244,245]. It is notable, however, that the selection of BCB positive oocytes is likely to only produce 1.2x and 1.7x more blastocysts compared to BCB negative oocytes at 166 and 191 hpf, respectively. In practical terms, one in three putative zygotes, selected from the BCB positive oocytes, placed in culture can develop into a blastocyst, while one in four putative zygotes, selected from the BCB negative oocytes, placed in culture can develop into a blastocyst. Although this difference is statistically significant, the effectiveness of this approach for the identification of oocytes that are more developmentally competent is questionable.

Given the limitations of the BCB approach, we reasoned that using a "two-step" approach would increase the odds of identifying developmentally competent embryos. We then used the separation of oocytes by BCB staining, followed by the separation of early cleaving embryos from late cleaving ones. This second approach was developed for the early identification of embryos that more likely to develop blastocysts[154,220,224-226,246,247]. Our results showed that the separation of early and late cleaving embryos reduced the differences between blastocyst yields produced from BCB positive or BCB negative oocytes.

Next, we carried out in vitro culture of embryos separated exclusively by their cleavage kinetics. Our results diverged from previous reports [154,225] that used similar time points to separate early cleaved embryos, and found that embryos that cleave as early as 24 hpf [225] or 27 hpf [154] produced more blastocysts that the slow cleaving embryos. However, our results closely resemble the observations by[167] who observed that embryos which cleaved at 24 hpf produced 25% blastocysts on day 8. Furthermore, our results are well aligned with the model proposed by[156] who proposed that embryos with moderate cleavage kinetics would have a greater likelihood of developing a blastocyst.

An increasing body of literature has indicated that the combination of the oocyte and spermatozoon influences embryo cleavage kinetics[156]. Embryos with different cleavage kinetics have different transcript abundance[157,248], metabolic profiles[249], and DNA methylation[249]. Despite these reports showing biological differences between these embryos, the underpinning molecular properties related to embryo developmental potential remain unknown.

One interesting result was that the separation of embryos in groups by cleavage kinetics not only allowed the identification of a group of embryos that produced significantly higher blastocyst yield, but produced higher blastocyst yield overall. One possible explanation is that embryos cultured in groups produce and secrete several biomolecules that induce autocrine embryotropic signaling[250], and the embryonic secretion of these biomolecules can improve their competence and promote their own development[251,252]. Under this point of view, the embryos in the Fast group would accumulate biomolecules that positively impact the overall developmental competence of the embryos withing the droplet. A second possible explanation is that the presence of putative zygotes or embryos that are prone to early developmental arrest may secrete factors that may impact neighboring embryos negatively[253]. The later explanation is less likely because the elimination of non-cleaved putative zygotes from the culture drop did not impact the developmental competence of the remaining embryos in that drop[252].

Although embryos with different cleavage kinetics have varied chances of reaching a blastocyst, we asked if developmental potential would extend into the survival of cryopreservation. When working with grade one[217] non-hatched blastocysts, the developmental potential to develop a blastocyst driven by the cleavage kinetics seems to be disconnected from their resistance to cryopreservation. This result corroborates previous findings that cleavage kinetics does not correlate with pregnancy establishment tested on gestation day 35 [154].

Conclusions

The presence or absence of a functional G6PDH enzyme, as measured by BCB staining, has a lower accuracy to predict blastocyst development when compared to embryo cleavage kinetics. Cleavage kinetics can reasonably identify early cleaving embryos with greater developmental potential; however, this approach cannot predict the survival of grade 1 blastocysts following cryopreservation.

CHAPTER 3

Sexing of cattle embryos using polymerase chain reaction based on the completed sequence of the cattle chromosome Y

Abstract

When necessary, polymerase chain reaction (PCR) assays can be used to determine the presence of the chromosome Y (ChrY) in samples. This information allows for biological variation due to sexual dimorphism to be studied. A recent publication of a complete sequence of the ChrY has removed limitations for the development of these procedures in cattle, otherwise imposed by the absence of a ChrY in the reference genome. Additionally, the completed sequence of the cattle ChrY has segments in the male-specific region that are not repeated. We designed a pair of oligonucleotides that targets one of these non-repeated regions in the male-specific sequence of the ChrY. Using this pair of oligonucleotides, in a multiplexed PCR assay with oligonucleotides that anneal to an autosome chromosome, we accurately identified the sex of cattle blastocysts. We developed efficient procedures for the sexing of samples in cattle using their DNA. The oligonucleotides used for the accurate sexing of samples using PCR are transferable to other cattle tissue samples.

Introduction

Determining sexual dimorphism has become increasingly important in biomedical studies[254]. Determining the sex of a sample collected from a mammalian individual before the development of gonads (for example, in pre-implantation embryos) requires the detection of DNA or RNA originating from the chromosome Y. Notably, however, the sequence of the cattle chromosome Y has not yet been added to the reference genome. This poses challenges for the identification of male specific sequences or genes for the development of assays or procedures to infer the sex of embryos or samples of unknown origin.

In cattle, sex determination of preimplantation embryos by polymerase chain reaction (PCR) of male-specific genomic regions has been successfully employed in previous studies [255-269] with an average success rate of approximately 90% [264,270,271]. This method has also been successfully used in sexing animal products, such as semen[272-275], blood[276], or meat[277-279]. In addition, maternal plasma[280,281] and fetal fluids[282,283] have undergone PCR for sexing of gestational fetuses. These studies, however, utilized oligonucleotide sequences which were designed based on an incomplete bovine chromosome Y sequence. Many of these oligonucleotides bind to repeated regions on the chromosome Y [255,258-260,263,264,266,284]. The amplification of repeated elements has been shown to negatively impact oligonucleotide binding specificity while increasing the instance of PCR artifacts, overall lowering assay efficiency and accuracy [285]. In attempts to combat this downfall, previous studies have relied on methods such as nested PCR[262,282,286] or loop-mediated isothermal amplification [256,268,269] to improve assay accuracy and sensitivity. However, replicability of the assays across laboratories has been challenging.

The recent publication of the complete sequence of the cattle chromosome Y [233], along with a gene annotation, motivated us to develop a procedure to determine sample sex using cattle DNA. We designed a new set of oligonucleotides that can accurately detect DNA from the chromosome Y in samples of unknown sex.

Materials and methods

Using NCBI Primer BLAST[234], oligonucleotides (F: 5’-AGGGTGAAGCAAATGGTCGT-3’, R: 5’-GGAGCAACAGTGTCCTGTGT-3’) were designed to target the male-specific region of the Y (MSY) chromosome (CM011803.1 [233]). These oligos produce a 279 nucleotide-long amplicon starting at the nucleotide 10308598 and ending at the nucleotide 10308776 of the sequence CM011803.1. Oligonucleotides targeting a portion of the gene CDK1 (F: 5’-GCCCAGACCCAGCATCATT-3’, R: 5’-GGGAGTGCCCAAAGCTCTAAA-3’), located on chromosome 28, were multiplexed in addition to those which targeted MSY. These oligonucleotides were designed to produce 590 nucleotide-long amplicons starting at the nucleotide 16498587 and ending at the nucleotide 16499156 of chromosome 28.

A total of 60 bovine blastocysts were collected individually in 1 µl phosphate buffered saline solution. The embryo’s DNA was exposed by the addition of 5 µl of Lucigen

QuickExtract DNA Extraction Solution (Lucigen, Middleton, WI) per embryo prior to incubation at 65ºC for 15 minutes then 98ºC for 2 minutes.

We obtained post-mortem blood samples from a heifer and a steer. We isolated the buffy coat by centrifugation as we have previously described[235,236]. Using a Zymo Direct-zol DNA/RNA Miniprep Kit (Zymo Research, Irvine, CA), we extracted DNA from the isolated peripheral white blood cells to serve as positive controls.

Using the total lysate of each blastocyst, we conducted PCR reactions comprised of 0.2 IU/µl Phusion Hot Start II DNA Polymerase (Thermofisher Scientific, Waltham, MA), 1X Phusion HF Buffer, 3% DMSO, 200 µM dNTPs (Promega, Madison, WI), and forward and reverse oligonucleotides (IDT, Coralville, Iowa) for CDK1 and MSY at 0.15 µM each, at a final volume of 20 µl in clear 0.2 mL PCR tubes (VWR, Philadelphia, PA). The cycling conditions used were as follows: 98ºC for 30s, then 35 cycles of 98ºC for 10s, 60ºC for 30s, and 72ºC for 30s, followed by a final extension of 72ºC for 5 minutes.

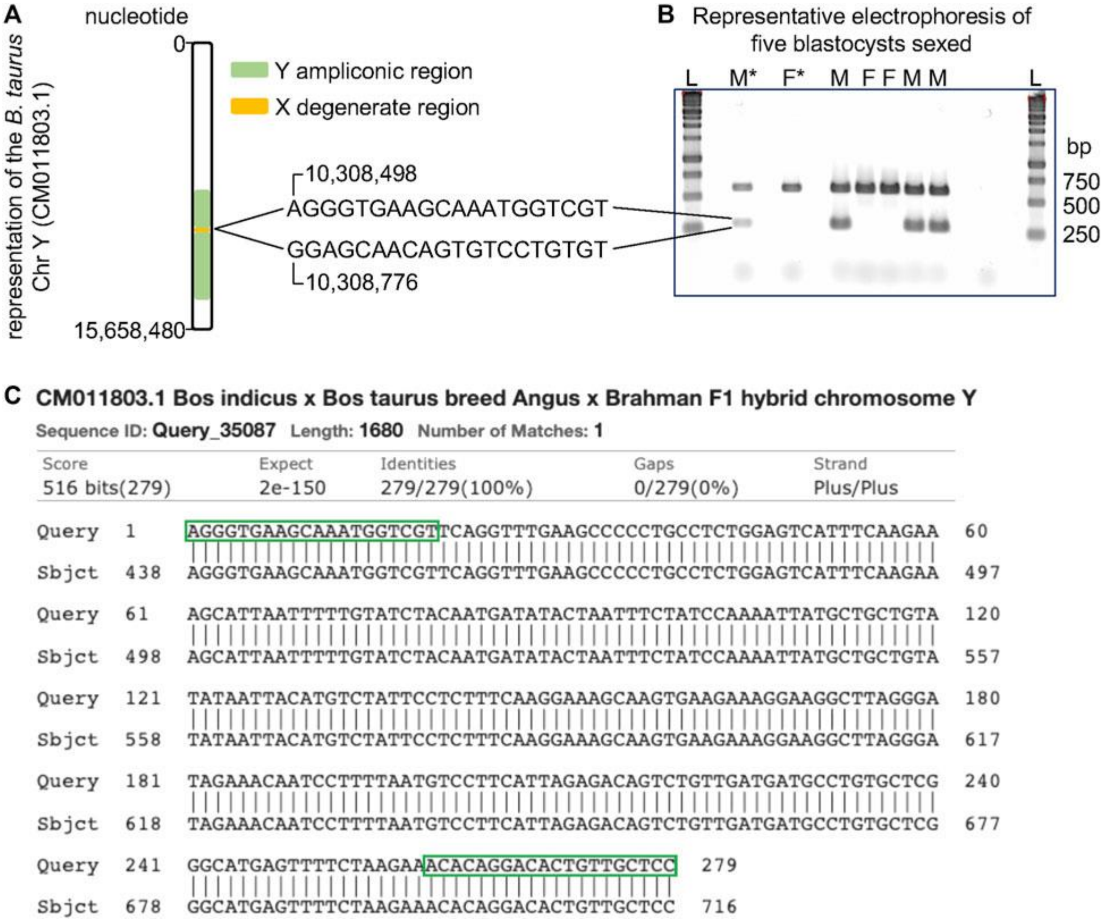
We determined the sex of each embryo by agarose gel electrophoresis. We loaded 10 µl of each PCR product in a 2% agarose gel (VWR, Philadelphia, PA) in 1x Tris-Acetate EDTE buffer (Thermofisher, Waltham, MA) and electrophoresis was carried out for 40 minutes at 90v. DNA was stained by submerging the gel into a solution of Promega Diamond Nucleic Acid Dye (Promega, Madison, WI) and 1x Tris-Acetate EDTE buffer for 30 minutes away from direct light. Using a BioRad Gel Doc XR and Image Lab program (BioRad, Hercules, CA), gels were imaged. Males were defined as those who presented bands from both CDK1 and MSY amplification, while females were defined as those who only presented a band for CDK1 amplification (Figure 5.).

To validate the identity of our amplicon, we proceeded with Sanger sequencing of PCR products resultant from MSY chromosome oligonucleotides. We conducted PCR on known male DNA extracted from peripheral white blood cells, using reaction and cycling conditions as described above. We then purified amplicon fragments with a Zymo DNA Clean and Concentrator kit (Zymo Research, Irvine, CA) by following the provided protocol. We quantified 2 µl of our purified product using a Nanodrop (Thermofisher Scientific, Waltham, MA). We submitted the purified fragment for Sanger sequencing by the Virginia Tech Genomic Sequencing Center. Resultant sequences were aligned to a segment of the Bos indicus x Bos taurus breed Angus x Brahman F1 hybrid chromosome

Y whole genome shotgun sequence (CM011803.1) to validate amplicon identity using BLAST from the NCBI[287,288] (Figure 5.).

Results

Using the complete sequence of the cattle chromosome Y, recently published [233], we identified a set of oligonucleotides spanning the nucleotides 10,308,490 and 10,308,776 that pair in the ampliconic region. Because these oligonucleotides did not align in a repeated region of the cattle chromosome Y, the amplified fragment is expected to be 279 base pairs long. Indeed, our tests using male DNA showed that these oligonucleotides produced a fragment of length close to the expected. More importantly, PCR assays using these two oligonucleotides did not produce amplicons when female DNA was used. We multiplexed this reaction with a pair of oligonucleotides mapping to the cattle chromosome 28, thus a reaction would have one product of amplification serving as a positive control. Therefore, using our oligonucleotides, one band indicates a female, and two bands indicate a male DNA. We further confirmed the amplicon by sequencing the fragment amplified using the Sanger method. The amplified product aligned to the sequence of the chromosome Y deposited in the NCBI and did not map to the reference genome (data not shown). Using the described PCR assay with two pairs of oligonucleotides, we unequivocally sexed 60 blastocysts, identifying 25 males and 35 females.



```
Sequence ID: Query_35087  Length: 1680  Number of Matches: 1

Score            Expect    Identities         Gaps         Strand
516 bits(279)    2e-150    279/279(100%)      0/279(0%)    Plus/Plus

Query  1    AGGGTGAAGCAAATGGTCGTTCAGGTTTGAAGCCCCCTGCCTCTGGAGTCATTTCAAGAA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  438  AGGGTGAAGCAAATGGTCGTTCAGGTTTGAAGCCCCCTGCCTCTGGAGTCATTTCAAGAA  497

Query  61   AGCATTAATTTTTGTATCTACAATGATATACTAATTTCTATCCAAAATTATGCTGCTGTA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  498  AGCATTAATTTTTGTATCTACAATGATATACTAATTTCTATCCAAAATTATGCTGCTGTA  557

Query  121  TATAATTACATGTCTATTCCTCTTTCAAGGAAAGCAAGTGAAGAAAGGAAGGCTTAGGGA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  558  TATAATTACATGTCTATTCCTCTTTCAAGGAAAGCAAGTGAAGAAAGGAAGGCTTAGGGA  617

Query  181  TAGAAACAATCCTTTTAATGTCCTTCATTAGAGACAGTCTGTTGATGATGCCTGTGCTCG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  618  TAGAAACAATCCTTTTAATGTCCTTCATTAGAGACAGTCTGTTGATGATGCCTGTGCTCG  677

Query  241  GGCATGAGTTTTCTAAGAAACACAGGACACTGTTGCTCC  279
            |||||||||||||||||||||||||||||||||||||||
Sbjct  678  GGCATGAGTTTTCTAAGAAACACAGGACACTGTTGCTCC  716
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Figure 5. Schematics of PCR assay for sexing samples from cattle. (A) Position of the oligonucleotides relative to the sequence of the cattle chromosome Y[233]. (B) Representative electrophoresis of the result of PCR assays for sexing cattle samples. (C) Alignment of the PCR product with a segment of the sequence of the cattle chromosome Y (CM011803.1). Green boxes indicate where the oligos align on the DNA sequence. L: ladder, F*: control DNA from a female, F: sample inferred as female, M*: control DNA from a male, M: sample inferred as male, bp: base pairs.

Discussion

Many PCR assays have been developed for the determination of sex in samples, including from cattle[264,270-283,286], however, most of the assays have relied on oligonucleotides that anneal to repeated regions on the Y-chromosome[255,258-260,263,264,266,284], or have limited efficiency[256,262,268,269,282,285,286]. The oligonucleotides we tested in this study have three important advantages. First, we used the complete sequence of the chromosome Y[233]; second, we intentionally focused our search of candidate oligonucleotide pairs on the segment of the chromosome Y that is unique to males; third, we avoided repeated regions of the DNA. Our strategy to narrow down our search to a segment of the chromosome Y combined with an efficient tool for primer design (NCBI Primer BLAST[234]) allowed us to identify a set of oligonucleotides that produce a single amplicon that is of the same length for any sample originated from males. Although we tested the oligonucleotides in DNA from single blastocysts and used DNA from white blood cells for controls, this pair of oligonucleotides is expected to work with a wide range of tissues and initial amounts of DNA that is free from PCR inhibitors[289]. The complete sequencing and annotation of genomes is essential for research in genomics and other disciplines. Working with the complete sequence of the cattle ChrY, we developed oligonucleotides that unequivocally determine the presence of the ChrY in a sample from cattle. A multiplex assay using oligonucleotides annealing to an autosome chromosome assures the assay efficiency in the absence of a chromosome Y in samples from females.

CHAPTER 4

Ablation of OCT4 function in cattle embryos by double electroporation of CRISPR-Cas for DNA and RNA targeting (CRISPR-DART)

Abstract

CRISPR-Cas ribonucleoproteins are important tools for gene editing in pre-implantation embryos. However, the inefficient production of biallelic deletions in cattle zygotes has hindered mechanistic studies of gene function. In addition, the presence of maternal RNAs that support embryo development until embryonic genome activation may cause confounding phenotypes. Here, we aimed to improve the efficiency of biallelic deletions and deplete specific maternal RNAs in cattle zygotes using CRISPR-Cas editing technology. Two electroporation sessions with Cas9D10A ribonucleoproteins targeting exon 1 and the promoter of *OCT4* produced biallelic deletions in 91% of the embryos tested. In most cases, the deletions were longer than 1000 nucleotides long. Electroporation of Cas13a ribonucleoproteins prevents the production of the corresponding proteins. We electroporated Cas9D10A ribonucleoproteins targeting exon 1, including the promoter region, of *OCT4* in two sessions with inclusion of Cas13a ribonucleoproteins targeting *OCT4* mRNAs in the second session to ablate OCT4 function in cattle embryos. A lack of OCT4 resulted in embryos arresting development prior to blastocyst formation at a greater proportion (13%) than controls (31.6%, P<0.001). The few embryos that developed past the morula stage did not form a normal inner cell mass.

Significance statement

CRISPR-Cas mediated DNA editing can revolutionize agriculture and biomedicine due to its simplicity of design and use. Modifications induced in embryos, though challenging to accomplish, are beneficial for the advancement of livestock production and the study of biological function. Here, we developed an approach using CRISPR-Cas enzymes to remove DNA segments of the cattle genome in one-cell embryos. Our results show major advancement in the efficiency of producing large deletions in the genome of cattle embryos. Using our approach, we removed the function of the *OCT4* gene. Our results confirmed *OCT4* as a major regulator of pluripotency genes during embryo development and its requirement for the formation of an inner cell mass in cattle.

Introduction

The driving force behind gene functionality studies is the targeted alteration of genomic sequences followed by observation of phenotypic deviations. The deletion of functional sequences in the genome, also called knockouts (KO), can be used to study the roles of genes during pre-implantation embryonic development[290]. Mechanistic studies of gene function provide information connecting genome and phenotype during early embryogenesis, and the data may be used to better understand biological function[291-294] or disease [295-297]. The CRISPR-Cas system has been the method of choice for most researchers wishing to alter genome sequences in somatic[298-300], germ [301-304], or embryonic cells[305-314]. CRISPR-Cas systems have gained traction due to the simplicity of design and synthesis of gRNAs with sequence complementarity to the target region [315] and improved efficiency when compared to other common methods for sequence alterations[316-319].

Despite recent advancements in protein engineering giving rise to CRISPR-Cas ribonucleoproteins of greater efficiency and specificity[320], biallelic deletion efficiency, or the deletion of targeted sequences in both chromosomes, remains low in CRISPR-Cas treated zygotes across many species, including cattle[321-326]. Interestingly, only four reports provide data on biallelic deletion efficiency in studies utilizing CRISPR-Cas introduced through electroporation of cattle zygotes [323-325,327]. These studies averaged 75% of sampled embryos containing partial deletions, with the presence of at least one wildtype allele, and 59% containing full deletions with no wildtype alleles. Some intrinsic factors of zygote biology, such as chromatin compaction and the timing of DNA replication, may impair deletion efficiency due to sequence inaccessibility for CRISPR-Cas binding or the increased number of target sites requiring DNA cleavage. Though the introduction of increased amounts of CRISPR-Cas by more intense electroporation conditions is shown to improve editing efficiencies in cattle zygotes, embryonic mortality increases in tandem [327]. Alternate methods for increasing CRISPR-Cas content in the zygote have been used, such as zona pellucida drilling prior to electroporation in cattle[324] or zona removal in swine[328]. These methods may improve CRISPR-Cas delivery but do not mitigate the setback of embryo mortality. Additionally, it has been suggested that maternally inherited mRNA, present in mammalian zygotes[329-332], may support sufficient protein production in the absence of a functional gene. The presence of mRNA resulting from the gene of interest likely hinders gene functionality studies in preimplantation embryos and may be responsible for inconsistent knockout phenotypes. To that end, Cas13a [333], a type of Cas that binds and cleaves ssRNAs[334], may be used to knockdown maternal or nascent mRNA and further obstruct protein production, but this element has not been accounted for in previous cattle studies. Altogether, many factors can influence the efficiency of CRISPR-Cas systems in pre-implantation embryos.

The gene *OCT4*, or octamer transcription factor 4, is thought to maintain pluripotency in early cattle[335,336] and human[337,338] embryos through its role as a transcription factor for many pluripotency related genes [337,339]. Additionally, it functions in the HIPPO signaling pathway[340] and is thought to be a key regulator of the first cell lineage differentiation event in cattle. The function of OCT4 has been studied in murine preimplantation embryogenesis models, and these studies show that normal blastocyst development and first cell lineage differentiation are possible in the absence of an *OCT4* gene[341,342], but one murine model results in the development of blastocysts with absent inner cell mass[337]. As HIPPO signaling processes vary between bovine and murine preimplantation development[340,343], these results may not provide adequate translation of information regarding human cell lineage differentiation. Studies to determine the role of *OCT4* have been completed by CRISPR-Cas mediated KOs in cattle zygotes, but these studies produced varying outcomes and inconsistent phenotypes[324,325,344,345]. Most studies report *OCT4* KO cattle embryos maintaining the ability to reach the blastocyst stage and effectively completing the first cell lineage differentiation event in the absence of this gene[100,325,345]. Conversely, one report showed developmental arrest at the morula stage, prior to cell lineage specification[324]. This variability may be due to unaccounted factors, such as maternal or pre-existing mRNA, the common presence of wildtype alleles in CRISPR-Cas genome edited cattle zygotes, and how zygotes were generated.

Here, we aimed to improve the efficiency of CRISPR-Cas mediated biallelic deletions in cattle zygotes while degrading preexisting RNAs transcribed from the target gene. We targeted the *OCT4* gene, given the inconsistency of results from previous reports. We hypothesized that paired ribonucleoproteins formed with CRISPR-Cas9D10A, a type of Cas9 that only cleaves the target strand and must be used in pairs[346], produces larger deletions at greater consistency and efficiency than CRISPR-Cas9, and CRISPR-Cas13a can efficiently knockdown mRNA in cattle zygotes. We also hypothesized that simultaneous targeting of DNA and RNA could ablate gene function in cattle zygotes in vitro. In this study, we have mitigated the barriers of poor deletion efficiency and the presence of preexisting mRNA while maintaining embryo survival. The dual delivery of CRISPR-Cas9D10A, six-hours apart, increases the incidence of gene editing and full deletions. Additionally, we targeted maternally inherited transcripts with CRISPR-Cas13a while simultaneously removing a targeted sequence of the genome. Altogether, we have developed a method for high efficiency genome and transcriptome editing in bovine zygotes using CRISPR-Cas editing technology. Our approach overcomes many limitations of gene editing for mechanistic studies of gene function in pre-implantation embryos. Although cattle blastocyst formation is possible in the absence of *OCT4*, these embryos lack an inner cell mass and present severe transcriptional dysregulation of several genes related to stemness.

Materials and methods

Unless otherwise specified, all reagents were purchased from Sigma-Aldrich.

In vitro production of embryos

All procedures and culture media composition for in vitro production of embryos are described in detail elsewhere[29,227]. Briefly, we obtained cattle ovaries from an abattoir (Brown Packing, Gaffney, SC) and washed them with anti-biotic anti-mycotic (Antibiotic-Antimycotic 100X, Thermofisher Scientific, Waltham, MA) and 0.9% saline solution. For the collection of cumulus-oocyte-complexes (COCs), we aspirated ovarian follicles 3-8mm in diameter using an 18g needle (Single-Use Needles BD Medical, VWR, Philadelphia, PA) connected to a regulated vacuum system and collection bottle containing oocyte collection medium (OCM, BoviPlus Oocyte Collection Medium, Minitube, Verona, WI) supplemented with gentamicin (50 µg/µl) and heparin (2 U/ml). We washed COCs twice in OCM, followed by three washes in oocyte maturation medium

(OMM). Then we selected COCs with homogeneous, non-granular oocyte cytoplasm and three or more compact layers of cumulus for in vitro maturation. COCs were placed in groups of 10 in 50 µl of OMM covered by light mineral oil. In vitro maturation plates were incubated for 22-24 hours at 38.5ºC and 5% CO2 humidified atmosphere. Following the incubation, we washed the mature COCs in synthetic oviductal fluid medium (SOF) containing N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES-TL, Thermofisher Scientific, Waltham, MA) and SOF for fertilization (SOF-FERT) before transferring into a final fertilization plate (100 COCs/ml). We thawed frozen semen straws and processed sperm prior to transfer into fertilization plates at a concentration of 1,000,000 spermatozoa/ml. COCs and spermatozoa were co-incubated for 12-13 hours under the same conditions described for in vitro maturation.

We removed putative zygotes (PZ) from fertilization medium at approximately 14 hours post fertilization (hpf) and denuded the cumulus cells by vortexing in 1% hyaluronidase for 5 minutes. Next, we moved PZ through three washes of SOF-HEPES and SOF culture medium (SOF-BE1). The PZs used for control groups were placed in their final culture dish immediately after the washes. Alternatively, the PZs used for electroporation were placed in temporary culture dishes containing 50µl SOF-BE1 covered with light mineral oil. After electroporation, we washed the PZs in SOF-BE1 before placing them in culture. PZs were cultured in groups of 25-30 in 50 µl SOF-BE1 covered by light mineral oil, incubated at 38.5ºC with 5% CO2, 5% O2 in a humidified Eve Benchtop Incubator (WTA, College Station, TX). For time-lapse image analysis, we cultured 8-cell embryos individually in 15 µl SOF-BE1 covered by light mineral oil, incubated at 38.5ºC with 5% CO2 and 5% O2 in a MIRI Time-Lapse Incubator (Esco Medical, Egaa, DK).

Guide RNA design

We designed sgRNAs to target the genomic DNA of the transcriptional start site and exon 1 of *OCT4* using the CRISPOR webservice[347]. We designed the sgRNAs for Cas13a using New York Genome's cas13designtool[348,349] to target the 4th exon of the *OCT4* mRNA. As an independent layer of in silico validation, we aligned all sgRNAs targeting the *OCT4* gene or transcript to the bovine genome with the BLAT software in the UCSC Genome Browser[350]. Additionally, Cas13a sgRNAs were designed to target CleanCap EGFP and mCherry mRNAs (5moU, TriLink Biotechnologies, San Diego, CA). The targeting sgRNAs used in this study were *OCT4* sgRNA1: CTTCGCCTTCTCGCCCCCGCCGG, *OCT4* sgRNA2: TGTCCCGCCATGGGGAAGGAAGG, *OCT4* mRNA sgRNA: ATGCTCTCCAGGTTGCCTCT, mCherry mRNA sgRNA: TCCTCGAAGTTCATCACCCG, EGFP mRNA sgRNA: CATGATATAGACGTTGTGG. We purchased all sgRNAs as a single RNA molecule comprised of both crRNA and tracrRNA sequences (Integrated DNA Technologies (IDT), Research Triangle Park, NC). We also purchased a scramble gRNA (Alt-R® CRISPR-Cas9 Negative Control crRNA #1) and tracrRNA (Alt-R® CRISPR-Cas9 tracrRNA) from IDT and combined them following the manufacturer's instructions.

Preparation of ribonucleoprotein and procedures for electroporation

We mixed Cas9 and sgRNAs for the formation of ribonucleoproteins in Opti-MEM™ Reduced Serum Medium (Thermofisher Scientific, Waltham, MA), and maintained the solution at room temperature for at least 30 minutes prior to electroporation. The specific concentrations and enzymes are detailed below.

As detailed above for control cultures, we removed the cumulus cells from the PZ and placed them in holding SOF-BE1 at 38.5ºC, 5% CO2, and 5% O2. We removed PZs in groups of 30-40 from a holding culture and briefly washed them in OptiMEM (previously equilibrated in the incubator at 38.5ºC and 5% CO2). Next, we mixed 3 µl of the solution containing ribonucleoproteins with 3 µl of OptiMEM containing PZs. We carried out the electroporation using a BTX oocyte petri dish with platinum electrodes (Harvard Apparatus, VWR, Philadelphia, PA). We transferred the final 6 µl to the electroporation chamber. Impedance was checked and, if necessary, adjusted to measure between 0.19 and 0.20 by the addition of OptiMEM or removal of the electroporation solution. The electroporation parameters were as follows: six poring pulses of 15 volts, with 10% decay, for two milliseconds with a 50-millisecond interval, immediately followed by five transfer pulses of 3 volts, 40% decay, for 50 milliseconds with a 50-millisecond interval, alternating the polarity. Following the electroporation, we washed the PZ with OptiMEM and SOF-BE1.

Cleavage assay of the targeted DNA

We carried out a cleavage assay to assess the formation and cleavage of DNA by ribonucleoproteins[351]. We amplified a segment of genomic cattle DNA to be targeted by the sgRNAs by assaying a PCR using the following oligonucleotides (forward: GGCAAGGAACTTGATGCACG and reverse: TGGCCAACCCACTGTTTGAT). The PCR reaction mix consisted of 0.2 IU/µl Phusion Hot Start II DNA Polymerase (Thermofisher Scientific, Waltham, MA), 1X Phusion HF Buffer, 200 µM dNTPs (Promega, Madison, WI), and forward and reverse oligonucleotides (IDT, Coralville, Iowa) at 0.10 µM each, in a final volume of 20 µl in 0.2 ml clear PCR tubes. The cycling conditions for this reaction were: 98ºC for 1 minute, followed by 40 cycles of 98ºC for 15 seconds, 55ºC for 45 seconds, and 72ºC for 1 minute, followed by a final extension of 4 minutes at 72ºC.

We incubated Cas9 (1 µM, Integrated DNA Technologies, Research Triangle Park, NC) with either sgRNA1 or sgRNA2 300 nM in OptiMEM for 30 minutes at room temperature to form the RNPs. Next, we incubated RNPs with DNA fragments containing the targeted sequence (1:10 (v:v) Cas9+sgRNA, 3 nM DNA, 1x NEB buffer 3.1) at 37ºC for 3 hours. Fragments were assessed by electrophoresis on a 1.5% Agarose I™ gel followed by staining with Diamond™ Nucleic Acid Dye and imaging.

Evaluation of electroporation efficiency

We evaluated the electroporation efficiency with RNPs formed by Cas9-RFP (Alt-R™ S.p.Cas9-RFP V3, Integrated DNA Technologies, Research Triangle Park, NC) at 800ng/µl and scramble gRNAs at 800ng/µl. After washing the PZs in OptiMEM, we imaged them using a fluorescent microscope (details below).

Assessment of sequence deletions by Cas9 or Cas9D10A

To test the pattern of deletions with either a double-cutting enzyme or a nickase, we carried out a single electroporation at approximately 15 hpf with ribonucleoproteins formed by either Cas9 or Cas9D10A (IDT) at 800ng/µl and sgRNAs at 800 ng/µl each. After washing the PZ in SOF-BE1, we placed them in culture as described for control PZs.

Assessment of mRNA cleavage by Cas13a

We carried out a single electroporation of PZs with one of the following solutions: a) mRNA of either mCherry or GFP at 400ng/µl; or b) mRNA of either mCherry or GFP at 400ng/µl and ribonucleoprotein formed by Cas13a (GenScript, Piscataway, NJ) at 400 ng/µl and the corresponding targeting sgRNA at 400 ng/µl. After washing the PZ in OptiMEM, we imaged them using a fluorescent microscope (details below) in SOF-HEPES.

CRISPR-DART

For CRISPR-DART, we carried out the first electroporation at approximately 14 hpf with 3µl of RNPs formed by Cas9D10A at 600ng/µl and sgRNAs at 800 ng/µl each mixed with 3µl of OptiMEM. The PZs were maintained in SOF-BE1 media in the incubator. Then, we electroporated them again at approximately 20 hpf with two solutions of RNP complexes prepared separately. One solution contained Cas9D10A at 600ng/µl and each sgRNA at 800 ng/µl and the other contained Cas13a at 1600 ng/µl and sgRNA at 800 ng/µl. At the time of electroporation, we mixed 1.5 µl of each RNP with 3 µl of OptiMEM containing the PZ. After washing the PZ in SOF-BE1, we placed them in culture as described for control PZs (Figure 6.).

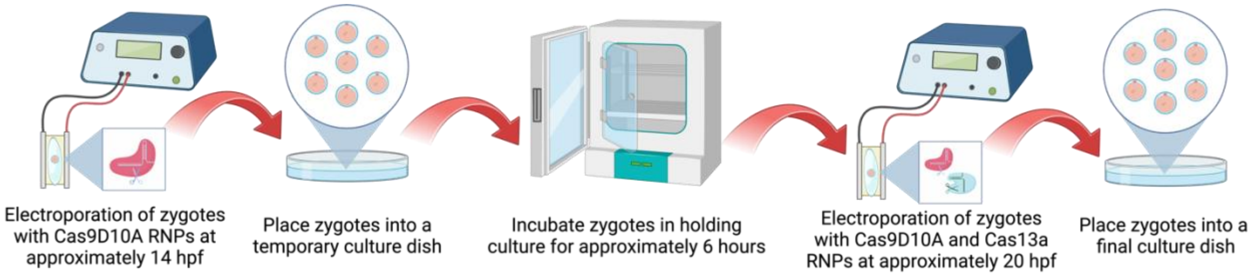

Figure 6. Schematic of CRISPR-DART procedure. Created with BioRender.com.

Targeted DNA sequencing

All embryos collected for DNA sequencing were washed in PBS 0.1% BSA fraction V, followed by removal of the zona pellucida by exposure to EmbryoMax® Acidic Tyrode′s Solution and gentle pipetting. Once the zona pellucida was removed, we washed the embryos in PBS 0.1% BSA fraction V twice and collected them individually in microtubes in approximately 1 µl PBS 0.1% BSA fraction V. We exposed the nucleic acids of each embryo with 5µl of QuickExtract™ DNA Extraction Solution (Lucigen, VWR, Philadelphia, PA), and incubated at 65ºC for 15 minutes followed by 2 minutes at 98ºC.

Sanger sequencing

We produced PCR amplicons using the procedures described for "High throughput long reads". When the amplification produced an amplicon, we treated the remaining PCR products with 3 µl ExoSAP-IT™ Express PCR Product Cleanup Reagent (Thermofisher Scientific, Waltham, MA) and incubated at 37 ºC for 15 minutes followed by 80 ºC for 15 minutes. The sequencing assay was carried out by the Genomics Sequencing Center at Virginia Tech using the same forward oligonucleotide used for the initial PCR.

Statistical analyses

Assessment of differences in embryo development

We recorded the number of embryos that developed to the blastocyst stage and the number of putative zygotes with arrested development prior to blastocyst formation at 164-166 hpf and 188-190 hpf for each culture drop. Culture drop was considered biological replicate. We analyzed count data (success of blastocyst development or developmental arrest) using a general linear model with a binomial family, which results in logistic regression analysis, using the "glm" function from the R package "stats". We used the number of blastocysts and the number of putative zygotes that failed to develop into blastocysts as the dependent variable, and the group (control, scramble, or Cas treated) was a fixed effect. The Wald statistical test was conducted with the function "Anova" from the R package "car"[242].Finally, we carried out a pairwise comparison using the odds ratio and two-proportion z-test employing the "emmeans" function of the R package "emmeans". The null hypothesis assumed that the odds ratio of the proportion (p) of two groups was not different from $(H_0: P_1/P_2 = 1)$ We adjusted the nominal P value for multiple hypothesis testing with the Bonferroni approach and inferred significance when adjusted P value < 0.05.

We analyzed data obtained from single embryo culture, with each embryo as a biological replicate, using the exact binomial test in R with the function "binom.test"[352]. Significance was inferred if the P value < 0.05.

Assessment of differences in fluorescence

First, we calculated corrected total cell fluorescence (CTCF]) using the standard formula: Integrated Density – (Area of selected cell X Mean fluorescence of background readings). We obtained the measurements necessary for the formula using the NIS-elements Imaging Software (v.5.02). Next, we fitted a linear model using the "lm" function of the R package "stats" where Log2(CTCF) was the dependent variable. Replicate and group (fluorescence protein mRNA or fluorescence protein mRNA + Cas13a and targeting sgRNA) were included as fixed effects. We assessed the significance of the variables using the "Anova" function of the R package "car". Next, we tested the pair-wise significance of the two groups by a t-score test employing the "emmeans" function of the R package "emmeans". The null hypothesis assumed that the difference between two averages ($\bar{x}$) was not different from zero $(H_0\colon \bar{x}_1 - \bar{x}_2 = 0)$, and significance was inferred at alpha = 0.05.

Results

First, we assessed the efficacy of electroporation and the cleavage function of the ribonucleoproteins (RNPs). Here, we used electroporation conditions modified from a previous publication[324], as follows: six poring pulses of 15 volts, with 10% decay, for two milliseconds with a 50-millisecond interval, immediately followed by five transfer pulses of 3 volts, 40% decay, for 50 milliseconds with a 50-millisecond interval, alternating the polarity. Fluorescence imaging showed that the RNP formed by Cas9-RFP + scramble guide RNAs (gRNAs) bypassed the zona pellucida in nearly all putative zygotes (PZ) electroporated. Next, we confirmed that the RNPs formed with Cas9 + OCT4 single guide RNA (sgRNA) 1 or Cas9 + OCT4sgRNA2 were able to cleave the targeted DNA in vitro.

Both CRISPR-Cas9 and CRISPR-Cas9D10A produce deletions in cattle zygotes

First, we asked if Cas9 and Cas9D10A would result in similar editing efficiencies and deletion patterns. High-throughput targeted sequencing revealed that 73.1% and 81.5% of embryos presented at least one segment of DNA deleted when we used Cas9 (N embryos = 26) or Cas9D10A (N embryos = 27), respectively. We observed that 15.4% and 25.9% of the embryos electroporated with Cas9 or Cas9D10A, respectively, and genotyped by sequencing, did not have a wild-type copy of the DNA in the targeted region.

The deletions resultant from Cas9 or Cas9D10A varied in their location and length. We observed that Cas9D10A RNPs produced longer deletions and removed the segment of DNA that included both sgRNAs, whereas Cas9 mostly produced small deletions in the region surrounding the sgRNAs but did not cause many deletions spanning both sgRNAs.

Although not significant (P=0.27, Fisher's Exact test), Cas9D10A produced 10.5 percentage points more full deletions, with no wildtype alleles, when compared to Cas9. Thus, we carried out the next experiments with Cas9D10A and *OCT4*-targeting sgRNAs. Also, considering that many of the Cas9-RFP RNPs remained in the membrane or perivitelline space, we reasoned that a second electroporation would increase the efficiency of full deletions in cattle presumptive zygotes (PZ). A second electroporation of PZ (approximately six hours after the first electroporation; see methods for details) with RNPs composed of Cas9D10A and associated sgRNAs resulted in no PCR amplification for most blastocysts when using the oligonucleotides designed for high-throughput short-read sequencing. This outcome, and prior reports that CRISPR-Cas9 can produce unexpected large deletions [353,354], prompted us to design oligonucleotides to flank a wider region of the DNA surrounding our sgRNA target sequences. Approximately 19% of the blastocysts tested with this long-range pair of oligonucleotides produced an amplicon. All blastocysts that had no amplicon produced with oligos surrounding our targeting sgRNAs were tested for amplification of a non-targeted autosomal region of the genome to confirm that an embryo was present in the tube.

We sequenced the PCR products from seven blastocysts using the Sanger procedure, and three of these samples produced electropherograms from only one fragment. The long-range PCR produced multiple amplicons in the other three samples, which is unsuitable for Sanger sequencing.

Embryo survival following one or two Cas9D10A electroporation sessions

We tested if the electroporation of Cas9D10A with scramble gRNAs would impact development to the blastocyst stage. One electroporation session with scramble gRNAs produced similar results to controls (164-166 hpf – Cas9D10A and scramble gRNAs: 17.1%±3.1, controls: 25.3%±3.2; 188-190 hpf – Cas9D10A and scramble gRNAs: 31.5%±3.8, controls: 30.8%±3.4, P>0.05). Two electroporation sessions with scramble gRNAs also produced similar results to controls (164-166 hpf – Cas9D10A and scramble gRNAs: 17.7%±2.6, controls: 25.3%±3.2; 188-190 hpf – Cas9D10A and scramble gRNAs: 28.2±3.1, controls: 30.8%±3.4, P>0.05). Therefore, one or two electroporation sessions with Cas9D10A and scramble gRNAs did not reduce blastocyst yield and maintained survival like that seen in non-electroporated embryos.

One electroporation session with Cas9D10A and *OCT4*-targeting sgRNAs reduced the blastocyst yield relative to scramble or control groups (164-166 hpf – Cas9D10A and targeting sgRNAs: 6.8%±1.4, controls: 25.3%±3.2; 188-190 hpf – Cas9D10A and targeting sgRNAs: 11.6%±1.7, controls: 30.8%±3.4, P<0.001). Two electroporation sessions with Cas9D10A and *OCT4*-targeting sgRNAs also reduced blastocyst development (164-166 hpf –Cas9D10A and targeting sgRNAs: 3.2%±0.7, controls: 25.3%±3.2; 188-190 hpf – Cas9D10A and targeting sgRNAs: 7.9%±1.1, controls: 30.8%±3.4, P<0.001). We also evaluated zygotes electroporated twice with Cas9D10A and *OCT4*-targeting sgRNAs (N=56), transferred into individual drops of media at the 8-cell stage and placed in a time-lapse incubator, along with controls that were not electroporated (N=28). A greater number of electroporated embryos arrested their development at the 8-cell (35.5% vs 17.8% controls, P=0.0013) and morula (51.8% vs 35.7% controls, P=0.0013) stages. Additionally, a lower proportion of the electroporated embryos developed to the blastocyst stage (12.5% vs 46.4% controls, P=1.06x10-7, exact binomial test). Thus, targeting the gene OCT4 by two electroporation sessions of Cas9D10A and sgRNAs caused partial developmental arrest at the 8-cell and morula stages with a sharp decline in the development to the blastocyst stage but did not eliminate embryo survival.

mRNA knockdown in cattle zygotes by Cas13a

To test whether Cas13a can target mRNAs in zygotes, first, we electroporated PZ with exogenous mRNAs of fluorescent proteins (red (RFP) or green (GFP)). Fluorescence imaging of embryos ~70 hpf showed successful introgression of exogenous mRNAs (GFP and RFP mRNAs) into PZ and expression of the corresponding proteins in cleavage embryos. By contrast, we quantified a significant reduction of fluorescence (1.37-fold for GFP, and 1.34-fold for RFP, P<0.001) when we electroporated PZ with the exogenous mRNA and Cas13a + targeting sgRNAs simultaneously. Since those PZ treated with Cas13a + targeting sgRNAs did not target an endogenous RNA, we tested whether Cas13a RNPs would impact embryo development. There were no statistical differences in the development to blastocyst stage at 188-190 hpf (30.8%, 37.7%, 32.1%, 33.3% for Cas13a+GFP mRNA sgRNAs, Cas13a+RFP mRNA sgRNAs, Cas13a+scramble gRNAs, controls, respectively, P>0.8, Figure 7.). Thus, Cas13a targets mRNAs efficiently in cattle zygotes with no alteration in their developmental potential.

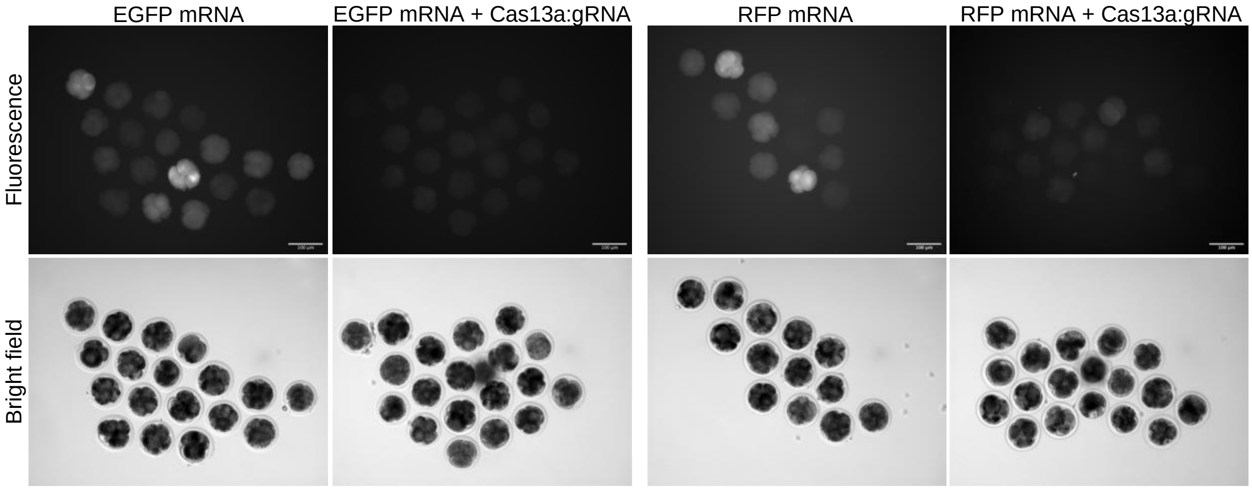

Figure 7. Knockdown activity of Cas13a in cleavage cattle embryos. Scale bar: 100μm.

Ablation of OCT4 function in cattle pre-implantation embryos by CRISPR-DART

We used CRISPR-DART to target the promoter (based on orthology with the human genome) and exon 1 of *OCT4*. The induced deletions significantly reduced embryo survival (164-166 hpf – CRISPR-DART: 6.1%±0.8, controls: 23%±2.3; 188-190 hpf – CRISPR-DART: 13%±1.2, controls: 31.6%±2.5, P<0.001). Using immunofluorescent staining, we determined that the putatively edited blastocysts (we estimated 91% editing success) did not produce OCT4 protein. Additionally, we detected a decrease of NANOG in the edited blastocysts. Thus, we confirmed that the deletion of the promoter and exon 1 of *OCT4* resulted in absence of OCT4 protein (Figure 8.).

Morphological examination showed an absence of a well-defined inner cell mass in blastocysts deemed *OCT4*-/-, whereas a well-defined inner cell mass is clearly visible in the control embryos. Time-lapse image analysis of the development of putative *OCT4*-/- embryos confirmed the formation of a blastocoel cavity and absence of a normal inner cell mass (Figure 8.).

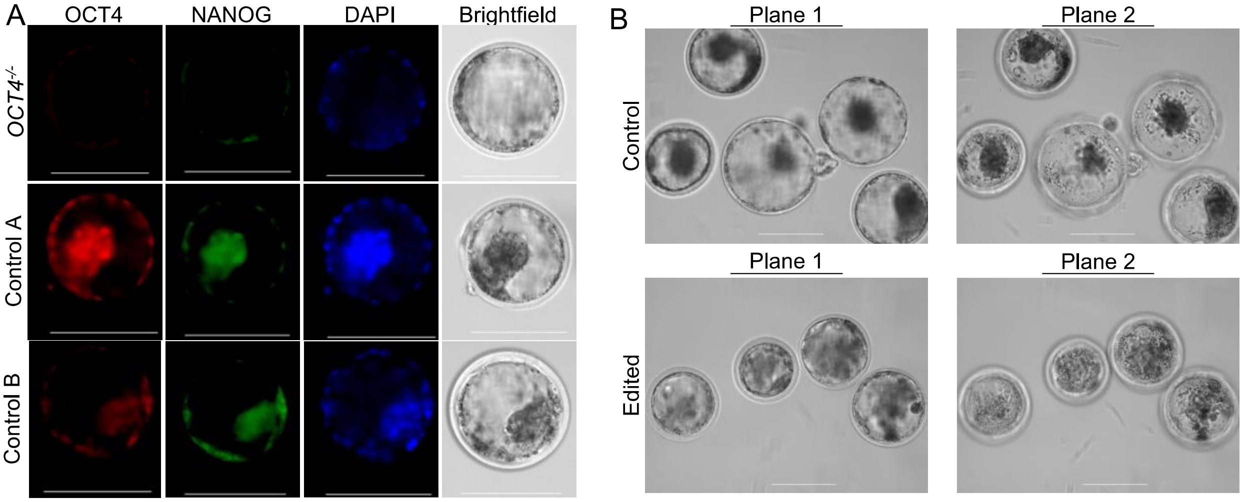

Figure 8. Impact of *OCT4* knockout in cattle pre-implantation embryos. (A) Immunofluorescence assay of OCT4 and NANOG in cattle pre-implantation embryos. Scale bar: 100 µm. (B) *In vitro* produced blastocysts 188-190 hpf. Images are presented in two focal planes for the visualization of the inner cell mass and blastocoel cavity. Scale bar: 100 µm.

Discussion

We developed an approach using Cas9D10A to delete targeted regions of the DNA and Cas13a to cleave targeted RNA for complete disruption of gene function in cattle zygotes at high efficiency. We used CRISPR-DART to target *OCT4* mRNAs and exon 1, including the promoter region. Our data provide several insights into the function of *OCT4* in cattle pre-implantation development. First, most *OCT4*-/- embryos arrest development before the blastocyst stage, but a minor proportion of edited zygotes do still survive. Second, *OCT4*-/- embryos that progress their development can form a blastocoel cavity with an outer layer of cells resembling trophectoderm but do not form an inner cell mass with similar morphology observed in control embryos. Finally, the ablation of *OCT4* significantly alters the transcript abundance of genes involved in pluripotency. Our results show that *OCT4* is necessary for the development of a cattle blastocyst with a morphologically normal inner cell mass.

Simultaneous deletion of DNA segments and cleavage of RNA in zygotes

Previous research has reported the use of CRISPR-Cas9 to delete DNA segments in cattle zygotes[324-327,355]. To build on this, we tested the efficacy of Cas9 and Cas9D10A with two sgRNAs targeting the exon 1 of OCT4. Although we did not test for off-targets, Cas9D10A produces single-strand DNA breaks and requires two sgRNAs targeting opposite strands to nick the DNA and induce faulty DNA repair [356]. This combination of factors significantly reduces mutation elsewhere in the genome. Our results confirmed that Cas9D10A RNPs produce large deletions beyond the region flanked by the sgRNAs [353]. However, we only detected deletions larger than 500 nucleotides when we electroporated the zygotes twice in an interval of six-hours between sessions. Two electroporation sessions allow for the introduction of greater quantities of RNPs in the zygote without causing toxicity. The combination of Cas9D10A targeting two sequences in the genome, and likely a higher quantity of RNPs entering the cell in two sessions of electroporation, increased the efficiency in producing full edits from 25.9 to 91%, which is higher than previous reports in cattle zygotes[324-327,355]. The timing of electroporation sessions takes intrinsic zygote biology, including onset and mean length of S phase[357], into account. It is likely that improved target sequence accessibility for Cas binding and deletion efficiency was achieved through appropriate timing of electroporation sessions.

The RNPs produced by the combination of CRISPR-Cas13a and an sgRNA can target and cleave single stranded RNAs[333]. These RNPs have been used in animal embryos[358] to target specific mRNAs. Here, we tested the efficacy of Cas13a in cattle zygotes by introducing and targeting mRNAs for either GFP or RFP. Our experiments showed that Cas13a could efficiently prevent protein synthesis from targeted mRNAs in cattle zygotes. One concern related to Cas13a is that it may cleave unintended mRNAs in the vicinities of targeted RNAs in a cell-dependent manner[359]. The introduction of Cas13a+sgRNAs targeting exogenous mRNAs (GFP or RFP) and the corresponding mRNAs into cattle zygotes did not reduce embryo survival, thus, if there are off-target effects, they are negligible in cattle pre-implantation embryos. Cas13a can knockdown specific mRNAs in zygotes in conjunction with Cas9D10A to target genomic DNA.

Effects of ablation of OCT4 in cattle embryos

Our CRISPR-DART approach efficiently deleted exon 1 and the promoter from the *OCT4* in most embryos. We expected that removing the promoter and transcript starting site would impair the production of *OCT4* mRNAs and proteins. Indeed, we confirmed that most of the embryos tested by immunofluorescence assays did not have detectable *OCT4*. The ablation of OCT4 function in cattle pre-implantation embryos severely reduced blastocyst development, but most of the blastocysts were confirmed to be fully edited. This finding aligns with reports that produced embryos from *OCT4*-/- somatic cells[344,345] or produced putative *OCT4*-/- embryos by introducing RNPs into zygotes[324,325]. The major blastocyst phenotype we observed was the absence of an inner cell mass, a phenotype previously reported in knockout mice (Pou5f1tm1Cgre/Pou5f1tm1Cgre, genotype id MGI:3040797[337]). By comparison, Simmet and colleagues showed that *OCT4* is necessary only for the second lineage differentiation[345], and it is possible that dysregulated genomic reprogramming due to somatic cell cloning could be the cause of the minor discrepancy in the phenotypes. Our results show that *OCT4* is required for the differentiation of inner cell mass in cattle embryos.

Limitations of the study

This study has limitations, mostly related to the editing efficiency and the inherent biology of DNA synthesis in zygotes. First, several technical factors, such as the concentration of ribonucleoprotein and electroporation conditions, may be finetuned to improve the efficiency of producing fully edited embryos. Here, we based our technical conditions on previous literature[322,324], but we are confident there is room for improvement. Second, although matured oocytes are exposed to sperm in the fertilization media simultaneously, there is a window of opportunity for oocytes to be fertilized. Thus, among hundreds of oocytes, the timing of fertilization is heterogeneous. Third, following fertilization, both pronuclei are formed of compacted chromatin, in which the RNPs are likely not accessible to the targeted sequence. The unwinding of the DNA for synthesis is an asynchronous process across zygotes, and DNA synthesis can happen in a window of approximately 10 hours[357,360]. Therefore, there is tremendous variability in the accessibility of the targeted DNA across zygotes. Fifth, we did not sequence the DNA of embryos that had deletions larger than the DNA sequence flanked by our oligonucleotides. We made several attempts to amplify very large fragments of 6 and 8 kilobases long, but nonspecific amplification hindered our ability to genotype the embryos accurately. Despite these limitations, our results show improvement in the efficiency of producing fully edited embryos, and the phenotype observed is coherent with the literature in mice and cattle.

Conclusion

The production of knockouts is essential for mechanistic studies of gene function in pre-implantation embryos. We showed that Cas9D10A is more efficient than Cas9 at producing biallelic deletions in zygotes. Two sessions of electroporation introduce greater quantities of Cas9D10A RNPs and increase the frequency of large biallelic deletions. The sequential introduction of RNPs does not impair embryo development as long sgRNAs targeting proximal sequences in the genome are not used. RNPs consisting of Cas13a prevent protein production from targeted mRNAs in cattle zygotes. Our CRISPR-DART approach increased the efficiency of producing knockout zygotes. Lastly, we show that *OCT4* is required for the regulation of several genes that control pluripotency and the formation of an inner cell mass in cattle blastocysts.

CHAPTER 5

Future directions for in vitro embryo production

IVP is a valuable technology for the study of embryo development, the improvement of livestock, or the treatment of infertility. Many limitations exist that hinder its effective use for these goals. Below, I have outlined the future direction for the improvement *in vitro* embryo production

1*.* **Refining selection methods:** Developing more accurate and reliable methods to assess embryo quality before transfer is critical. These methods ensure that only the highest quality embryos, with the best chance of survival, are maintained in culture and transferred to a recipient. The advancement of time-lapse imaging has opened avenues for computer-mediated selection of competent embryos. These data may provide morphological parameters for embryos selection to use without time-lapse imaging, or artificial selection of embryos by computer algorithms using time-lapse data. These methods have much room for improvement before their widespread use, as algorithm accuracy remains low and is difficult to reproduce across laboratories.

2. **Enhancement of culture conditions:** Optimizing culture conditions to reduce stress on embryos is vital. This could involve modifications in media composition, gas concentrations, and culture duration to mimic the natural environment more closely and avoid excess cellular stress of the embryo. Current conditions provide moderate success, but improvements could lead to the production of a large quantity of high-quality embryos.

3. **Improving similarity to in vivo development:** Striving to create in vitro conditions that mirror the molecular and physiological environment of in vivo development. This could involve adjusting nutrient compositions or growth factors within the culture system to improve the translation of results. Large gaps remain in our ability to produce embryos with equivalent molecular features to those produced in vivo, and this directly impacts the success rate of IVP or embryo transfer.

4. **Utilizing biological insight:** Leveraging information from the study of biological factors during preimplantation development can guide the refinement of embryo production techniques. This might involve deeper exploration of the factors influencing successful pregnancy outcomes and applying those insights to culture methods. I believe that to

improve cattle reproduction efficiency, we must understand the divergence of development between successful and unsuccessful pregnancy.

5. **Interdisciplinary Collaboration:** Collaborating with experts from various fields, such as genomics, reproductive biology, and engineering, could offer innovative perspectives and solutions to improve the efficiency of *in vitro* embryo production.

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