Germline-ablation achieved via CRISPR/Cas9 targeting of *NANOS3* in bovine zygotes

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Germline-ablation achieved via CRISPR/Cas9 targeting of *NANOS3* in bovine zygotes

ABSTRACT

The transmission of genetic information across generations depends on the integrity of the germline. Primordial germ cells (PGCs) are the embryonic precursors of mature germ cells, spermatozoa and oocytes, and their proper development is essential for reproductive success. PGCs are initially formed in extraembryonic regions and migrate to the developing gonads. Through a series of mitotic divisions, meiosis, and differentiation, PGCs transform into fully mature gametes that carry the genetic material required for the creation of a new individual upon fertilization. The *NANOS* gene family has been found to play a critical role in germ cell development across various organisms, including mammals, with specific expression in germ cells. Through murine studies, *Nanos3* has been identified as a crucial regulator of germ cell development in both sexes, protecting germ cells from apoptosis during migration and colonization of the gonadal ridge. In contrast, *Nanos2* is specifically involved in male germ cell differentiation and maintenance of the spermatogonial stem cell population. These genes exert their control through translational repression of target mRNAs, influencing the expression of key factors involved in meiosis and germ cell differentiation. Recent studies in pigs, sheep, and cattle have shown that *NANOS2* knockout (KO) animals replicate the male-specific germline ablation observed in mice, while female germline development remains unaffected. Similarly, *NANOS3* KO livestock, including male and female pigs and a female bovine fetus, exhibited a complete loss of germ cells but normal gonadal development. Notably, live *NANOS3* KO cattle have not been generated to date, and the specific role of *NANOS3* in male cattle has yet to be explored. Collectively, these findings suggest that *NANOS3* KO livestock could serve as potential hosts for germline complementation, a technique where donor cells from one genetic background replace

the germline of a sterile host with a different genetic background. Germline complementation in livestock offers the potential to generate germ cells from highly genetically valuable donor animals in the gonads of sterile host animals, thereby increasing the availability of gametes from desirable dams and sires (i.e., surrogate sires, or more generally, surrogate hosts). Studies have demonstrated the achievement of germline complementation and the production of live, donor-derived offspring in rodents as well as non-mammalian food species, including chickens and fish.

To facilitate the application of germline complementation technology in livestock, it is crucial to develop efficient methods for generating germline-ablated hosts that retain the function and architecture of their somatic cell gonadal support tissues. One promising method involves using genetic tools like gene editing (GnEd) to inactivate essential genes for germline production at the embryo stage. This creates a vacant germline niche within the host, providing an opportunity for donor cells to colonize and develop in the germline in the absence of competition from endogenous germ cells.

Among the genes targeted in mammals to achieve germline ablation thus far, the RNA-binding protein gene, *NANOS3*, stands out as a promising target in cattle for two primary reasons. The disruption of *NANOS3*, being one of the earliest germline-specific genes expressed, is anticipated to result in the early elimination of PGCs. This would create a favorable environment for exogenous donor cells to migrate to and colonize the gonadal ridge, offering the possibility of germline complementation at an early stage of embryogenesis. Furthermore, if *NANOS3* is confirmed to play an essential role in both male and female germ cell development, as hypothesized, the generation of *NANOS3* KO cattle could serve as hosts for introducing donor-derived germ cells in both sexes, thus expanding the potential for novel breeding schemes.

This study aimed to investigate the consequences of eliminating *NANOS3* in bovine germline development and evaluate the potential for *NANOS3* KO cattle to serve as hosts for germline complementation. To accomplish this, an approach to achieve a CRISPR/Cas9-mediated KO of *NANOS3* via co-injection of two selected guide RNA (gRNA)/Cas9 ribonucleoprotein complexes at 6 hours post fertilization in *in vitro* produced bovine zygotes was successfully optimized. This efficient and repeatable *NANOS3* KO method allowed the generation of *NANOS3* KO cattle and investigation of the impact of *NANOS3* elimination on bovine germline development at different stages of development and at reproductive age.

Through embryo transfer, eight pregnancies with *NANOS3*-presumptively-edited embryos were produced, which were collected at four different timepoints for analysis. These timepoints included during sexual differentiation (41 days of fetal age (d)), after sexual differentiation (90d), at the perinatal stage (283d), and post-puberty (15 months of age (mo)). These samples were comprehensively evaluated using DNA, RNA, protein, and physiological assessments. Long-read sequencing analyses showed the achievement of a remarkable 75% KO rate, with all but one of the edited *NANOS3* alleles resulting in a predicted KO (i.e., loss-of-function). Immunofluorescence analyses revealed that male *NANOS3* KO bovine gonads exhibited the elimination of PGCs as early as 41d. Further analyses using single-cell RNA sequencing confirmed a complete loss of germ cells in the *NANOS3* KO fetal and perinatal testes, while maintaining normal testis cord formation and the presence of somatic support cell populations.

Ultimately, three live, healthy *NANOS3* edited calves were born without assistance, a heifer calf #854, and two bull calves, #838 and #3964. Heifer #854 and bull #838 were determined to be *NANOS3* mosaic KOs. On the other hand, bull #3964 showed editing without complete KO, as he carried an allele with only small, in-frame deletions, that did not result in a complete loss-

of-function mutation. The live *NANOS3* edited animals exhibited normal growth patterns and were ultimately harvested at 15mo to enable collection of meat samples and their reproductive tracts. The edited bulls both exhibited normal pre-pubertal reproductive hormone profiles. At sexual maturity, KO bull #838 was found to have normal libido and an anatomically normal reproductive tract, although no spermatozoa were present in his ejaculate. Additionally, histological analysis of his testes confirmed the absence of germ cells and the presence of gonadal somatic support cells, indicating successful ablation of the germline while preserving the integrity of the somatic gonad. In contrast, the sexually mature *NANOS3* edited bull #3964 exhibited fertility as evidenced by the presence of spermatozoa in his ejaculate and cross-sections of his testes. These findings suggest that bovine *NANOS3* is a haplosufficient gene, and that the allele with small, in-frame deletions produced a functional protein.

The *NANOS3* KO heifer #854 also had a complete loss of germ cells, as confirmed by the absence of oogenesis in her ovarian tissue. However, she also exhibited an anatomically abnormal reproductive tract and irregular gonad development. Additionally, her hormone profiles indicated a lack of reproductive cycling and functional granulosa cells, consistent with the absence of germ cells and follicles in this animal. The relationship between germ cells and supporting somatic cells in the gonad is crucial for the successful development and function of gametes, so any disruption to germ cells can have detrimental effects on the coordinated processes and functions of both germ and somatic cells involved in gametogenesis. In this study, the absence of germ cells in *NANOS3* KO cattle had a greater impact on the normalcy of ovarian development as compared to testes development. Finally, the meat composition from these *NANOS3* KO cattle at 15mo was within the bounds of the normal variation seen in international meat compositional databases, which

is not surprising as *NANOS3* is a germline specific gene, and the lack of germ cells would not be expected to have an impact of meat composition.

Overall, this study provides insights into the role of *NANOS3* in bovine germline development and indicates the potential of *NANOS3* KO cattle to serve as hosts for donor-derived exogenous germ cell production in both sexes. This could unlock both an opportunity to reduce the genetic lag between elite seedstock animals and the genetic merit of commercial cattle, and additionally provide an efficient means for the generation and dissemination of genetically improved GnEd donor genetics.

CHAPTER 1

Literature review: Germline development and complementation

1. INTRODUCTION

The germline plays a fundamental role in the transmission of genetic information from one generation to the next. During fetal development, primordial germ cells (PGCs) are specified as the embryonic precursors of mature germ cells, which ultimately give rise to spermatozoa or oocytes. The establishment of the germline begins with the specification of PGCs in extraembryonic regions, followed by their migration to the developing gonads. After a period of mitotic proliferation, PGCs undergo meiosis and differentiate into fully mature gametes. Following mating, the fusion of sperm and egg triggers the start of embryogenesis. As the embryo develops new PGCs are formed, marking the beginning of a new germline cycle. Through this cycle, genetic information is perpetuated across successive generations, ensuring the continuity of species (Dechiara et al., 2009).

Members of the *NANOS* gene family are known to be critical for normal germline development in several diverse organisms. In *Drosophila* embryos, the *NANOS* (nos) gene encodes a single protein that is required for the development of both the male and female germlines (Wang and Lehmann, 1991). However, in other species there are multiple *NANOS* gene homologs (Nanos C2HC-Type Zinc Finger 1, 2, and 3), which vary in their effects on germ cell fate and developmental pathways (Tsuda et al., 2003). One homolog, *Nanos1,* is not detected in mouse PGCs, and despite the fact it is observed in the seminiferous tubules of mature testis, it was found to be dispensable for murine reproduction (Haraguchi et al., 2003). Conversely, in humans *NANOS1* mutations are associated with a male infertility disorder caused by spermatogenesis defects (Kusz-Zamelczyk et al., 2013).

NANOS2 is predominantly expressed in male germ cells and is required for the maintenance of the spermatogonial stem cell (SSC) population; however, it has not been found to

be requisite for female germline development or fertility (Tsuda et al., 2003). Recently, *NANOS2* knockout (KO) pigs, sheep, and cattle were found to phenocopy *NANOS2* KO mice with male specific germline ablation and normal female germline development (Ciccarelli et al., 2020, Park et al., 2017).

In contrast, murine *Nanos3* expression is found at an earlier stage, in migrating PGCs. The elimination of this factor in mice results in the complete loss of both male and female germ cells (Tsuda et al., 2003). Murine *Nanos3* was found to play an essential role in protecting the migrating PGCs from apoptosis (Suzuki et al., 2007). Additionally, decreased levels of *NANOS3* in human cells, resulted in a reduction in germ cell numbers and reduced expression of genes involved in the regulation of germ cell properties (Julaton and Reijo Pera, 2011).

In livestock, *NANOS3* KO male and female pigs and a *NANOS3* KO female bovine fetus (190 days of fetal development) were shown to have a complete loss of germ cells but otherwise normal gonadal development (Ideta et al., 2016, Kogasaka et al., 2022, Park et al., 2023, Wang et al., 2023). However, in the cattle study, where researchers attempted to produce *NANOS3* KO heifers through somatic cell nuclear transfer (SCNT) cloning, only one homozygous *NANOS3* KO female fetus was successfully generated (Ideta et al., 2016), due in part to the low efficiency of SCNT cloning (Keefer, 2015). Notably, live *NANOS3* KO cattle have not yet been produced, and to date the specific role of *NANOS3* in male cattle has not been reported in the literature. Based on these findings, it is anticipated that *NANOS3* KO bulls would also exhibit a complete loss of germ cells while maintaining normal gonadal development, which would make them suitable candidates for germline complementation studies (Ledesma et al., 2023, Oback and Cossey, 2023).

Germline complementation is the concept of using donor cells from one genetic background to complement or replace the germline of an otherwise sterile host of a different

genetic background. In livestock, germline complementation could be used to generate germ cells from high genetic merit donor animals exogenously in the gonads of otherwise sterile host animals, thereby expanding the availability of gametes from genetically desirable dams and sires (Gottardo et al., 2019). This concept is often referred to as surrogate sires, or more generally, surrogate hosts. Additionally, donor cells could be gene edited (GnEd) to introduce beneficial traits. Importantly for livestock that are raised in extensive-management systems, like beef cattle, surrogate sires would be able to pass on desirable donor genetics through natural service mating, which could unlock both an opportunity to reduce the genetic lag between the seedstock and commercial sectors, and provide an efficient means for the generation and distribution of cattle genetically improved through GnEd (Bishop and Van Eenennaam, 2020, Gottardo et al., 2019, Ledesma et al., 2023, Mueller and Van Eenennaam, 2022, Oback and Cossey, 2023).

Successful germline complementation requires two components, donor cells that are capable of becoming gametes, and a germline ablated host capable of supporting gametogenesis. Therefore, an important step in enabling germline complementation technology in livestock is efficiently generating germline-ablated hosts that retain reproductive capabilities. Historically, various physicochemical approaches, such as toxic drug treatment, irradiation, and heat shock were used to attempt to generate hosts. However, these methods are not efficient and impractical in livestock because they either fail to completely eliminate the endogenous germline, or the treatment has undesirable side effects on animal health (Giassetti et al., 2019). An alternative approach is to use genetic tools, like GnEd, to inactivate essential genes for germline production at the host embryo stage to create a germline developmental niche. Creating a developmental niche

in the host is advantageous to allow the donor cells unfettered access to a specific cell lineage, in this case the germline (Ledesma et al., 2023, Oback and Cossey, 2023).

GnEd in zygotes provides an efficient method for targeted gene disruption, avoiding the use of cell lines and SCNT cloning of reconstructed embryos that were historically employed for the targeted disruption of specific genes in livestock species (McFarlane et al., 2019). GnEd refers to the use of site-directed nucleases to precisely introduce double-stranded breaks (DSBs) at predetermined locations in the genome (Gaj et al., 2013). Cells have evolved two primary pathways to repair DSBs: non-homologous end joining (NHEJ) and homology-directed repair (HDR). The NHEJ pathway often results in errors, which can lead to the disruption, or KO, of a gene. Currently, the most efficient, versatile, and cost-effective gene editing tool is the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system, which uses a guide RNA (gRNA) paired with a reprogrammed Cas9 nuclease (Ferreira and Choupina, 2022, Sander and Joung, 2014).

2. EUTHERIAN GERMLINE DEVELOPMENT

The transmission of genetic information from one generation to the next is an essential aspect of life that is made possible through the germline. The germline is established during fetal development via PGCs, which are the embryonic precursors of the mature germ cells that give rise to gametes - spermatozoa or oocytes. In vertebrates, the development of PGCs occurs through two main mechanisms. The first mechanism involves germ plasm inheritance, where maternal proteins and RNAs carrying germ cell determinants are directly passed from the egg to the developing embryo after fertilization (Seydoux and Braun, 2006). This mechanism is observed in various vertebrates such as teleost fish, frogs, and birds (Hansen and Pelegri, 2021). The second mechanism, known as epigenesis or inductive signals, involves the induction of PGCs from a

competent niche formed from a mesodermal cell type. This mechanism is observed in other species including mammals (Kojima et al., 2017).

Particularly, in eutherians, PGCs are induced at an early stage of embryonic development. They are among the first cell types to differentiate in the embryo, responding to extrinsic signals from the surrounding environment. This induction of PGCs sets the foundation for the subsequent development of the germline and the transmission of genetic information to future generations. Our understanding of the developmental processes involved in eutherian germline formation is primarily derived from studies in mice. Therefore, this review will focus on findings from murine experiments, with a particular emphasis on the male germline, which aligns with the primary focus of this project. Additionally, known differences observed in livestock, specifically cattle, will be highlighted.

2.1. Early embryonic developmental potency

The germline cycle begins with the fusion of highly specialized male and female gametes during fertilization to form an embryo. During the early stages of embryonic development, these cells possess high developmental potency. In mice, embryonic genome activation (EGA) occurs after the first cleavage division, at the 2-cell stage, and the embryonic cells, or blastomeres remain totipotent up until the 4-cell stage (2 days post-conception (dpc)). Individual blastomeres at this stage can be isolated and give rise to fully formed, fertile individuals when placed in a uterus of a pseudopregnant female, thereby exhibiting totipotency (Maemura et al., 2021, Tarkowski, 1959). The capacity to form all embryonic, including the germline, and extraembryonic lineages defines totipotency. Similarly, in cattle, totipotency exists up to the 4-cell stage. Bovine blastomeres from *in vitro* derived four-cell embryos were able to be cultured until blastulation and transferred into synchronized recipients to produce live calves (Johnson et al., 1995). In mice, the first lineage

decision occurs after the 8-cell stage, when the embryo begins to compact and the outside cells become polarized with an apical domain, whereas the inside cells remain apolar (Johnson and Ziomek, 1981). This change in polarity biases the inside cells towards the inner cell mass (ICM) of the blastocyst and outside cells towards the trophectoderm (Hillman et al., 1972). The next lineage differentiation occurs when the epiblast and hypoblast arise from the ICM by the late blastocyst stage in mice (4.5 dpc). The epiblast will primarily form the embryonic lineages, thus exhibiting pluripotency (i.e., the ability to develop into the three primary germ cell layers of the early embryo, but not extra-embryonic tissues), while the hypoblast will contribute to the extraembryonic tissues (Evans and Kaufman, 1981, Gardner and Rossant, 1979). In bovine embryos, EGA and compaction begin later in embryonic development at the 8-cell stage (3 dpc) and after the fifth division (5 dpc; 16-32-cell stage), respectively (Halstead et al., 2020, Van Soom et al., 1997, Van Soom et al., 1992).

2.2. Germline Specification

As mammalian embryonic cells continue to differentiate, a small group of cells is induced to form the germline lineage by extrinsic signals produced from neighboring cells (i.e., based on spatiotemporal position). In mice, the blastocyst implants at approximately 5 dpc, and the epiblast cells develop into a cup-shaped monolayer, continuing to multiply until gastrulation begins at approximately 6 dpc (Wolpert et al., 2011). Regardless of their position, murine epiblast cells at this stage remain germline competent. This was demonstrated by successfully inducing PGCs from cells transplanted from the distal to the proximal regions of the epiblast (Tam and Zhou, 1996). At 6.5 dpc, the murine extraembryonic ectoderm induces proximal epiblast cells to the germ cell fate via bone morphogenetic protein (BMP) 4 and BMP8B signaling (Fujiwara et al., 2001, Lawson et

al., 1999, Winnier et al., 1995, Ying et al., 2001). BMPs are key cell signaling molecules for controlling tissue organization in the early embryo (Wolpert et al., 2011).

The extrapolation of these mechanisms to other mammals is challenging due to the distinctive morphology of the mouse epiblast and extraembryonic tissues at this stage. Most other eutherian species (e.g., rabbits, pigs, sheep, and cattle), form a flat disc-shaped epiblast with only two layers - the epiblast and the hypoblast. As a result, they lack the third layer of contact with the trophoblast lineage, known as the extraembryonic ectoderm, which is unique to the mouse (Sheng, 2015). However, BMP induction mechanisms appear to be conserved in rabbits, pigs, and cattle (Hopf et al., 2011, Kobayashi et al., 2017, van Leeuwen et al., 2015). In a functional experiment using pig epiblasts, it was demonstrated that BMP4 is essential for inducing the program that leads to the specification of PGCs from competent epiblast cells (Kobayashi et al., 2017). Unlike in mice, the timeframe for PGC specification in pig embryos is relatively extended, lasting for at least 4 days. PGCs first emerge in the posterior epiblast around 11.5 dpc in a region expressing T-Box transcription factor T (TBXT, also known as Brachyury). This process continues until just before the initiation of primitive streak formation, which occurs near 15 dpc. During this stage, PGCs are non-replicative, and they are derived from PGC-competent mesoderm cells located in the posterior region of the embryo (Kobayashi et al., 2017).

In peri-gastrulating bovine embryos, BMP4, Brachyury, Eomesodermin (EOMES), and members of the Wingless/Integrated (WNT) signaling pathways have also been detected and therefore may have roles in determining germline fate in cattle (Pfeffer et al., 2017, van Leeuwen et al., 2015). After specification, bovine PGCs have been shown to express well-known pluripotency markers, octamer-binding transcription factor 4 (OCT4, also known as POU5F1) and nanog homeobox (NANOG), and well-conserved germline markers, PR/SET Domain 1 (PRDM1,

also known as Blimp1), NANOS3, KIT Proto-Oncogene, Receptor Tyrosine Kinase (KIT), Deleted In Azoospermia Like (DAZL), and DEAD-box helicase 4 (DDX4, a VASA homolog) (Bartholomew and Parks, 2007, Hummitzsch et al., 2013, Ideta et al., 2016, Kritzenberger and Wrobel, 2004, Planells et al., 2019, Soto and Ross, 2021). NANOS3 and KIT are known markers of early PGCs (i.e., prior to gonadal colonization), and KIT is not specific to bovine germ cells as it can also be present in hematopoietic cells and somatic cells of the developing gonad (Kritzenberger and Wrobel, 2004, Lavoir et al., 1994, Ohno and Gropp, 1965, Soto and Ross, 2021). DAZL and DDX4 have primarily been found to be expressed in bovine PGCs at advanced, or late, stages of differentiation (Guo et al., 2015, Li et al., 2017, Soto and Ross, 2021).

2.3. PGC migration, occupation of the gonad, and commitment to the germ cell fate

During gastrulation, the newly specified PGCs move towards the primitive streak and into the extraembryonic region of the wall of the yolk sac, where they proliferate. Then as the hindgut of the embryo invaginates (murine 8.5-9.5 dpc), the PGCs are passively carried back into the embryo proper. The PGCs then actively migrate through the developing gut to their final destination in the developing gonads, which arise as genital ridges budding from the mesonephros (murine 10.5-11.5 dpc) (Saitou and Yamaji, 2012). In cattle, PGCs have been first identified migrating between 18 to 25 days of fetal development in the yolk sac wall towards the mesonephros. Approximately 10 days later, around 27 days of fetal development, bovine PGCs have been observed to first enter the gonadal anlagen (Wrobel and Süß, 1998). Once PGCs reach the genital ridge they lose their motility and begin proliferating. From this stage until shortly after birth, male PGCs are referred to as gonocytes, or prospermatogonia, and oogonia in females (Culty, 2013, Dechiara et al., 2009).

During the PGC migration and proliferation period, PGCs undergo many epigenetic modifications to prepare them for germ cell fate. The expression of *Sox2*, *Oct4*, and *Nanog* activate the genetic pluripotency network to a limited degree (Spiller and Bowles, 2019). Murine PGCs isolated at 11.5 dpc were unable to contribute to early embryo chimeras (Durcova-Hills and Capel, 2008). Additionally, the re-acquisition of pluripotency includes genome-wide DNA demethylation, resetting of genomic imprinting, reactivation of silenced X-chromosome in females, and decondensation of histone chromatin (Hajkova et al., 2008). Shortly after PGCs arrive at the developing gonad, they undergo a critical transition activating widely conserved germ cell-specific factors while down-regulating the expression of pluripotency factors. This transition marks the commitment to germ cell fate (murine 12 dpc). DAZL, an RNA-binding protein, has been found to be necessary for the correct acquisition of germ cell fate in both sexes of mice and pigs (Nicholls et al., 2019). During normal germ cell differentiation, male germ cells with XY chromosomes enter a mitotic arrest at the G0/G1 phase, while female germ cells with XX chromosomes initiate meiosis (Western et al., 2008). In murine *Dazl* mutants, male germ cells are unable to progress into the G0/G1 phase, while female germ cells are unable to initiate oogenesis. These affected germ cells instead retain the genetic and epigenetic characteristics of migrating PGCs and are eventually lost to apoptosis (Gill et al., 2011, Nicholls et al., 2019, Ruggiu et al., 1997). Therefore, DAZL is suggested to be a "licensing factor" that is required for PGC sexual differentiation (Gill et al., 2011). Around the same time (murine 11.5 dpc), the differentiating PGCs to express another key RNA-binding protein, DDX4 (Lin and Page, 2005, Toyooka et al., 2000). In cattle at 50 dpc, fetal germ cells were found to still be in a relatively undifferentiated state (i.e., still expressing pluripotency and early PGC markers) (Soto and Ross, 2021). The late

PGC markers, DAZL and DDX4 were first observed in bovine fetal gonads of both sexes around 60-70 dpc and 80-90 dpc, respectively.

2.4. Gonadal sexual differentiation

When the genital ridge first arises, it is sexually bipotential (murine 10.5-12.5 dpc). The sex chromosomes in the somatic cells of the genital ridge will determine which type of gonad the ridge becomes. Specifically, a single gene on the Y chromosome has an especially important role in this decision. In males, the sex-determining region (SRY) gene will begin expressing in a subset of XY somatic cells (murine 10.5-12 dpc), which activates the expression of transcription factor SRY-box transcription factor 9 (SOX9) to differentiate those cells into pre-Sertoli cells (Koopman et al., 1991, Sekido et al., 2004). In mice, peak SRY expression occurs at 11.5 dpc leading to gonadal sexual differentiation by 12 dpc. Murine SRY expression completely stops by 12.5 dpc (Hacker et al., 1995). In cattle, peak SRY expression occurs at 40 dpc leading to gonad sexual differentiation, with testis cords being distinguishable by 42-44 days (Planells et al., 2019, Ross et al., 2009). In contrast to murine, bovine SRY decreases around 42 dpc, but remains at low levels until approximately 60 dpc, and it is subsequently reactivated in adult bulls (Planells et al., 2019, Ross et al., 2009).

In males, after gonadal sexual differentiation, gonocytes begin to cluster with developing Sertoli cells to start forming testicular cords (Ross et al., 2009, Skinner and Anway, 2005). As fetal development continues, distinct seminiferous cords form with Sertoli cells fully enclosing populations of gonocytes (Planells et al., 2019, Ross et al., 2009). At birth, the mitotically arrested gonocytes are separated from the basement membrane of the seminiferous cords by immature Sertoli cells (Culty, 2013). During male fetal development gonocytes proliferate, but they do not start meiosis until after puberty. In contrast, throughout fetal development female oogonia remain

dispersed across the gonad, start meiosis I, and develop primordial follicles. At birth, primary oocytes are arrested in prophase I and will resume meiosis after puberty (DeFalco and Capel, 2009).

2.5. Germ cell sex determination

Before their arrival at the genital ridge, XX and XY PGCs do not exhibit any known differences (Bowles and Koopman, 2010). However, following the completion of their migration and germ cell determination, these cells start to differentiate based on the specific signals they receive from the somatic cells of the developing genital ridges. Retinoic acid emanating mainly from the mesonephros, the embryonic kidney from where the genital ridges arose, acts as a signal to the gonocytes to enter meiosis (Bowles et al., 2006). During this stage, germ cells in both sexes are primed to enter meiosis, but their pathways differ. Female oogonia undergo DNA replication prior to meiosis, and enter meiosis under the influence of retinoic acid (Bowles and Koopman, 2010). As development progresses, somatic pre-granulosa cells surround the oocytes, resulting in most or all oocytes being enclosed in primordial follicles by the time of birth. On the other hand, male gonocytes undergo mitotic arrest. Initially, fibroblast growth factor 9 (FGF9) is expressed in the gonads of both sexes, but its expression significantly increases in the developing testis shortly after the activation of SRY and SOX9 in pre-Sertoli cells (Bowles et al., 2010). In addition, male Sertoli cells produce the enzyme cytochrome P450 family 26 subfamily B member 1 (CYP26B1), which degrades retinoic acid, leading to low levels of retinoic acid in the testis and high levels of FGF9 (Bowles et al., 2006). In the ovary, retinoic acid is not degraded, and FGF9 levels are low. Retinoic acid directly stimulates germ cells to increase the expression of stimulated by retinoic acid 8 (STRA8*)*, which is essential for meiotic entry. Conversely, FGF9 directly acts on germ cells to inhibit *Stra8* expression, maintain the expression of pluripotency markers (*Oct4* and *Sox2*), and

induce male germ cell fate markers (*Nanos2,* and DNA methyltransferase 3 like *(Dnmt3L)*) (Bowles et al., 2010). *Nanos2,* an RNA-binding protein, is upregulated around the same time that the retinoic acid-degrading enzyme *Cyp26b1* is downregulated, ensuring the prevention of premature meiotic entry. Male germ cells remain undifferentiated until puberty, whereas primary oocytes in females enter meiosis and are arrested in prophase I at birth.

After birth, male gonocytes resume their proliferation and migrate towards the basement membrane using pseudopods, and are then referred to as spermatogonia (Culty, 2013). SSCs, which are located in the basal compartment of the seminiferous tubules, do not directly produce sperm. Instead, they generate progenitor cells that undergo proliferation, differentiation, and meiosis to eventually produce haploid cells, which mature into sperm (Kubota and Brinster, 2018). Undifferentiated spermatogonia can be categorized into different types based on their morphology and arrangement. In mice, these types include single spermatogonia (As), paired spermatogonia (Apr), and aligned spermatogonia (Aal) (de Rooij, 2009). The As cells serve as a reservoir of germline stem cells and proliferate to become Apr cells, which are connected in pairs by intercellular bridges. Subsequently, the Apr cells further differentiate into Aal cells, where 4, 8, 16, and sometimes 32 cells are interconnected. As, Apr, and Aal are collectively known as undifferentiated spermatogonia. Within this population, As cells are considered the SSCs, while the transition from Apr to Aal represents the progenitor population. Aal cells then begin to differentiate into A, Intermediate, and B spermatogonia, which eventually undergo meiosis to become primary spermatocytes. It is important to note that each cell division during spermatogenesis is incomplete, as the cells remain connected through cytoplasmic bridges to ensure synchronized development. Throughout spermatogenesis, specific signals and

environmental cues play a vital role in coordinating the differentiation of germ cells (de Rooij, 2009).

2.6. The role of *NANOS* in eutherian germline development

NANOS, an evolutionarily conserved group of RNA-binding proteins, play crucial roles in germ cell development across various organisms (De Keuckelaere et al., 2018). First discovered in *Drosophila,* the *NANOS* (nos) gene encodes a single protein that is required for the development of both the male and female germlines (Wang and Lehmann, 1991). In Drosophila, the maternally supplied Nanos protein is involved in the migration of PGCs into the gonad and it plays a crucial role in maintaining germ cell development by interacting with the Pumilio RNA-binding protein and employing translational repression mechanisms (Kobayashi et al., 1996, Sonoda and Wharton, 1999). Also, in species where maternal factors are pivotal in germ cell specification (e.g., Drosophila, C. elegans, and zebrafish), Nanos has specifically been implicated in the migration and maintenance of PGCs (Kobayashi et al., 1996, Köprunner et al., 2001, Sonoda and Wharton, 1999).

While mechanisms to maintain germ cells are highly conserved across the animal kingdom, the mechanisms of determining germ cell fate are distinct, especially in mammals, where zygotic genes play a more prominent role (Tsuda et al., 2003). In mice, three *Nanos* gene homologs have been identified, with varying effects on germ cell fate and developmental pathways (Tsuda et al., 2003). *Nanos1*, although observed in the seminiferous tubules of mature mouse testes, is not detected in mouse PGCs and is dispensable for murine reproduction (Haraguchi et al., 2003). In contrast, *NANOS1* mutations in humans are associated with male infertility due to spermatogenesis defects (Kusz-Zamelczyk et al., 2013). *Nanos2* is predominantly expressed in male germ cells and is essential for maintaining the SSC population but is not required for female germline

development or fertility (Tsuda et al., 2003). On the other hand, murine *Nanos3* is present in migrating PGCs of both sexes, and its elimination leads to the complete loss of male and female germ cells (Tsuda et al., 2003).

Nanos genes encode proteins that contain a conserved carboxy-terminal zinc finger motif (CCHC) (De Keuckelaere et al., 2018). This zinc finger domain is essential for NANOS function as it facilitates RNA binding and interactions with other proteins (Jaruzelska et al., 2003, Sonoda and Wharton, 1999). Remarkably, the CCHC zinc-finger motif is 100% conserved across phyla, from lower organisms like fruit flies and roundworms to mammalian NANOS family members (Beer and Draper, 2013, Bhandari et al., 2014, De Keuckelaere et al., 2018). Additionally, NANOS proteins of all vertebrates and a few invertebrates (e.g., jellyfish) have a similar N-terminal region of 17 amino acids (AA) called NOT1 interacting motif (NIM), but the sequences of the N-terminal domains of the various NANOS proteins are not conserved. The evolutionary conservation of NANOS across diverse species, including mammals, and its specific expression in germ cells make it a highly intriguing gene family to study.

2.7. NANOS1

In mice, maternally derived, N*anos1* was observed in substantial amounts in oocytes and transient zygotic *Nanos1* expression was observed at eight-cell, morula, and blastocyst stages (Haraguchi et al., 2003). Additionally, *Nanos1* expression re-emerges in the central nervous system during later embryonic stages and continues to be expressed in the adult brain. In the testis, *Nanos1* is detected in the seminiferous tubules but not in PGCs. Surprisingly, *Nanos1* KO mice develop normally without any detectable abnormalities and maintain fertility, indicating that murine *Nanos1* is not essential for normal germline development (Haraguchi et al., 2003). In humans, the role of *NANOS1* appears to differ. NANOS1 has been shown to interact with the human PUMILIO-

2 protein, a known RNA binding protein. NANOS1 and PUMILIO-2 form a stable complex and are co-expressed in spermatogonia, which suggests that NANOS1 may play a role in post-transcriptional regulation of gene expression in human spermatogonia (Jaruzelska et al., 2003). Furthermore, mutations in the *NANOS1* gene have been associated with male infertility in humans, specifically due to defects in spermatogenesis (Kusz-Zamelczyk et al., 2013). These findings suggest that *NANOS1* may have a significant role in human male fertility, in contrast to its dispensability in mice.

2.8. NANOS2

Nanos2 is predominantly expressed in male germ cells, and the elimination of this gene results in a complete loss of spermatogonia. In the first mammalian studies, RT-PCR of isolated murine PGC fractions revealed that murine *Nanos2* was expressed only in developing male PGCs on embryonic day 13.5-16.5 (E; equivalent of dpc) (Tsuda et al., 2003). To investigate the consequences of *Nanos2* deficiency, Tsuda et al. (2003) generated both heterozygous and homozygous *Nanos2* KO mice of both sexes, which were all viable and showed no apparent developmental abnormalities. Both heterozygous and homozygous *Nanos2* KO female mice were fertile with morphologically and functionally normal ovaries. Additionally, heterozygous *Nanos2*+/- KO male mice were fertile with morphologically and functionally normal testes. In contrast, significant germ cell deficiency was observed in homozygous *Nanos2*-/- KO male mice. In these *Nanos2*-/- KO male mice, at E14.5 the testicular cords were well organized with germ cells normally localized. However, from E15.5 some germ cells started to localize outside the seminiferous tubules, and their numbers gradually decreased. By four weeks of age, no germ cells were observed in the testes of *Nanos2*-/- KO male mice. Additionally, TUNEL assays showed apoptosis in germ cells from E15.5 onwards, coinciding with the gradual loss of germ cells.

Furthermore, the testes of four-week-old *Nanos2*-/- KO mice were significantly reduced in size and weight, measuring only about 30% of that observed in wild-type mice.(Tsuda et al., 2003).

Further studies revealed that murine *Nanos2* plays an important role in leading germ cells to male-type differentiation in the embryonic stage, including inhibiting premature meiosis (Kato et al., 2016, Niimi et al., 2019, Saba et al., 2014, Sada et al., 2009, Suzuki et al., 2010, Suzuki et al., 2016, Suzuki et al., 2012, Suzuki and Saga, 2008, Suzuki et al., 2007).

Nanos2 was found to be a key regulator of meiosis in male gonocytes by inhibiting *Stra8* expression, which is required for premeiotic DNA replication, after *Cyp26b1* is decreased during fetal development (Suzuki and Saga, 2008). *Nanos2*-null male gonocytes were observed to undergo normal mitotic arrest at E14.5 but failed to maintain the arrest and reinitiated proliferation from E15.5 (Saba et al., 2014, Suzuki and Saga, 2008). In addition to preventing premature meiosis, Suzuki and Saga (2008) found that *Nanos2* activates a male-specific genetic program. This was evident when *Nanos2* was artificially expressed in female gonocytes, resulting in the failure of Stra8 expression in response to retinoic acid signaling and the adoption of characteristics associated with male germ cell fate (Suzuki & Saga, 2008).

Murine *Nanos2* was found to be expressed in a small subset of undifferentiated spermatogonia and through lineage-tracing found that these cells were able to self-renew and generate the entire spermatogenic cell lineage (Sada et al., 2009). In mammals, SSC pools in postnatal testes are maintained through self-renewal in order to continuously generate spermatozoa. Since eliminating *Nanos2* results in the complete loss of spermatogonia (Tsuda et al., 2003), to elucidate the function of *Nanos2* during spermatogenesis, Sada et al. (2009) used a conditional KO (cKO) approach to disrupt *Nanos2* postnatally. Most of the *Nanos2*-cKO tubules were devoid of any germ cells shortly after birth (12 weeks-old). Additionally, by only 2-weeks-

old the number of undifferentiated spermatogonia (identified by the marker Promyelocytic Leukemia Zinc Finger or PLZF) declined rapidly. Conversely, when *Nanos2* was overexpressed in postnatal testes, the number of undifferentiated spermatogonia (PLZF positive) significantly increased, while differentiated spermatogonia (identified by the marker KIT) were rarely observed. These findings demonstrated that murine *Nanos2* played a vital role in maintaining the immature state of SSCs by supporting their self-renewal properties and suppressing differentiation (Sada et al., 2009).

Suzuki et al. (2010) began to elucidate *Nanos2* mechanisms in mice. They initially observed that NANOS2 localized to processing (P)-bodies, which are RNA degradation centers that are abundantly accumulated in male gonocytes. Then through immunoprecipitation assays, they discovered that components of the CCR4-NOT (CNOT; carbon catabolite repression 4–negative on TATA-less) deadenylation complex coprecipitated with NANOS2 from male gonadal extracts. The CNOT deadenylation complex comprising of at least 10 CNOT proteins, regulates gene expression by shortening the poly(A) tails of targeted mRNAs, leading to their rapid degradation (Bartlam and Yamamoto, 2010). In the testes of mice, CNOT proteins were found to colocalize with NANOS2 in P-bodies, suggesting that NANOS2 promotes the localization of CNOT proteins to P-bodies *in vivo* (Suzuki et al., 2010). Importantly, they demonstrated that NANOS2/CNOT complex retains its deadenylase activity *in vitro*. They also discovered that certain RNAs implicated in in meiosis, such as *Stra8, Sycp3*, and *Dazl*, interacted with NANOS2 and accumulated in its absence. These findings indicated that meiosis-promoting RNA molecules are normally suppressed through a NANOS2-mediated mechanism involving recruitment to P-bodies and degradation by the enzymes present within, facilitated by NANOS2-mediated deadenylation.(Suzuki et al., 2010).

Suzuki et al. (2012) next demonstrated the crucial role of the interaction between NANOS2 and the CNOT Deadenylation Complex in the biological functions of *Nanos2 in vivo*. They identified CNOT1, the largest protein and scaffold component of the CNOT deadenylation complex, as a direct factor mediating the interaction with NANOS2. To investigate the interaction between NANOS2 and CNOT1, they systematically deleted different regions of the *Nanos2* gene and examined the resulting mutated NANOS2 protein's interaction with CNOT1 (Suzuki et al., 2012). They found that the deletion of the first 10 amino acids (AA) from the N-terminal region of NANOS2 abolished its interaction with CNOT1. Moreover, when transgenic mice expressing a *Nanos2* variant lacking the first 10 AAs (NANOS2-ΔN10) were generated, these mice were unable to rescue the *Nanos2*-null phenotype, despite this variant retaining the highly conserved CCHC-type zinc finger motif. This led to the conclusion that the first 10 AA of the N-terminal region of murine NANOS2 are essential for binding with CNOT1 and for the subsequent functional role of NANOS2 in the male testis.

To investigate the broader role of *Nanos2* in the sexual differentiation of male germ cells beyond its function in meiosis suppression, Saba et al. (2014) conducted a study using *Nanos2*/*Stra8* double KO (dKO) mice. These mice lacked both *Nanos2* and *Stra8*, which successfully prevented meiosis in male germ cells. The researchers observed that, similar to *Nanos2* KO mice, the expression of male-specific genes was still reduced in the dKO mice. Furthermore, the germ cells in the dKO mice exhibited characteristics more akin to undifferentiated PGCs rather than quiescent male germ cells (Saba et al., 2014). These results suggest that *Nanos2* has pivotal roles in the sexual differentiation of male germ cells, extending beyond its function as a meiosis suppressor. One possible function of *Nanos2* is to terminate the undifferentiated state of PGCs. Importantly, even in the absence of *Stra8*, male gonocytes of the

dKO mice were observed to abnormally resume of the cell cycle. This indicates that *Nanos2* also contributes to the maintenance of mitotic quiescence independently of its role in meiosis suppression.

Given that additional deletion of *Stra8* in a *Nanos2*-deficient background did not rescue the defective male-specific gene expression, Kato et al. (2016) aimed to uncover the specific targets regulated by *Nanos2* to gain insight into its role in the sexual differentiation of XY germ cells. Through a comprehensive microarray analysis, they identified *Dazl* as a crucial target of NANOS2. *Dazl* is a germ cell-specific gene encoding an RNA-binding protein involved in translation. The researchers demonstrated that NANOS2 exerts direct post-transcriptional repression on *Dazl* expression in sexually differentiating XY germ cells (Kato et al., 2016). Since DAZL is implicated in activating translation of its target RNAs, they hypothesized that NANOS2 could counteract this activity of the DAZL protein in order to regulate other target RNAs. They found that many mRNAs, including those of genes involved in meiosis and oogenesis, were commonly associated with both NANOS2 and DAZL, suggesting that NANOS2 could act as an antagonist of the DAZL protein. Based on their findings, Kato et al. (2016) proposed a dual system of NANOS2-mediated suppression of *Dazl* expression and its target RNAs as a pivotal molecular mechanism promoting the sexual differentiation of XY germ cells.

Suzuki et al. (2016) aimed to further understand the molecular mechanisms underlying the target specificity of the NANOS2/CNOT deadenylase complex. They employed immunoprecipitation (IP) coupled with mass spectrometry to identify proteins interacting with NANOS2 in male gonadal extracts. Their investigation led them to discover that Dead end1 (DND1), another RNA-binding protein, directly interacts with NANOS2 and plays a role in loading specific RNAs into the CNOT complex (Suzuki et al., 2016). To investigate the interaction

between NANOS2 and DND1, the researchers performed systematic mutations in different regions of the *Nanos2* gene, focusing on the conserved zinc finger motifs. They found that substitution of the first cysteine residues in the two CCHC motifs of *NANOS2* (C61 and C96) with alanine, yielding NANOS2 (C61A, C96A), drastically reduced and almost abolished the interaction with DND1. Moreover, single mutations in either CCHC motif (C61A or C96A) also drastically reduced this interaction (Suzuki et al., 2016). This indicates that both CCHC-type zinc finger motifs are structurally essential for the interaction between NANOS2 and DND1 and demonstrate that the two conserved, consecutive CCHC-type zinc finger motifs of NANOS2 constitute a protein-interacting domain for DND1 (Suzuki et al., 2016). Additionally, in undifferentiated spermatogonia in postnatal murine testes, DND1 was found to associate with NANOS2 and interact with known NANOS2-target mRNAs (Niimi et al., 2019). This study provided evidence that DND1 acts as a partner of NANOS2 not only in male embryonic germ cells, but also in undifferentiated spermatogonia and this interaction is proposed to play a crucial role in supporting the survival of differentiating spermatogonia (Niimi et al., 2019).

2.9. NANOS3

Nanos3 is primarily expressed in migrating PGCs, and its absence leads to a complete loss of germ cells in both male and females. Early studies using RT-PCR analysis on isolated PGC fractions demonstrated that murine *Nanos3* expression is detected in migrating PGCs during the developmental period of E9.5-14.5 in both sexes (Tsuda et al., 2003). Subsequent research employing transgenic mouse models revealed that *Nanos3* expression initiates as early as E7.25 in newly induced PGCs (Yamaji et al., 2010). In female germ cells, *Nanos3* expression persists until approximately E14.5, coinciding with the onset of meiosis. After this stage, *Nanos3* expression diminishes and is no longer detectable in fetal female germ cells. Conversely, in male germ cells,

Nanos3 expression continues throughout the fetal period, albeit at declining levels after E16.5, which corresponds to the time when male germ cells typically enter mitotic arrest (Yamaji et al., 2010).

The consequences of *Nanos3* deficiency were investigated by Tsuda et al. (2003) through the generation of heterozygous and homozygous *Nanos3* KO mice in both sexes. The *Nanos3* KO mice were viable and did not exhibit any apparent developmental abnormalities. Analysis of PGCs during early embryonic stages revealed that *Nanos3* deficiency did not affect the specification and derivation of PGCs, as a comparable number of PGCs were observed in *Nanos3* KO and wild-type embryos at E7.5. However, a significant reduction in the number of PGCs was observed during the migration phase (E8.5-9.5) in *Nanos3* KO embryos compared to wild-type embryos. This decline continued during the settlement phase (E10.5-11.5), resulting in a near absence of PGCs in the gonadal ridge of *Nanos3* KO embryos, while PGCs in wild-type embryos continued to proliferate. Additionally, the few PGCs that managed to reach the genital ridge in *Nanos3* KO embryos eventually underwent cell death and failed to develop into germ cells. These findings indicate that *Nanos3* is crucial for the maintenance and survival of PGCs during their migration and gonad colonization (Tsuda et al., 2003).

In the study by Suzuki et al. (2008), two potential mechanisms were considered for the loss of PGCs in *Nanos3* KO embryos: apoptotic cell death or abnormal differentiation into somatic cells. The researchers speculated that apoptosis was the most likely reason for the PGC loss since apoptosis is a common mechanism for eliminating abnormal cells during development. Previous studies have shown that during PGC migration, a significant number of cells deviate from their normal migratory path and are subsequently eliminated through apoptosis (Runyan et al., 2006, Stallock et al., 2003). Even among the germ cells that successfully localize in the gonad, a

considerable proportion undergo apoptosis. In male gonadal germ cells, apoptosis occurs in waves between E13.5 and E17.0, followed by a second wave around the time of birth (Wang et al., 1998). Similarly, female gonadal germ cells also experience apoptotic cell death at different stages, including around E13.5, between E15.5 and birth, and after birth (Bakken and McClanahan, 1978, Beaumont and Mandl, 1963, Borum, 1961). To investigate the role of *Nanos3* in preventing PGC apoptosis during migration, Suzuki et al. (2008) employed immunostaining of activated Caspase3, which revealed increased apoptosis in migrating PGCs of *Nanos3* KO embryos. This finding provided evidence that *Nanos3* is necessary to protect PGCs from undergoing apoptosis during their migration. Furthermore, lineage analysis confirmed that the PGCs in *Nanos3* KO embryos underwent cell death rather than abnormal differentiation into somatic cells.

To further investigate the apoptotic mechanism, Suzuki et al. (2008) generated double KO embryos lacking both *Bax* and *Nanos3*. Previous studies have shown that ectopic PGCs, which are located outside of the genital ridges, are eliminated through *Bax*-dependent apoptotic mechanisms (Runyan et al., 2006). However, the researchers discovered that the elimination of *Bax* did not fully rescue PGC apoptosis in *Nanos3*-null embryos. Only a fraction of the PGCs survived in the double KO embryos. These rescued PGCs, present in small numbers, were able to persist and differentiate into male and female germ cells in the adult gonads. The surviving female germ cells displayed normal differentiation based on their morphology and expression of marker genes. In contrast, the surviving male germ cells exhibited abnormal proliferation and were observed in central regions of the tubules rather than being confined to the periphery, indicating disrupted spermatogenesis. Additionally, mature sperm were not detected in the dKO mice. Based on these findings, Suzuki et al. (2008) concluded that while *Nanos3* is not directly involved in germ cell

differentiation itself, it plays a critical role in maintaining the germ cell lineage by suppressing both *Bax*-dependent and *Bax*-independent apoptotic pathways.

Suzuki et al. (2010) investigated the regulatory mechanisms that restrict the expression of *Nanos3* to germ cells in mice. They observed that while *Nanos3* transcripts were present in both germ and somatic cell lineages, efficient translation only occurred in the germ lineage. The researchers sought to understand the mechanism behind this selective translation by focusing on the 3' untranslated region (3'UTR) of *Nanos3*, which has been implicated in spatial and temporal regulation of *Nanos* expression in other species (Kuersten and Goodwin, 2003). To examine its role in mice, Suzuki et al. (2010) generated transgenic mice with an exogenous 3'UTR (Bovine growth hormone poly(A) signal - BghpA) replacing the endogenous *Nanos3* 3'UTR, along with a red fluorescent protein (RFP) marker. They confirmed that this transgene was able to rescue the *Nanos3* KO phenotype, indicating that the construct contained regulatory elements sufficient to maintain endogenous *Nanos3* expression, and the NANOS3-mRFP protein was functional. In embryos carrying the transgene with the exogenous 3'UTR, the expression of NANOS3-mRFP gradually increased in somatic tissues as embryonic development progressed. Ultimately, the entire body exhibited NANOS3-mRFP expression. These findings indicate that *Nanos3* is transcribed in multiple embryonic tissues, but the presence of the *Nanos3*-3'UTR is crucial for suppressing translation in somatic tissues. Further experiments with additional transgenic mice confirmed that the *Nanos3*-3'UTR specifically restricted mRFP expression to germ cells, even when driven by a strong ubiquitous promoter (CAG promoter). This finding led to the conclusion that the *Nanos3*-3'UTR plays an essential role in translational control during mouse embryonic development (Suzuki et al., 2010).

In postnatal mice, the expression pattern of *Nanos3* differs between males and females. In females, *Nanos3* is not detected, while in males, it exhibits robust expression. During prepubertal stages, *Nanos3* is strongly expressed in undifferentiated spermatogonia (Lolicato et al., 2008, Suzuki et al., 2009, Yamaji et al., 2010). This expression is observed in newborn male gonocytes and by the age of one week is present in almost all stages of undifferentiated spermatogonia. However, 1-week later, *Nanos3* expression becomes restricted to a subset of spermatogonia and is not observed in spermatocytes undergoing meiotic divisions (Yamaji et al., 2010). Undifferentiated type-A spermatogonia in mice are classified into three subtypes: single (As), paired (Apr), and aligned (Aal) spermatogonia, based on their topographical arrangements (Russell et al., 1990). *Nanos3* is detectable in most undifferentiated spermatogonia (As to Aal) as well as differentiating A1 spermatogonia. However, its expression is gradually downregulated after differentiation into A2 spermatogonia (Suzuki et al., 2009; Yamaji et al., 2010). The appearance of A1-A2 spermatogonia coincides with the expression of KIT, a marker of differentiating spermatogonia. While most *Nanos3*-positive undifferentiated type-A spermatogonia did not co-express c-Kit, the *Nanos3*-positive Aal spermatogonia strongly expressed c-Kit, which aligns with the stage at which Aal spermatogonia begin to differentiate into A1 spermatogonia (Suzuki et al., 2009, Yamaji et al., 2010). In contrast, *Nanos2* is predominantly expressed in As to Apr cells, indicating a distinct expression pattern from *Nanos3*. Overall, *Nanos3* exhibits predominant expression in later stages of undifferentiated spermatogonia, suggesting specific functions in spermatogonial progenitor cells (Suzuki et al., 2009, Yamaji et al., 2010).

To investigate the distinct functions of *Nanos2* and *Nanos3* during germline development, Suzuki et al. (2007) conducted a study using transgenic mice. They generated a transgenic mouse line that expressed *Nanos2* under the control of the *Oct4* proximal enhancer promoter, resulting in

a similar expression pattern to endogenous *Nanos3*. By ectopically expressing *Nanos2* in a *Nanos3*-null background from E8.0 onwards, they aimed to determine if *Nanos2* could rescue the *Nanos3*-null defects. The study revealed that when *Nanos2* was ectopically expressed (from E8.0 onwards) in the absence of *Nanos3*, it partially rescued the *Nanos3*-null phenotype. Although the number of PGCs that reached the genital ridge was still lower than in wildtype mice, the presence of ectopic *Nanos2* led to a significant increase in the number of PGCs compared to the *Nanos3* KO alone. Furthermore, these PGCs appeared to be maintained in the developing gonads of both male and female transgenic embryos. This finding indicated that *Nanos2* can functionally substitute for *Nanos3* during early PGC development. In contrast, the study found that *Nanos3* was not able to rescue the defects observed in *Nanos2*-null mice. Therefore, the study by Suzuki et al. (2007) demonstrated that *Nanos2* and *Nanos3* perform overlapping functions during early PGC development, but *Nanos2* plays a unique role in male germ cell development.

Several studies have provided insights into the mechanism of *Nanos3* in mice. NANOS3 was found to co-localize with mRNA degradation machinery, such as stress granules and P-bodies, similar to NANOS2 (Yamaji et al., 2010). Both NANOS2 and NANOS3 have been found to be associated with the CNOT deadenylation complex. However, NANOS3 was found to interact directly with a small component CNOT8, whereas NANOS2 interacts with the large scaffolding protein CNOT1. Additionally, NANOS3 exhibited lower deadenylase activity compared to NANOS2 *in vitro* (Suzuki et al., 2014). These findings suggest that the different interactions of NANOS2 and NANOS3 with the CNOT complex may contribute to their functional redundancy and differences (Suzuki et al., 2014).

To further understand the functional differences, Wright et al. (2021) conducted a study examining the structure of NANOS2 and NANOS3 proteins. They generated chimeric mice

expressing proteins with swapped N-terminal and zinc finger motifs between NANOS2 and NANOS3. However, neither chimeric protein fully rescued the male differentiation pathway, indicating that the combination of both the N-terminal and zinc finger motif of NANOS2 are essential for male-type differentiation. Previous experiments demonstrated that DND1 directly interacts with NANOS2 for RNA target specificity and that the zinc finger motifs are crucial for this interaction (Suzuki et al., 2016). Therefore, Wright et al. (2021) investigated the binding between DND1 and NANOS proteins. They found that DND1 strongly bound to the NANOS2 zinc finger motif but not the NANOS3 zinc finger motif. Interestingly, the chimeric protein with the NANOS3 N-terminal but NANOS2 zinc finger motif showed comparable binding to DND1 as NANOS2. These results indicate that the zinc finger domain of NANOS2 plays a crucial role in its binding ability to DND1. Consequently, the inability of *Nanos3* to rescue *Nanos2* function may be attributed to poor DND1 recruitment and CNOT1 binding and lower deadenylation activity (Suzuki et al., 2014, Wright et al., 2021).

To determine whether *Nanos3* plays any role in male germ cell differentiation, Wright et al. (2021) conducted a study using cKO mice. They specifically eliminated *Nanos3* at the time when its expression is normally downregulated (E11.5), while *Nanos2* is upregulated. The expression of *Nanos2* was unaffected in the cKO *Nanos3* mice, and male germ cell differentiation proceeded normally even in the absence of *Nanos3*. This suggests that *Nanos2* alone can protect against germ cell death and promote male differentiation. However, when the researchers generated conditional dKO *Nanos2*/3 mice, they observed a more rapid loss of germ cells during the sexual differentiation stage compared to the cKO of *Nanos2* alone. In the absence of *Nanos2*, *Nanos3* was upregulated and allowed the germ cells to survive for a longer period, but the cells did not undergo male-type differentiation. This indicates that *Nanos3* may have a role in

suppressing apoptosis when *Nanos2* is absent, but it cannot fully rescue the male differentiation phenotype (Wright et al., 2021).

As *Nanos3*-deficient mice lose all germ cells before birth, in order to assess the role of *Nanos3* in spermatogenesis, Inoue et al. (2022) produced *Nanos3*-RFP labeled cKO mice. When *Nanos3* was eliminated at the perinatal stage (E13.5), the cKO mice were able to produce functional sperm, but their testis size was noticeably reduced, and there was a progressive decrease in the number of germ cells over time. At 4 weeks of age, the relative number of undifferentiated spermatogonia (marked by PLZF immunostaining) in the cKO testes was significantly lower compared to the control testes. Subsequently, the numbers of differentiating spermatogonia (marked by KIT immunostaining) and meiotic spermatocytes (marked by SYCP3 immunostaining) were also reduced in the cKO testes (Inoue et al., 2022)

To determine which population of undifferentiated spermatogonia was affected by *Nanos3* depletion, the researchers classified the cells into subtypes and counted only the *NANOS3*-deficient cells (marked by the absence of RFP). Interestingly, the number of one subtype, Apr spermatogonia, was not significantly different between the control and cKO mice. However, the number of Aal spermatogonia was significantly reduced in the cKO mice, suggesting that *Nanos3* is specifically required for the expansion of Aal spermatogonia or spermatogonial progenitors. Surprisingly, the decrease in spermatogonial progenitors in the cKO mice was not due to apoptosis, but rather premature differentiation, as indicated by an increased proportion of STRA8-positive undifferentiated spermatogonia in the cKO testes. Overall, the study by Inoue et al. (2022) suggests that *Nanos3* plays an important role in regulating the proper timing of progenitor expansion during spermatogenesis.

2.10. Summary of *NANOS* in mammalian germline development

In summary, the evolutionarily conserved RNA-binding proteins, NANOS2 and NANOS3, play crucial roles in mammalian germ cell development and differentiation. While *Nanos3* is involved in protecting germ cells from apoptosis during migration and colonization in both sexes, *Nanos2* is male-specific and necessary for male germ cell differentiation during fetal development. Both proteins become expressed again during spermatogenesis, during which *Nanos2* is exclusively expressed in SSCs (As – Apr) and *Nanos3* is primarily expressed in later Aal spermatogonia or progenitor cells. These proteins have distinct expression patterns and functions. However, there is uneven functional redundancy given that ectopic *Nanos2* can compensate for the loss of *Nanos3* in embryonic PGC development and postnatal spermatogenesis, but ectopic *Nanos3* cannot rescue or compensate for the loss of *Nanos2* in spermatogenesis.

NANOS2 and NANOS3 both share common conserved zinc-finger domains and similar N-terminal regions that are essential to their roles in germ cell development. The zinc-finger domain is crucial for their RNA-binding ability and their interactions with other proteins involved in translational regulation. Through their RNA-binding activity, NANOS2 and NANOS3 exert translational repression of target mRNAs to control germ cell development via recruitment of deadenylases, such as the CNOT complex. By repressing the translation of specific transcripts, NANOS2 and NANOS3 play pivotal roles in regulating the expression of key factors involved in meiosis and germ cell differentiation.

3. GERMLINE COMPLEMENTATION STRATEGIES

Germline complementation is the process of using donor cells from one genetic background to complement or replace the germline of an otherwise sterile host of a different genetic background. Successful germline complementation requires two components, donor cells

that are capable of becoming gametes and a germline ablated host capable of supporting gametogenesis (Ledesma et al., 2023, Oback and Cossey, 2023). An application that is particularly enticing to the livestock industry is the possibility of using germline complementation to generate germ cells from elite donor animals (e.g. sires used in artificial insemination programs) exogenously in the gonads of otherwise sterile host animals that have inferior genetics (e.g. herd or commercial sires), thereby expanding the availability of gametes from genetically desirable dams and sires (Gottardo et al., 2019).

Ideal hosts for germline complementation are animals that lack an endogenous germline but provide an intact gonadal niche to support donor-derived gametogenesis. A promising method is to use genetic tools, like GnEd, to inactivate essential genes for germline production at the host embryo stage to create a germline developmental niche. Targeted gene disruption in a developing embryo can lead to the loss of germ cells through failure of PGC specification, migration, proliferation, or commitment to the germ cell fate. Potential sources of germline competent donor cells are blastomeres, stem cells, including embryonic stem cells (ESC), induced pluripotent stem cells (iPSC), and SSC, and primordial germ cell-like cells (PGCLCs) (Bishop and Van Eenennaam, 2020, Ledesma et al., 2023, Mueller and Van Eenennaam, 2022, Oback and Cossey, 2023). Preimplantation embryos can be dissociated to yield individual blastomeres that are totipotent, but not self-renewing (McLean et al., 2020). In contrast, stem cells are self-renewing. SSCs can be isolated from mature or juvenile testes and are capable of spermatogenesis (i.e., unipotent), but are not pluripotent (Ciccarelli et al., 2020, Giassetti et al., 2019). ESCs are derived from the ICM of preimplantation blastocysts, while iPSCs are somatic cells that have been reprogrammed by exogenous expression of the Yamanaka factors (Kumar et al., 2021, Takahashi and Yamanaka, 2006). ESCs and iPSCs, which are both pluripotent stem cells (PSC), can be

induced in culture to become PGCLCs (Hayashi et al., 2012). In mice, PGCLCs have been further induced to form *in vitro* gametes, that were capable of producing live, fertile offspring (Hayashi et al., 2012, Ishikura et al., 2016, Yoshino et al., 2021). Although bovine PGCLCs have yet to be produced, the ability to derive bovine ESCs now makes this strategy a possibility (Goszczynski et al., 2018, Ledesma et al., 2023).

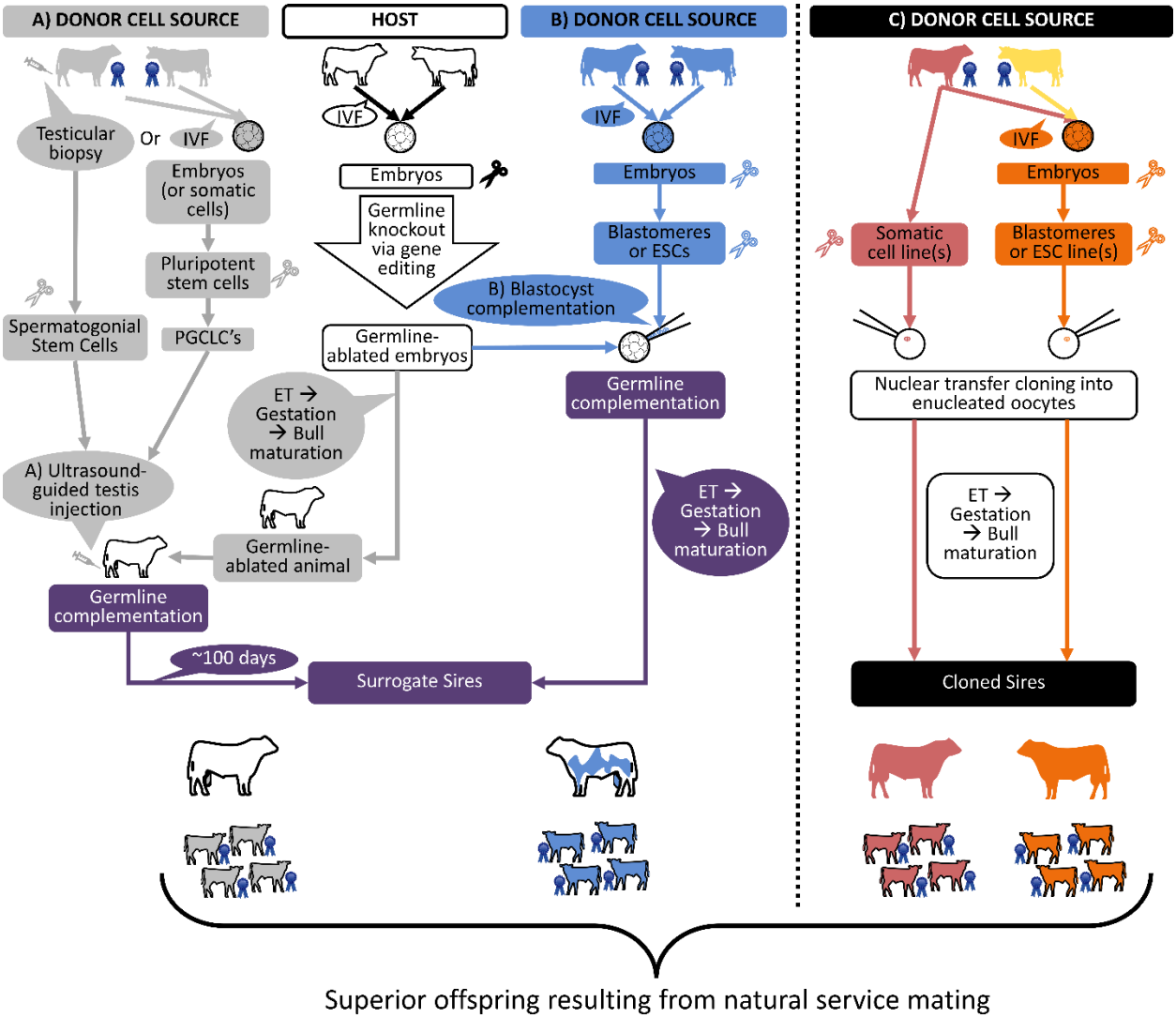

Figure 1.1. Schematic comparing potential surrogate (A-B) and cloned (C) sire production systems to ultimately result in superior offspring produced via natural service mating. A-B) For surrogate sire production, the steps to generate the host animal are in white with black outlines. A) grey and B) blue represent potential alternative sources and steps for generating donor cells. Dark

purple represents the germline complementation steps and the resulting final surrogate sire product. Key differences are that in the A path (grey), germline complementation would take place in a live, juvenile or adult, animal so the host would be non-mosaic. Alternatively, in the B path (blue), germline complementation would take place at the embryo stage and the resulting host could be mosaic. C) For cloned sire production, red steps represent standard somatic cell nuclear transfer (SCNT) cloning, which would result in a clone of the donor sire (i.e., the current generation). Alternatively, the orange steps represent nuclear transfer cloning of embryonic cells, which would result in a clone of an embryo (i.e., the next generation). Blue ribbons represent elite genetics and scissors represent steps that require gene editing (solid fill) or where gene editing could potentially be introduced (outline only). IVF: *in vitro* fertilization. PGCLC: primordial germ cell-like cells, ESC: embryonic stem cell. ET: embryo transfer. Adapted from Mueller and Van Eenennaam (2022) and reproduced from Ledesma et al. (2023) under a CC-BY license.

The timing of germ cell loss in the host and donor cell source determines which complementation strategy can be used (Figure 1.1). The two different germline complementation strategies that currently exist are testis complementation or embryo complementation, and both result in the formation of chimeras that are derived from more than one genotype (Ledesma et al., 2023, Oback and Cossey, 2023). Testis complementation involves injecting donor SSCs or PGCLCs into a juvenile or adult host's germline-deficient gonad, resulting in the production of a 'secondary' chimeras, where tissues are combined after organogenesis. Alternatively, embryo complementation involves combining donor blastomeres or PSCs with a germline-ablated host during embryo development stage, thus producing 'primary' chimeras, where the genetically different cell populations already coexist during embryogenesis (Ledesma et al., 2023, Oback and Cossey, 2023). Embryo complementation is commonly referred to as blastocyst complementation,

which got its name from the original embryo complementation studies that typically injected donor cells into the host at the blastocyst stage. However, host embryos can be injected with donor cells at different stages of embryo development (8-cell to blastocyst), depending on the species and complementation goal (Dechiara et al., 2009). Germline complementation has also been accomplished in non-mammalian species, including fish and chickens. In chickens, the isolation of PGCs from blood or gonadal tissues during embryonic development allows for their subsequent injection into the bloodstream of developing embryos, resulting in the generation of germline chimeras (Ballantyne et al., 2021, Hu et al., 2022, van de Lavoir et al., 2006). Similarly, transplantation of fish PGCs and gonial cells into sterile hosts at different developmental stages, including blastula, larvae, and adults, has successfully achieved donor gametogenesis in various fish species (Goto and Saito, 2019).

Both testis and embryo complementation, the two strategies for germline complementation in mammals, present distinct challenges related to donor cell isolation, culture, genetic modification, and integration with the host (Oback and Cossey, 2023). In rodents, donor animals are castrated to isolate SSCs from their testes through enzymatic digestion and cell sorting. However, castration of elite sires is not feasible in livestock breeding, necessitating the collection of testicular biopsies and *in vitro* culture of SSCs to obtain sufficient quantities for transfer. The lack of universal SSC markers and optimized culture media for multiplying elite donor SSCs has limited their availability and application in livestock species. Additionally, obtaining testicular biopsies requires technical expertise and handling of live animals. Selection of the optimal injection site in the host testis varies based on the reproductive anatomy of the host species, impacting the ease and efficiency of SSC transfer. Successful SSC transfer in livestock has been achieved through ultrasound-guided injections into the rete testis and has been more successful

when immature rather than mature hosts are used (Ciccarelli et al., 2020). SSC transfer effectively represents germline cloning of the current generation of sires, whereas PGCLCs derived from ESCs would represent germline cloning of the next generation. Regardless of the donor cell source, the testis complementation method is invasive and requires handling of juvenile animals, potentially requiring multiple procedures to ensure continuous donor-derived spermatogenesis. An advantage to testis complementation is that the somatic component of the host is entirely host-derived, eliminating any potential challenges associated with primary chimeras.

Embryo complementation offers several advantages in livestock production, including reduced animal handling as the process occurs *in vitro* prior to embryo transfer (Oback and Cossey, 2023). Additionally, donor cells, either blastomeres or ESCs, are derived from embryos, allowing for germline cloning of the next generation and the potential for a diverse founder population, given that producing a large number of donor embryos is more feasible compared to live animals. Embryo complementation of germline-ablated hosts produces primary chimeras. So, although the germline is expected to be 100% donor-derived, the somatic component of the host is likely to be chimeric, meaning the surrogate host may have a mixed genetic background and phenotype that includes traits encoded by both the donor and host cell lines. A potential complication unique to embryo complementation is the unintentional creation of sex chimeras when female donors are combined with male hosts, or vice versa. This can result in hermaphrodite phenotypes. To avoid complications related to incomplete sex conversion, it is recommended to use only hosts and donors of the same sex. This can be achieved by utilizing sexed sperm for generating host and donor embryos, or by employing PCR-based sexing methods for both complementation partners. By ensuring the sex of host and donor cells are concordant, the challenges associated with sex chimeras can be eliminated. Embryo complementation via blastomere aggregation, a simpler

technique which requires less specialized equipment than embryo microinjection has been accomplished in livestock, but blastomeres have limited proliferation capacity (Ideta et al., 2016). In contrast, ESCs have unlimited self-renewal capacity, enabling sequential gene editing for trait stacking. ESCs would also allow for DNA extraction without harming the viability of the remaining stem cells, facilitating the use of genomic selection to select superior lines and the confirmation of targeted alterations introduced using GnEd. While germline transmission through ESC-based embryo complementation has not yet been achieved in livestock, the increasing availability and variety of embryonic-derived pluripotent stem cells in livestock species brings this approach closer to realization (Goszczynski et al., 2018, Ledesma et al., 2023).

An alternative method to germline complementation for producing donor-derived offspring is nuclear transfer cloning (Figure 1.1). Traditional SCNT allows for cloning of the current generation, while nuclear transfer cloning using cells from an embryo (such as ESCs or blastomeres) would enable cloning of the next generation (McLean et al., 2020). However, SCNT is inefficient and is often associated with abnormalities in offspring, limiting its use in livestock (Keefer, 2015). On the other hand, blastomere cloning has shown reduced incidence and severity of abnormal phenotypes compared to somatic clones, but its potential for multiplication is limited due to the small number of blastomeres per embryo (Heyman et al., 2002, McLean et al., 2020, Misica-Turner et al., 2007). Recent experiments have demonstrated that nuclear transfer cloning of ESCs yields similar blastocyst development rates compared to SCNT, with the potential for higher pregnancy rates and fewer offspring abnormalities (Bogliotti et al., 2018, McLean et al., 2020, Zhao et al., 2021).

3.1. Eutherian germline ablation & rescue

DAZL, an RNA-binding protein that is necessary for the correct acquisition of germ cell fate in both sexes, was the first gene targeted in mice to result in a complete loss of germ cells, with an intact somatic gonad (Rilianawati et al., 2003, Ruggiu et al., 1997, Saunders et al., 2003). In mice lacking *Dazl*, PGCs fail to commit to a germ cell lineage instead retaining their undifferentiated state and they are eventually lost to apoptosis, which results in sterile adult male and female mice (Chen et al., 2014, Gill et al., 2011, Lin and Page, 2005, Nicholls et al., 2019, Rilianawati et al., 2003, Ruggiu et al., 1997, Saunders et al., 2003). Using the testis complementation approach, Rilianawati et al. (2003) injected wildtype murine germ cell suspensions containing SSCs into the testis of *Dazl* KO mice and successfully produced donor-derived spermatozoa. *Dazl*-deficient, or knockdown, rat testes were found have an apparently intact SSC compartment, but clear failure to produce mature haploid gametes resulting in infertility (Dann et al., 2006, Richardson et al., 2009). However, when donor rat SSCs were transplanted into *Dazl*-deficient rats (i.e., testis complementation), they produced function spermatozoa resulting in 100% donor germline transmission to progeny by natural mating (Richardson et al., 2009). Elimination of *DAZL* in pigs also resulted in germline-ablation of both sexes, but the *DAZL* KO female pigs also developed spontaneous ovarian teratomas at a high rate, similar to murine observations (Nicholls et al., 2019). Most recently, the testes of *DAZL* null neonatal sheep were shown to lack prospermatogonia, or gonocytes, but maintain normal somatic cell morphology and known-marker expression (McLean et al., 2021). To date no studies have tested germline complementation of *DAZL* KO pigs or sheep.

Murine Ets-variant transcription factor 5 (*Etv5*), which is expressed by Sertoli cells, was found to be essential for SSC self-renewal and absence of *Etv5* severely impaired SSC

development resulting in male infertility (Chen et al., 2005). In *Etv5* KO mice testes, germ cells were gradually lost with age and a Sertoli cell-only phenotype was observed by 12-weeks of age. The KO of *Etv5* impairs the ability of Sertoli cells to form an optimal testicular environment to support spermatogenesis due to a defect in the stem cell niche. However, the overall structure of seminiferous tubule remains intact and remarkably Zhang et al. (2021) successfully obtained donor-derived spermatozoa via SSC testis complementation of *Etv5* KO mice, albeit at low efficiency. Additionally, *Etv5* KO mice were observed to be smaller in body size and weight compared with WT mice, indicating that *Etv5* has an influence on overall growth. *Etv5* mRNA has been detected in a variety of tissues, including the heart, lungs, thymus, lymphocytes, kidneys, and skeletal muscles and this widespread expression of *Etv5* during development may be crucial for growth (Liu et al., 2016). Therefore, *Etv5* is not an ideal target in livestock and thus far has not been studied in any livestock species.

In contrast, the RNA-binding protein gene, NANOS2, which is expressed specifically in male germ cells, has been targeted in multiple livestock species. As described in detail previously, *Nanos2* homozygous KO female mice are fertile, while homozygous KO male mice are infertile due to failure of male germ cell differentiation during fetal development (Suzuki et al., 2007, Tsuda et al., 2003). *NANOS2* KO pigs, goats, and cattle have all been found to phenocopy mice with male specific germline-ablation (Park et al., 2017). Testis complementation with allogenic donor SSCs has successfully produced motile spermatozoa with normal morphology, albeit at low efficiency, in *NANOS2* KO boars and bucks. Moreover, donor mice SSC testis complementation of prepubertal *Nanos2* KO mice successfully regenerated spermatogenesis to obtain natural fertility and produced donor-derived progeny via natural mating. Most recently, it was reported that an SSC-transplanted *NANOS2* KO bull produced semen with normal sperm concentration and

motility (Latham et al., 2023). Additionally, the donor-derived bull sperm successfully *in vitro* fertilized bovine oocytes resulting in donor-derived embryos, and pregnancies were achieved via natural mating, but to date no live donor-derived offspring have been reported (Latham et al., 2023).

Additionally, PGCLCs generated from mouse ESCs have been used in testis complementation of neonatal mice lacking germ cells to achieve donor-derived spermatogenesis. Furthermore, the PGCLC donor-derived spermatozoa was used to fertilize oocytes via intracytoplasmic sperm injection (ICSI), resulting in donor-derived offspring that grew into fertile adults (Hayashi et al., 2012).

For embryo complementation studies, the RNA-binding protein gene, *NANOS3*, which is primarily expressed in PGCs, has been targeted. As described in detail previously, *Nanos3* homozygous KO mice of both sexes are infertile, as PGCs fail to survive during migration and colonization of the fetal gonad (Suzuki et al., 2008, Tsuda et al., 2003). Moreover, Miura et al. (2021) injected donor mouse ESCs into *Nanos3*-null mouse blastocysts, and the resulting mice had a 100% donor-derived germline, were fertile, and were able to produce donor-derived offspring. Additionally, a *NANOS3* KO female bovine fetus was found to phenocopy mice with a complete loss of germ cells, but otherwise normal ovarian development. Moreover, embryo complementation via microinjection of bovine donor blastomeres into *NANOS3*-null bovine host morulas resulted in donor-derived primary oocytes in the ovaries of a bovine female fetus (Ideta et al., 2016). Most recently, *NANOS3* KO pigs were also shown to have complete germline ablation with otherwise intact gonads (Kogasaka et al., 2022, Park et al., 2023, Wang et al., 2023). Furthermore, donor-derived spermatozoa was successfully produced in *NANOS3* KO boars after testis complementation with donor SSCs (Wang et al., 2023).

Two additional genes that have been exclusively targeted in mice and result in a complete loss of germ cells, with an intact somatic gonad are PR Domain Zinc Finger Protein 14 (*Prdm14*), and TSC22 Domain Family Member 3 (*Tsc22d3*).

In rodents, *Prdm14* is expressed exclusively in pluripotent cells and nascent PGCs and is a key transcriptional regulator essential for the specification of PGCs. Rodents lacking *Prdm14* lose PGCs during specification due to a failure in the reacquisition of potential pluripotency and lack of epigenetic reprogramming, resulting in infertile males and females (Kobayashi et al., 2021, Kobayashi et al., 2020, Yamaji et al., 2008). Kobayashi et al. (2021) recently achieved both allogenic and xenogeneic germline complementation when rodent ESCs were used in embryo complementation of *Prdm14*-null rats. First, rat ESCs were injected into *Prdm14* KO rat blastocysts (i.e., allogenic), resulting in complete germline transmission of donor rat ESCs without any host-derived germ cells. Furthermore, the rat ESC donor-derived spermatozoa was used to fertilize rat oocytes via round spermatid injection (ROSI), resulting in donor-derived offspring. Additionally, Kobayashi et al. (2021) injected mouse ESCs into *Prdm14* KO rat blastocysts (i.e., xenogeneic) and remarkably produced adult rat-mouse chimeras that contained mouse-derived germ cells undergoing normal spermatogenesis. Even though the xenogeneic-derived mouse sperm had impaired motility, they were able to *in vitro* fertilize mouse oocytes via round spermatid injection (ROSI) and produce normal offspring. Given that rodents lacking *Prdm14* are infertile but have no other developmental abnormalities, *Prdm14* could be a suitable target for generating livestock hosts for germline complementation.

However, *PRDM14* function may not be conserved across mammalian species. In human fetal gonads, the knockdown of *PRDM14* did not affect PGC-like cell specification, thus it has been suggested that *PRDM14* is dispensable for human PGC fate (Sugawa et al., 2015). In contrast,

Sybirna et al. (2020) induced a rapid and comprehensive loss of endogenous PRDM14 protein in human PGCLCs and observed significantly reduced specification efficiency and an aberrant transcriptome demonstrating its critical role in human PGC fate. In bovine fetal gonads, *PRDM14* was only detected in a small subset of bovine PGCs, indicating a potentially different role in bovine PGC fate (Soto and Ross, 2021).

Tsc22d3 is an x-linked gene that is crucial for spermatozoa generation in mice. Donor-derived spermatozoa have successfully been generated in *Tsc22d3* KO mouse testes via both testis and embryo complementation methods. Zhou et al. (2019) transplanted mouse SSCs into *Tsc22d3* KO mouse testes and produced donor-derived spermatozoa and offspring via ICSI. Koentgen et al. (2016) and Zvick et al. (2022) injected mouse ESCs into *Tsc22d3* KO mouse blastocysts (i.e., allogenic) and produced donor-derived spermatozoa and offspring via natural mating. Additionally, Zvick et al. (2022) injected rat ESCs into *Tsc22d3*-mutated mouse blastocysts (i.e., xenogeneic) and remarkably produced donor-derived rat spermatozoa that morphologically appeared indistinguishable from normal rat sperm cells. Although the xenogeneic-derived rat sperm had impaired motility, it was used to *in vitro* fertilize rat oocytes via ROSI and produced blastocysts. However, the fertilization rates with xenogeneic-derived rat sperm were noticeably lower than those of rat sperm cells produced in rats, and live rat offspring could not be produced (Zvick et al., 2022). It is important to note, that continuous propagation of the X-linked genotype in *Tsc22d3* KO male mice requires a complex conditional KO strategy, which is impractical for livestock applications (Koentgen et al., 2016).

Among the genes targeted in mammals for germline ablation thus far, the RNA-binding protein genes, *DAZL*, *NANOS2*, and *NANOS3* have emerged as ideal candidates for livestock. In contrast, other genes targeted specifically in rodents have limitations that make them less suitable.

For instance, *Etv5* targeting results in impaired Sertoli cell function which may not robustly support donor-derived gametogenesis, and targeting *Tscd22d3* requires a complicated conditional KO strategy to maintain the genotype, which is not practical in livestock. *Prdm14*, on the other hand, could be suitable in livestock, but its specific function in germline development outside of mice is less clear, making it a less favorable as a target.

While research in rodents has demonstrated the feasibility of generating germline chimeras and provided insights for their development, the application of such strategies in livestock has been slow. Although the successful birth of primary chimeras and the establishment of germline transmission through blastocyst complementation in mammalian livestock have not yet been reported, studies in non-mammalian food species, including chickens and fish, have demonstrated the achievement of germline complementation and the production of live, donor-derived offspring. Furthermore, the combination of the rapidly growing field of livestock PSCs and the successful generation of germline-ablated hosts through GnEd brings us closer to realizing embryo complementation in livestock breeding. Germline complementation offers a transformative opportunity in livestock breeding by enabling the generation of germ cells from elite donor animals within the gonads of genetically inferior and sterile host animals. This approach could not only expand the availability of gametes from genetically desirable parents but also potentially facilitate the widespread distribution of beneficial traits through gene editing of donor cells. Therefore, the realization of germline complementation has the potential to revolutionize livestock breeding and is a crucial area of research.

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CHAPTER 2

Synergistic power of genomic selection, assisted reproductive technologies, and gene editing to drive genetic improvement of cattle

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ABSTRACT

Genetic improvement of cattle around the globe has been, and will continue to be, an important driver of animal agriculture sustainability. There are several reproductive and molecular biotechnologies that are used in genetic improvement of cattle, and their impact on the rate of genetic progress is maximized when combined synergistically in a structured breeding program with a clear breeding objective. One of the most recently developed and increasingly popular tools, gene editing, allows animal breeders to precisely add, delete, or replace letters in the genetic code so as to influence a specific trait of interest (e.g., disease resistance), in as little as one generation. However, for gene editing to be an important factor for genetic improvement, it must integrate smoothly into conventional cattle breeding programs to maintain or accelerate rates of genetic gain. This review first summarizes the current state of key reproductive and molecular biotechnologies available for the genetic improvement of cattle, and then discusses potential strategies for effectively incorporating gene editing into cattle genetic improvement programs and methods for disseminating traits improved via gene editing. Moreover, it examines how genetic improvement strategies, including the use of gene editing, will differ depending on the cattle industry sector (i.e., dairy or beef), and the region of the world in which they are being deployed.

1. INTRODUCTION

Genetic improvement is a powerful tool for improving animal agriculture sustainability because the results are permanent and cumulative. Unlike nutritional and animal health interventions, which require continuous inputs, genetic improvements made in one generation are passed onto the next. Moreover, genetic solutions for animal health and welfare issues often require less labor and material inputs than chemical or mechanical methods. For example, polled, or hornless, genetics can eliminate the need for physical dehorning of animals, which is undertaken

to ensure both worker and animal safety, can save livestock producers both time and money, in addition to addressing an animal welfare concern (Gottardo et al. 2011; Thompson et al. 2017).

Sustainable agriculture and increased production efficiency go hand-in-hand. Efficiency is defined as achieving maximum productivity with minimum waste, or in other words, producing more product with the same or even fewer resources. Livestock genetic improvement programs, beginning with selective breeding using statistical prediction methods, such as estimated breeding values (EBVs), and more recently genomic selection (GS), in combination with assisted reproductive technologies (ART) have enabled more accurate selection and intense utilization of genetically superior parents for the next generation to accelerate rates of genetic gain. Genetic gain is the amount of increased performance, or the improvement in average genetic value, in a population that is achieved annually through selection. Increased animal performance based on genetic improvement results in more product produced per animal, so fewer animals are required to meet the same amount of demand, which reduces the environmental impact per unit of livestock product. Therefore, increasing rates of genetic gain can improve livestock production efficiency and ultimately the sustainability of animal agriculture.

The power and scale of genetic improvement is well-illustrated by the increased efficiency of the United States (U.S.) dairy cattle population from 1944 to today, which now produces over 80% more milk with 65% fewer cows. This was enabled by a more than four-fold increase in milk production per cow, from 2000 kg/cow in 1944 to 10,000 kg/cow in 2017 (Capper and Cady 2019; Capper et al. 2009). It is estimated that approximately 50% of the increased productivity per animal observed can be attributed solely to the increased rate of genetic gain obtained by the widespread use of artificial insemination (AI) over natural service breeding alone (Bertolini and Bertolini, 2009). Overall, the dramatic decrease in the number of dairy cows (25.6 million to 9 million)

required to meet the demand, due to increased productivity per animal largely from improved genetics, reduced the current environmental impact of a glass of milk to approximately one third of that associated with the same glass of milk in 1944 (Capper and Cady 2019; Capper et al. 2009).

In livestock breeding programs, the breeder's equation is used to measure the rate of genetic gain (ΔG) towards the breeding objective of a given production system. It consists of four components: $\Delta G = \frac{i \times r \times \sigma A}{L}$, where i is selection intensity (how extensively the most elite animals are used as parents of the next generation); r is selection accuracy (how well the EBV represents the true breeding value of selection candidates); σ_A is genetic diversity (as measured by the additive genetic standard deviation of the population); and L is the generation interval (interval length calculated as the average age of parents when progeny are born) (Lush 1937).

Strategies to improve rates of genetic gain in a population involve increasing the components of the breeder's equation in the numerator and decreasing the denominator, or generation interval. It is important to note that the foundation of genetic improvement is a well-structured breeding program with a clear breeding objective, and routine recording of pedigree and performance information on the population under selection. Genomic information can additionally improve the accuracy of the relationship matrix compared to pedigree information alone. Within a structured breeding program, reproductive and molecular biotechnologies, such as ART and GS, can be applied to further accelerate rates of genetic gain by influencing one or more of the components of the breeder's equation.

To increase selection intensity, ART [e.g., AI and embryo transfer (ET)] have been incorporated into cattle breeding schemes. Concurrently, the development of high-throughput genotyping of single nucleotide polymorphisms (SNPs), has enabled GS to predict the genetic merit of an animal based on its DNA (Meuwissen et al. 2001). Using GS has both improved the

accuracy of selection and reduced the generation interval. Additionally, GS can provide information on traits that are recorded late in life, or that are difficult or expensive to record (García-Ruiz et al. 2016; Hayes et al. 2013; Meuwissen et al. 2013). Moreover, the benefits of each of these tools, GS and ART, can be maximized when used synergistically to accurately select young animals, which can markedly reduce the generation interval and ultimately accelerate genetic gain (Fig. 2.1) (Kadarmideen et al. 2015; Loi et al. 2016).

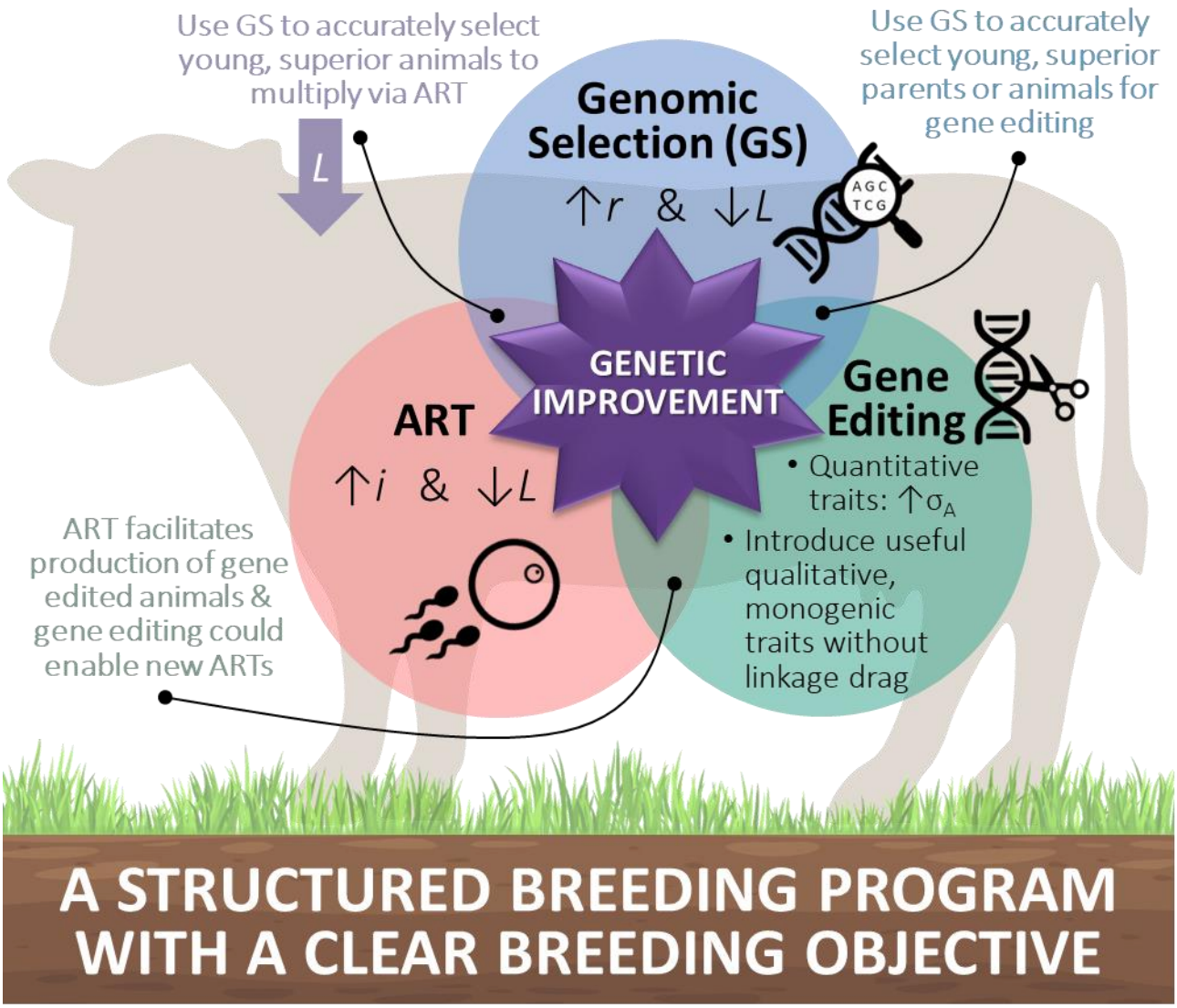

Figure 2.1. Schematic illustrating the synergistic relationships between genomic selection (GS), assisted reproductive technologies (ART), and gene editing for the genetic improvement of cattle.

The foundation of genetic improvement is a well-structured breeding program with a clear breeding objective. Within a structured breeding program, reproductive and molecular biotechnologies, such as ART and GS, can be applied to further improve rates of genetic gain by effecting one or more of the components of the breeder's equation (Lush 1937): (1) increase selection intensity (i), (2) increase selection accuracy (r), (3) decrease the generation interval (L), and (4) increase genetic variation (σ_A)

Genome or gene editing (GnEd) is one of the most recently developed tools for genetic improvement. This advanced biotechnology allows animal breeders to very precisely target the addition, deletion, or replacement of base pairs in the genetic code to influence traits of interest. Specifically, GnEd refers to the use of site-directed nucleases (i.e., nucleic acid cleaving enzymes) to precisely introduce double stranded breaks (DSB) in the DNA at a targeted location in the genome (Gaj et al. 2013). When the cell attempts to repair the DSB, it can result in the disruption (knockout) of a gene, or if a donor repair nucleic acid template is provided, the insertion (knock-in) of an allele or gene from the same species (intraspecies or cisgenic) or possibly a different species (interspecies or transgenic).

In cattle breeding programs, GnEd offers promising opportunities to introduce useful genetic variation from one breed of cattle to another in the absence of undesired linkage drag, or even beneficial traits from different species. Currently, GnEd research in cattle has focused on and is well-suited for improving monogenic, or Mendelian, traits. Mendelian traits are controlled by one to a few loci that each have large effects, and most are qualitative traits, such as horned/polled or coat color. Although, there are a few known single genes that have large effects on important quantitative traits. For example, a naturally occurring mutation in the myostatin (*MSTN*) gene present in some cattle breeds like Belgian Blue, results in a substantial increase in the quantitative

trait, muscle yield (Kambadur et al. 1997; McPherron and Lee 1997). If GnEd is used to target a gene that has a large effect on a quantitative trait, like *MSTN*, then GnEd has the potential to increase genetic variation of that trait in the population, thus accelerating the rate of genetic gain. It should be noted that complete *MSTN* knockouts have also resulted in increased birth weights, which can cause dystocia issues (i.e., calving difficulties), so more precise *MSTN* mutations will likely be required for practical applications of this target (Proudfoot et al. 2015).

However, most of the traits that animal breeders want to improve are polygenic and quantitative (e.g., marbling, growth, feed efficiency, etc.). For these traits, quantitative genetics and GS have been, and will continue to be, the major driver for genetic improvement. Additionally, GnEd in livestock is only possible through the use of ART. Therefore, the potential of GnEd can only be fully realized when used in conjunction with ART and GS in a structured breeding program with a clear breeding objective to accelerate genetic gain by concurrently altering multiple components of the breeder's equation (Fig. 2.1) (Bishop and Van Eenennaam 2020; Jenko et al. 2015; McLean et al. 2020; Van Eenennaam 2017).

Given that there are a wide variety of tools for genetic improvement in cattle, this review first summarizes the current state of key reproductive and molecular biotechnologies, and then discusses their synergistic potential when employed jointly. There is a primary focus on how the increasingly popular modern biotechnology, GnEd, is being used for genetic improvement of cattle and strategies for effectively incorporating it into existing cattle breeding programs. Moreover, we discuss how genetic improvement strategies, including the use of GnEd, will differ depending on the cattle industry sector (i.e., dairy or beef) being targeted, and the region of the world in which they are being deployed.

2. CONSIDERATIONS FOR GENETIC IMPROVEMENT OF CATTLE IN BEEF VERSUS DAIRY SYSTEMS

Advanced reproductive and molecular biotechnologies are often easier to cost effectively implement in the breeding pyramid of vertically integrated, "high-input" (intensive), industries. In such systems, external inputs such as supplementary feeds, veterinary medicines and ART are relatively easy to obtain and widely used. Additionally, in vertically integrated programs the return on investment in performance recording of each nucleus animal can be recouped through thousands, or even millions, of genetic descendants (Van Eenennaam et al. 2014).

Compared to other livestock species, cattle have a long generation interval and low fecundity, which slows genetic progress. Nevertheless, the dairy industry was well-positioned for rapid adoption of GS due to its industry-wide selection goal (e.g., Lifetime Net Merit, NM$ in the U.S.), widespread use of AI, large number of high accuracy AI sires, primary use of purebred animals (e.g., Holstein), extensive and uniform phenotype data collection, and central evaluation program to receive genotypes. Moreover, large breeding organizations were willing to fund genotyping because they received a clear cost savings in terms of identifying AI sires at a young age (< 1 year-old) compared to previous progeny testing schemes (> 5 years-old) (Wiggans et al. 2017).

In contrast, genetic progress in beef cattle selection programs has been slower and industry-wide rates of genetic gain lag well below what is possible (Banks 2005). This is due to a multitude of factors including the difficulty of developing an industry-wide breeding objective in large part because of industry segmentation. The beef industry has a large number of ranches/decision makers raising animals in very diverse environments and selection decisions are made at the seedstock level without good linkages to performance metrics in the commercial cow-calf, feedlot,

or processing sector. Also, the beef industry is comprised of multiple breeds and breed associations all collecting separate data, has limited to no data recording on several economically relevant traits (e.g., female reproduction and feed efficiency), has lower producer adoption of economic indexes, and a limited use of AI (Van Eenennaam et al. 2014). Moreover, a large proportion of the world's beef cattle are located in tropical and subtropical environments, which requires additional traits, such as tolerance or resistance to environmental stressors, to be included in the breeding objective and those traits are typically very difficult or expensive to effectively record for genetic improvement purposes and they may have antagonistic relationships with productive attributes.

3. GENOMIC SELECTION (GS) OPPORTUNITIES

The development of high-throughput genotyping of SNPs enabled the development of approaches to predict an animal's genetic merit based on its DNA (Meuwissen et al. 2001). In GS, SNP effects are estimated using genotyped individuals that are phenotyped for the characteristics of interest (i.e., training population), and then genomic estimated breeding values (GEBVs) can be predicted for any genotyped individual by using only its SNP genotypes and estimated SNP effects. GS has been used in cattle to improve accuracy of selection, reduce the generation interval, and to provide useful information on traits that would otherwise be difficult to measure (García-Ruiz et al. 2016; Meuwissen et al. 2013). Genetic improvement in cattle, using GS for hard to measure traits like feed efficiency, cow longevity and fertility, has the potential to reduce the environmental footprint per unit of production (Barwick et al. 2019; Fennessy et al. 2019; Hayes et al. 2013; Pryce and Haile-Mariam 2020; Quinton et al. 2018).

Furthermore, improving efficiency of cattle production through exploitation of genomics can be considered a public good (Berry et al. 2016). For example, in Ireland this concept has been recognized by public support of genotyping cattle to facilitate GS. In 2016, a multibreed genomic

evaluation in beef cattle was launched and a monetary incentive was provided for beef producers to genotype females, more extensively phenotype females, and to retain genomically tested high-index females as herd replacements to increase the efficiency of the national herd. To date, the Irish Cattle Breeding Federation (ICBF) has genotyped almost 2 million animals. This program provided the data required to validate that higher maternal index females, on average, calved for the first time at a younger age, had shorter calving intervals, survived longer, and were also expected have a lower mature weight. An accelerated rate of genetic gain in the Irish maternal index was observed following the deployment of genotyping incentives and genomic predictions (Twomey et al. 2020). All of these improvements would be expected to reduce the environmental impact per unit of beef production in this system.

4. ASSISTED REPRODUCTIVE TECHNOLOGIES (ART) ADOPTION

ART is the term used to describe treatments and procedures which involve the manipulation of reproductive cycles, gametes, or embryos. In cattle breeding schemes, ART including AI, cryopreservation of sperm or embryos, estrus synchronization, multiple ovulation ET (MOET), ovum-pick up (OPU) and *in vitro* embryo production (IVP), sex determination of sperm or embryos, and nuclear transfer (NT) have been incorporated to increase selection intensity, which can accelerate rates of genetic gain. Globally, the most widely used ART in cattle is AI (Baruselli et al. 2018). AI allows females around the world to be inseminated by genetically superior bulls via cryopreserved semen, which increases the selection intensity of males and thus accelerates rates of genetic gain. India, which is the country with the second largest number of cattle in the world in 2019 (193 million head, not including over 110 million Buffalo, Mithun and Yak), behind Brazil (215 million head), currently has the world's largest AI infrastructure. This consists of 49 semen stations producing 66.8 million doses of frozen semen annually. Additionally,

there has been an increase in the uptake of sexed semen in India to reduce the number of male calves born into dairy herds (Ojango et al. 2016).

In some countries, the adoption of AI has been markedly skewed towards the dairy sector. For instance, while AI has been widely adopted by the U.S. dairy industry (> 80%) (Capper and Cady 2019; VanRaden 2007), to date it has seen limited uptake in the U.S. beef industry (USDA 2020). Only 12% of U.S. beef producers report using AI, and even fewer (7%) use estrus synchronization. In 2017, this resulted in less than 10% of all females being bred via AI. A larger portion of beef heifers (19%) were bred via AI compared to only 7% of cows (USDA 2020). Additionally, in Northern Australia, which accounts for over 50% of Australia's total beef cattle population, it is estimated that AI is used by less than 1% of breeding herds (MLA 2015). This low adoption rate in the beef industry is largely due to the difficulty in extensive systems of identifying females in estrus and constraining them to allow AI (USDA 2020).

To eliminate the burden and challenge of estrus detection, timed AI (TAI) was developed (Pursley et al. 1995). Additionally, TAI allows anestrous cows to be inseminated and has enabled conception to be more clustered to the beginning of the breeding season, thus increasing the reproductive and productive efficiency of farms (Baruselli et al. 2018). South America has widely adopted TAI. In 2017, more than 15 million breeding females were inseminated using TAI in Brazil, Argentina and Uruguay (Mapletoft et al. 2018). Specifically in Brazil, the widespread adoption of TAI resulted in a remarkable 220% increase in the Brazilian market for bovine semen units, from 7 million doses in 2002 to 15.5 million in 2018 (Fig. 2.2) (Baruselli et al. 2019). Over this time period (2002–2018), the percentage of female cattle in Brazil that were inseminated using AI more than doubled from 5.8% to 13.1%, totaling approximately 9.5 million head (13.6% of beef and 10.8% of dairy). Importantly, the large majority (86.3%) of these inseminations were via

TAI (Fig. 2.2). Overall, it has been estimated that TAI returns more than half a billion U.S. dollars per year to the Brazilian beef production chain due to genetic improvement in economically important traits, such as growth and carcass merit, as compared to natural service (Baruselli et al. 2018).

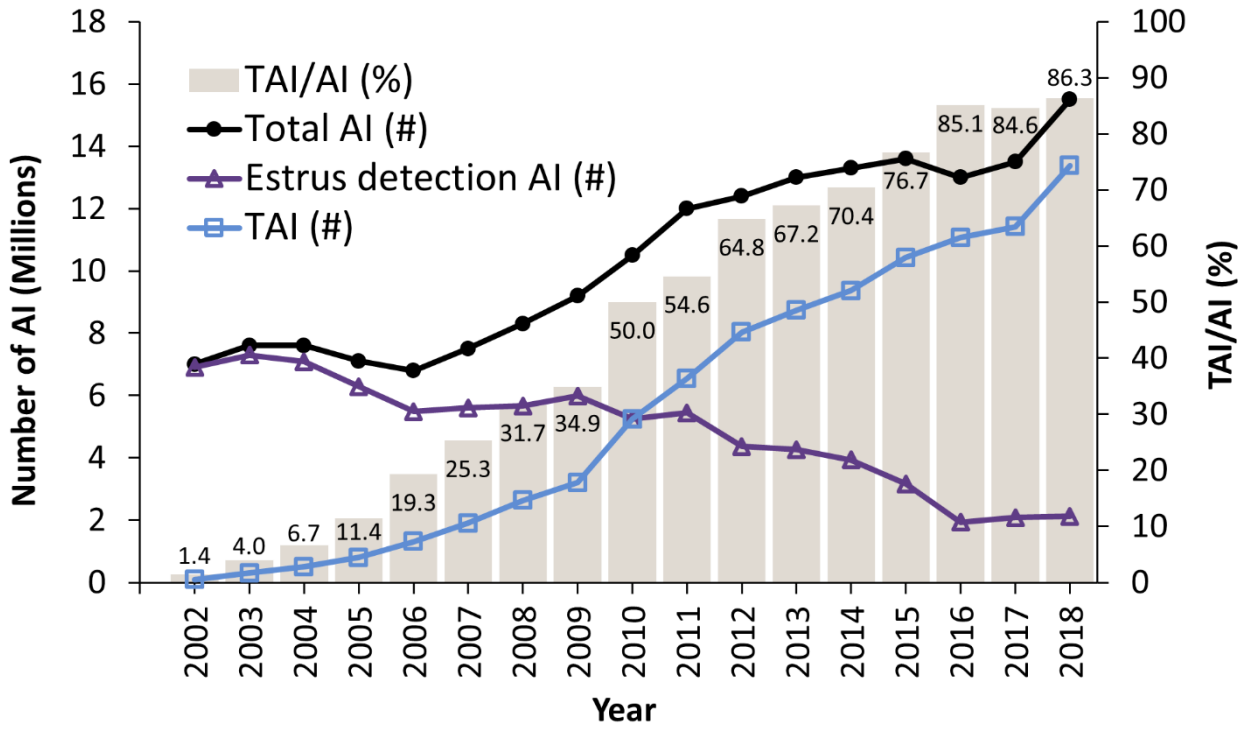

Figure 2.2. Comparison of timed artificial insemination (TAI) and artificial insemination with estrous detection in cattle in Brazil from 2002 to 2018. Reproduced from Baruselli et al. (2019) under a CC-BY license.

While AI and TAI enable increased selection intensity of males, MOET and OPU-IVP have allowed for increased selection intensity of females. In livestock, ET is the process of placing an embryo (usually at day 7 of development) into the uterus of a synchronized (in estrus 7 days prior to the transfer) recipient female that is typically not related to the embryo. Additionally, the development of synchronization techniques for timed embryo transfer (TET), has significantly increased the number of recipients suitable for receiving an embryo (Nasser et al. 2004).

Historically, most embryos for ET were produced through MOET, also known as "flushing" or *in vivo* production. In a MOET program, a genetically superior donor female is typically superovulated prior to AI and then the resulting embryos are flushed from the uterus of the donor (i.e., genetic dam) 7 days after AI. Alternatively, embryos can be generated via IVP. In an IVP program, unfertilized oocytes are collected from the donor cow's ovaries by transvaginal, ultrasound-guided needle aspiration of multiple follicles per ovary, also known as OPU. The collected oocytes then undergo *in vitro* maturation (IVM), followed by *in vitro* fertilization (IVF) and then *in vitro* culture (IVC) for 7 days until they reach the blastocyst stage and are ready for cryopreservation or ET. IVP is advantageous because donors can be collected repeatedly for most of the year, even while pregnant, thereby keeping them in synchrony with an annual calving cycle. Furthermore, in a process known as juvenile *in vitro* ET (JIVET), oocytes for IVP can also be collected from prepubertal heifers (< 7 months old), but with decreasing embryo development rates at younger ages (Brogliatti and Adams 1996; Duby et al. 1996; Torres et al. 2014). Using JIVET could decrease the female generation interval to one year (Duby et al. 1996; Granleese et al. 2015).

Globally, the number of IVP embryos has increased dramatically overtime (Fig. 2.3). This increase has occurred predominately in North and South America and to a lesser extent in Europe, with almost no uptake of this technology in Asia and Africa. World-wide, more than one million bovine IVP embryos were produced in 2018 and 742,908 were transferred, of which more than 50% were transferred in South America. In Brazil specifically, over 270,000 IVP embryos were transferred in 2018. Baruselli et al. (2019) concluded that the uptake of reproductive biotechnologies in Brazil "increases productivity per unit of land and significantly contributes to improve the efficiency of livestock. Therefore, with the intensification of the use of reproductive biotechnologies it is possible to enhance production with reduced environmental impact." The

challenge to continued adoption of these technologies is, according to these authors, dependent on an increase in extension services for producers and specialists, development of more efficient/cost-effective products and practical protocols, increased integration between universities, research institutes, veterinarians and industry, and market demand for the production of animal protein with higher quality, efficiency and environmental and economic sustainability (Baruselli et al. 2019).

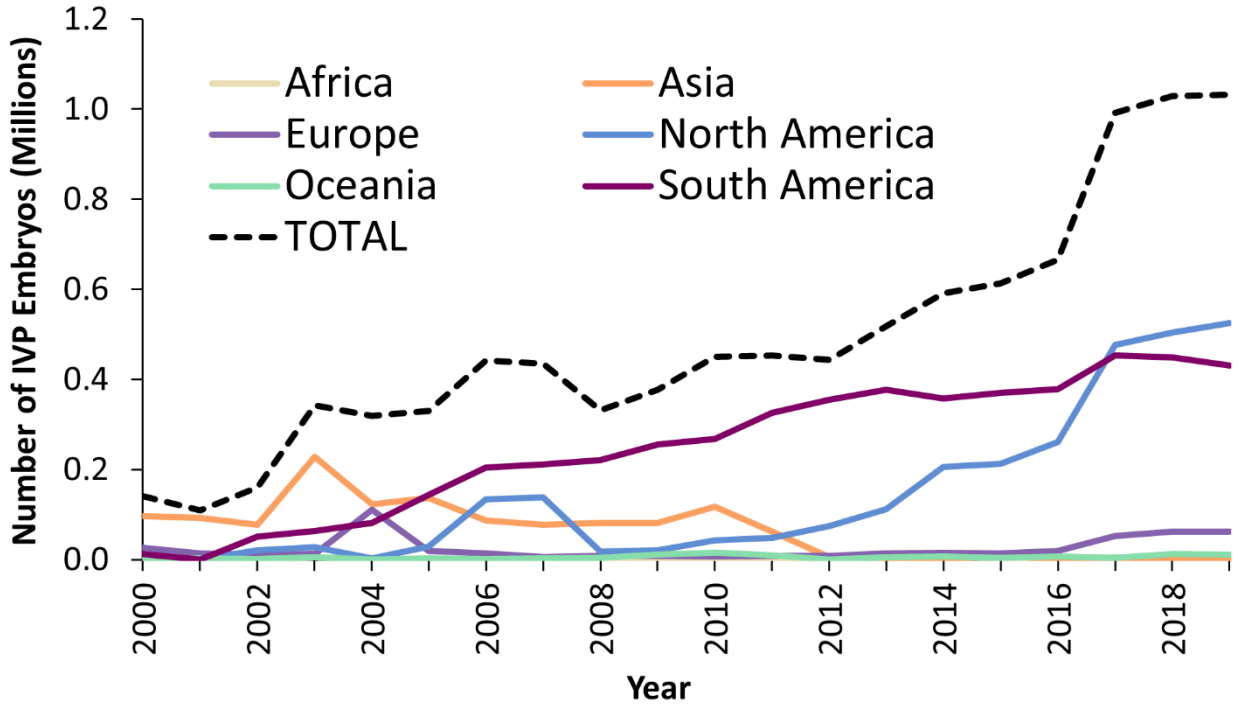

Figure 2.3. Number of *in vitro* produced (IVP) bovine embryos from 2000 to 2019, by continent. Data from IETS (2000–2019) Data Retrieval Committee Reports

Another way to increase selection intensity is through embryo multiplication procedures, including embryo splitting and cloning by embryonic cell NT (ECNT) (Heyman et al. 1998; Lopes et al. 2001). Alternatively, adult somatic cell NT (SCNT) cloning can be used to multiply unique genotypes (Oback and Wells 2003; Wilmut et al. 1997). Unfortunately, due to faulty or incomplete epigenetic reprogramming of the donor cell genome, SCNT cloning often results in high rates of pregnancy loss and can also negatively affect the viability of live-born calves (Akagi et al. 2013; Galli and Lazzari 2021; Keefer 2015). Therefore, SCNT cloning is primarily used for research or

to produce “back-ups” of individual animals with unique genetic features (Bousquet and Blondin 2004; Loi et al. 2016). On the other hand, ECNT cloning has been shown to greatly reduce the incidence and severity of abnormal phenotypes compared to somatic clones, but has limited multiplication potential due to the small number of embryonic cells, or blastomeres (Heyman et al. 2002; McLean et al. 2020; Misica-Turner et al. 2007).

One advanced reproductive biotechnology that has been invaluable for rodent and primate research, but until recently was not available for livestock species, is embryonic stem cells (ESCs) (Blomberg and Telugu 2012; Evans and Kaufman 1981; Ezashi et al. 2016; Li et al. 2008; Soto and Ross 2016). ESCs are derived from the inner cell mass (ICM) of preimplantation blastocysts. The ICM is the tight cluster of cells inside a blastocyst that will eventually give rise to the definitive structures of the fetus. ESCs are a unique cell type because they are self-renewing (able to replicate indefinitely) and pluripotent, meaning they can differentiate into all three primary germ layers: ectoderm, endoderm, and mesoderm (Wu and Belmonte 2015; Ying et al. 2008). Given that ESCs are derived from pre-implantation embryos, they could provide a potentially unlimited source of elite genetics from the next generation of animals for multiplication, which could further increase the selection intensity of both males and females in livestock production.

Unfortunately, derivation and stable propagation of pluripotent ESCs from domestic ungulates, including cattle, has been challenging (Blomberg and Telugu 2012; Ezashi et al. 2016; Soto and Ross 2016). Although there have been reports of the development of bovine ESC lines, they did not pass the standard pluripotency tests (i.e., *in vitro* embryoid body formation, *in vivo* teratoma assay, and/or chimera formation). Moreover, they showed poor derivation efficiencies, limited proliferation capacities, and loss of pluripotency markers after extensive passages (Kim et al. 2017; Saito et al. 1992). Consequently, cattle research has been limited to investigation of

induced pluripotent stem cells (iPSC), which can be derived from the epigenetic reprogramming of somatic cells (Heo et al. 2015; Kawaguchi et al. 2015).

However, in 2018, after decades of research, Bogliotti et al. (2018) reported the successful derivation of pluripotent bovine ESCs with stable morphology, transcriptome, karyotype, population-doubling time, pluripotency marker gene expression, and epigenetic features. Moreover, the authors reported that stable bovine ESCs can be established quickly in 3–4 weeks and were simply propagated by trypsin treatment (Bogliotti et al. 2018). More recently, Zhao et al. (2021) reported the successful derivation of another type of bovine pluripotent stem cell, expanded potential stem cells (EPSCs). Currently, the production of a live calf from ESCs would require NT using an ESC as the nuclear donor. Experiments have shown that ESC-NT results in similar blastocyst development rates to SCNT, but there could potentially be higher pregnancy rates and less offspring abnormalities (Bogliotti et al. 2018; McLean et al. 2020; Zhao et al. 2021).

In the future, ESCs could enable *in vitro* breeding (IVB) schemes, which could drastically decrease the generation interval (Goszczynski et al. 2018). IVB would involve repeated cycles of deriving gametes (i.e., sperm and eggs) in culture from ESCs and IVF (Goszczynski et al. 2018). In mice, ESCs have been induced in culture to become primordial germ cell-like cells (PGCLCs) and subsequently induced to form gametes. Furthermore, these *in vitro* gametes have successfully produced live, fertile offspring (Hayashi et al. 2011, 2012; Ishikura et al. 2016; Yoshino et al. 2021). Although bovine PGCLCs have yet to be produced, the ability to derive bovine ESCs now makes this strategy possible (Goszczynski et al. 2018). However, IVB will only be a useful tool to improve genetic gain if combined with GS (see discussion below).

5. SYNERGISTIC POWER OF GS & ART

When GS and ART are used concurrently, the benefits of each act synergistically to accurately select genetically superior, young animals, thereby substantially reducing the generation interval and accelerating rates of genetic gain (Fig. 2.1) (Granleese et al. 2015; Kadarmideen et al. 2015; Loi et al. 2016). For example, GS can be used to accurately select high-genetic-merit young donor females for MOET or IVP and bulls for semen collection. The embryos produced from these matings will also have high genetic merit. However, due to Mendelian sampling variance, not all full-sibling embryos have the same genetic merit and there is a large cost and natural resource drain in gestating ET calves of unknown genetic merit only to later cull the genetically inferior animals (Segelke et al. 2014). Therefore, methods to produce and identify genetically superior embryos before ET have been highly sought after.

The idea of combining GS with the manipulation of sex cells and embryos to accelerate genetic gain, coined "velogenetics," was first proposed by Georges and Massey (1991). Briefly, velogenetics is a breeding scheme based on the collection of fetal oocytes for IVP followed by genomic testing of the resulting embryos, with the possibility to reduce the generation interval to 3–6 months (Georges and Massey 1991). Although this scheme would provide a substantial decrease in the generation interval, the low efficiency and practical complications of having to slaughter the dam for fetal collection, have inhibited further development of this specific scheme (Chohan and Hunter, 2004; Figueiredo et al., 1993). However, alternative approaches with the same goals have been developed.

Genomic screening of embryos (GSE), sometimes referred to as embryo genotyping, is the process of genotyping cells collected from a biopsy of a preimplantation embryo (i.e., before ET into a recipient female). GSE can be used to predict an embryo's genetic merit so that only the

embryos with the highest genetic merit are used for ET. Moreover, since a larger number of embryos can be generated via IVP compared to live-born animals, GSE can be used to select a small number of animals (in their embryo stage) from a large pool of candidates for ET, which will further increase the selection intensity (Fisher et al. 2012; Kadarmideen et al. 2015; Yudin et al. 2016). Although GSE holds great potential, there are currently several technical limitations to overcome.

There is an inverse relationship between the viability of a biopsied embryo and the ability to obtain enough DNA sufficient for genotyping (Ponsart et al. 2013). DNA extracted from embryo biopsies can be used for genetic diagnosis [i.e., genotyping of a few specific loci via polymerase chain reaction (PCR)], for GS, or a combination of both. DNA from one to several biopsied cells has been used successfully for genetic diagnosis (primarily, sex identification) of preimplantation bovine embryos (Cenariu et al. 2012; de Sousa et al. 2017; Ponsart et al. 2013; Tominaga and Hamada 2004). Moreover, de Sousa et al. (2017) took biopsies of a limited number of cells (10–20 blastomeres) from the trophectoderm of both *in vivo* derived and IVP bovine embryos on day 7 of development. They demonstrated that the biopsies were sufficient for embryo sexing via PCR and that there was no significant ($P > 0.05$) difference on day 60 pregnancy rates of fresh transfer, biopsied embryos compared to control, non-biopsied embryos. It is important to note that this study did not investigate pregnancy rates of embryos that had been both biopsied and cryopreserved. Due to the limited amount of time between being able to biopsy an embryo and needing to transfer the fresh embryo (i.e., both on day 7 of IVP development), the ability to cryopreserve biopsied embryos will likely be a critical process for applying GSE on a commercial scale (Mullaart and Wells 2018).

While embryo biopsies for sex determination have been routinely used in ET programs (Bondioli 1992; Lopes et al. 2001; Ponsart et al. 2013), GS of embryos has been limited since a much larger number of cells (minimum of 30–40 cells) must be biopsied and genotyped to make accurate selection decisions (Fisher et al. 2012; Ponsart et al. 2013). Although taking a biopsy of more than ~ 20 cells will drastically decrease embryo viability, alternatives to generate a sufficient amount of DNA for GS from only a small number of biopsied cells have been investigated, such as growing biopsied cells in culture (Ramos-Ibeas et al. 2014; Shojaei Saadi et al. 2014), and using whole genome amplification of biopsied cells in combination with imputation from known parental and population genotypes (Allan 2019; Lauri et al. 2013; Shojaei Saadi et al. 2014).

An adaption to traditional GSE was developed by Kasinathan et al. (2015) to genomically screen unborn bovine fetuses rather than embryos. Their strategy utilized multiple ET's and subsequent embryo flushing (21–26 day fetuses) to generate fetal fibroblast lines. DNA was extracted from the fibroblast lines for GS and the resulting GEBVs for NM$ (U.S. dairy) were used to select the line with the highest genetic merit. Cells from the selected elite fibroblast line were used as donor cells for SCNT cloning. Following ET of the cloned embryos, five healthy calves with elite dairy genetics were born (Kasinathan et al. 2015). While this scheme overcomes the challenges of taking embryo biopsies for GS, it still relies on the inefficient process of SCNT cloning to produce live offspring (Akagi et al. 2013; Keefer 2015).

Bovine pluripotent stem cells (Bogliotti et al. 2018; Zhao et al. 2021) have the potential to open a whole new avenue for GSE. Given that pluripotent stem cells are self-replicating, a sufficient amount of DNA could be extracted without harming the viability of the remaining stem cells, which would allow for the use of GS to determine the genetic merit of each line. The genetically superior stem cell lines could then be used for ECNT, similar to the Kasinathan et al.

(2015) method. Alternatively, the genetically superior stem cell lines could be *in vitro* differentiated (as described above) to produce gametes which would enable IVB schemes (Goszczynski et al. 2018). Goszczynski et al. (2018) anticipates that one round of IVB could be completed in 3–4 months, which would drastically reduce the generation interval. These authors estimate that in the same time that it takes a GS program to obtain its first generation (2.5 years), an IVB program would instead allow 10 generations of mating and selection in this same period, ultimately enabling substantial genetic improvements to be made in a short amount time (Goszczynski et al. 2018).

6. GENE EDING (GnEd) POTENTIAL

A potentially ground-breaking tool for genetic improvement is GnEd, which offers promising opportunities to inactivate targeted gene function (i.e., knockout genes), knock-in genes from other species, and achieve intraspecies allele introgression in the absence of undesired linkage drag. GnEd refers to the use of site-directed nucleases to precisely introduce DSB at predetermined locations in the genome (Gaj et al. 2013). Cells have evolved two primary pathways to repair DSBs: non-homologous end joining (NHEJ) and homology-directed repair (HDR). The underlying principle is that the cell's endogenous repair factors will identify and congregate at the site of the DSB to repair the DNA in an efficient manner.

When using the NHEJ pathway, the cell's natural DNA repair pathway fuses the broken DNA ends back together through blunt-end ligation. NHEJ is referred to as "non-homologous" because the ligation occurs without the use of a homologous nucleic acid template (e.g., sister chromatid) (Moore and Haber 1996). Consequently, this pathway is error-prone and often introduces variable-length insertion and deletion mutations (indels) at the DSB site (Sander and Joung 2014). In other words, the NHEJ pathway allows for the efficient disruption or knockout of

a gene by targeting breaks to the coding region of the gene, where indels can result in frameshift or nonsense mutations.

On the other hand, the cell can use the HDR pathway if a nucleic acid donor template is provided. HDR templates can be designed to include desired modifications between regions of homology flanking either side of the targeted DSB, and templates are generally provided to the cell in the form of single-stranded or double-stranded DNA. The cell's DNA repair enzymes can use the template as a model for precise repair by homologous recombination. The HDR pathway can be used to introduce, or knock-in, a range of gene edits, from point mutations to allelic substitutions, to entire transgenes (Sander and Joung 2014). However, in most cell types a lower frequency of HDR than NHEJ has been observed (Sonoda et al. 2006).

There are currently three primary site-directed nucleases used for GnEd in livestock: (1) zinc finger nucleases (ZFN); (2) transcription activator-like effector nucleases (TALENs); and (3) clustered regularly interspersed short palindromic repeats and associated protein 9 (CRISPR/Cas9). Since 2012, all three GnEd systems have been used to perform both gene knockouts and knock-ins in livestock cells and zygotes (Bishop and Van Eenennaam 2020; Tait-Burkard et al. 2018; Tan et al. 2016). Most recently, the high efficiency, technical simplicity of design, and cost-effectiveness of the CRISPR/Cas9 system has greatly advanced the potential for GnEd in livestock (Petersen 2017).

GnEd experiments in cattle have primarily focused on three main areas of improvement (1) animal health and welfare, (2) product yield or quality, and (3) reproduction or novel breeding schemes (Table 2.1). All three of these areas are highly aligned with the goals of conventional breeding programs (Rexroad et al. 2019; Tait-Burkard et al. 2018; Van Eenennaam 2017).

Table 2.1. Publications using gene editing in cattle for agricultural applications, grouped by category of genetic improvement goals.

Goal	Genome target and function	Editing approach	Editor[a]	Live edited offspring	Reference(s)
Animal health/welfare					
Prevent horn growth	Horn/Poll	Replaced bovine horned allele (p) with bovine *POLLED*, Celtic allele (P_C)	TALEN	Yes	Carlson et al. (2016), Tan et al. (2013)
		Generated deletions in the horned loci	CRISPR/Cas9	No	Hennig et al. (2021a, b)
Disease resistance: Mastitis	*CSN2* (Beta-Casein): milk protein gene	Inserted Staphylococcal lysostaphin (antimicrobial) gene	ZFN	Yes	Liu et al. (2013)
		Inserted human lysozyme (antimicrobial) gene	ZFN	Yes	Liu et al. (2014)
Disease resistance: Tuberculosis	*ITGB2* Intergenic region between *SFTPA1* (Surfactant Protein A1) and *MAT1A* (Methionine Adenosyltransferase 1A)	Inserted mouse *Sp110* (SP110 Nuclear Body Protein) gene	TALEN	Yes	Wu et al. (2015)
	Intergenic region between *FSCN1* (Fascin Actin-Bundling Protein 1) and *ACTB* (Actin Beta)	Inserted human *NRAMP1* (Natural Resistance-Associated Macrophage Protein 1) gene (controls Tuberculosis infections)	CRISPR/Cas9	Yes	Gao et al. (2017)

Goal	Genome target and function	Editing approach	Editor[a]	Live edited offspring	Reference(s)
Disease resistance: BRD	(Integrin Subunit Beta 2): encodes the leukocyte signal peptide CD18	Substituted glycine in place of glutamine to cause cleavage of CD18	ZFN	No	Shanthalingam et al. (2016)
Disease resistance: BSE	*PRNP* (Prion Protein): susceptibility to BSE	Disrupted the *PRNP* gene	TALEN	No	Choi et al. (2015)
			CRISPR/Cas9	No	Bevacqua et al. (2016)
		Substituted valine in place of glycine at position 127 to confer resistance	CRISPR/Cas9	Yes	Park et al. (2020)
Repair mutation: IARS syndrome	*IARS*	Substituted a single base pair to correct the mutation	CRISPR/Cas9	Yes	Ikeda et al. (2017), Ishino et al. (2018)
Thermo tolerance	*PMEL* (Premelanosomal Protein): coat color	Introduced a 3 bp deletion associated with diluted, or silver, coat-color	CRISPR/Cas9	Yes	Laible et al. (2020)
	PRLR (Prolactin Receptor): hair coat length	Disrupted *PRLR* gene to generate a SLICK (short, sleek hair coat) phenotype	CRISPR/Cas9	Yes	Rodriguez-Villamil et al. (2021)

Goal	Genome target and function	Editing approach	Editor[a]	Live edited offspring	Reference(s)
Product yield or quality					
Eliminate a milk allergen	*BLG* (Beta-Lactoglobulin): whey protein gene	Disrupted the *BLG* gene	ZFN	Yes	Yu et al. (2011)
		Disrupted the *BLG* gene by inserting 5 bp with single stranded oligonucleotide template	ZFN or TALEN	No	Wei et al. (2015)
			TALEN	Yes	Wei et al. (2018)
	CSN2: milk protein gene	Inserted *LacS* gene (sulfolobus solfataricus beta-glycosidase) to digest lactose	TALEN	Yes	Su et al. (2018)
Increase lean muscle yield	*MSTN* (Myostatin): negative regulator of muscle growth	Disrupted the *MSTN* gene	TALEN	No	Carlson et al. (2012)
			ZFN	Yes	Luo et al. (2014)
			TALEN	Yes	Proudfoot et al. (2015)
			CRISPR/Cas9	No	Namula et al. (2019)

Goal	Genome target and function	Editing approach	Editor[a]	Live edited offspring	Reference(s)
Reproduction and novel breeding schemes					
Generate host for germ cell transfer	*NANOS2* (Nanos C2HC-Type Zinc Finger 2): necessary for male germline development	Disrupted the *NANOS2* gene to eliminate germ cell production	CRISPR/Cas9	Yes	Ciccarelli et al. (2020), Miao et al. (2019)
Increased frequency of male offspring	Safe harbor loci, H11	Inserted an additional copy of the bovine *SRY* (Sex Determining Region Y protein) gene	CRISPR/Cas9	Yes	Owen et al. (2021)

[a]Editor: zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs), and clustered regularly interspersed short palindromic repeats (CRISPR)-associated protein 9 (Cas9). Note: BRD, bovine respiratory disease; BSE, bovine spongiform encephalopathy; IARS, Isoleucyl-tRNA synthetase

In particular, a highly anticipated application of GnEd in livestock is to enable breeders to tackle animal health and welfare issues at a genetic level in a way that is either not currently possible, or would result in decreased rates of genetic gain, if pursued through conventional breeding. For example, GnEd enabled Wu et al. (2015) and Gao et al. (2017) to precisely insert genes from other species (mouse *Sp110* (SP110 Nuclear Body Protein) and human *NRAMP1* (Natural Resistance-Associated Macrophage Protein 1), respectively) into an intergenic region of the bovine genome to decrease susceptibility to tuberculosis. This scientific feat would not have been possible through conventional breeding methods alone. GnEd has also enabled researchers to replicate a beneficial mutation in the prolactin receptor (*PRLR*) gene, first found in Senepol cattle and hypothesized to result in a SLICK phenotype (i.e., short, sleek hair coat), in Angus cattle to increase thermotolerance (Rodriguez-Villamil et al. 2021). Although the Senepol *PRLR* mutation could be introgressed into another breed, such as Angus, through conventional breeding methods alone, the process would require multiple generations of backcrossing to restore genetic merit to pre-introgression levels, due to linkage drag (Tan et al. 2012). In a species like cattle, with a long generation interval, backcrossing is a time-consuming and expensive process (Gaspa et al. 2015; Visscher et al. 1996). Additionally, it is important to note that genetic solutions for animal health and welfare issues are often more sustainable and require less labor for livestock producers than chemical or mechanical methods (e.g., polled genetics versus dehorning) (Gottardo et al. 2011; Thompson et al. 2017). It is also anticipated that GnEd could be used to repair defective genes, such as recessive lethal or heritable disease variations in high genetic merit animals (Ikeda et al. 2017; Ishino et al. 2018).

Overall, the potential for GnEd to improve livestock sustainability is clearly evident. As illustrated by the 2018 National Academies of Sciences, Engineering, and Medicine (NASEM)

study, “Science Breakthroughs 2030: A Strategy for Food and Agricultural Research,” which identified “the ability to carry out routine gene editing of agriculturally important organisms” as one of the five most promising scientific breakthroughs that are possible to achieve in the next decade to increase the U.S. food and agriculture system’s sustainability, competitiveness, and resilience (NASEM 2018). However, strategies for routinely incorporating GnEd into existing animal breeding programs, especially for species with long-generation intervals, like cattle, are less evident.

6.1. ART enables production of live GnEd offspring

For GnEd to be an important factor for genetic improvement, it must reliably edit the germline of breeding stock, so the edits can be passed on to the next generation. To date, it has been challenging to produce live, homozygous, non-mosaic, GnEd offspring. There are currently two primary methods to generate GnEd bovine embryos and each has associated tradeoffs (Fig. 2.4).

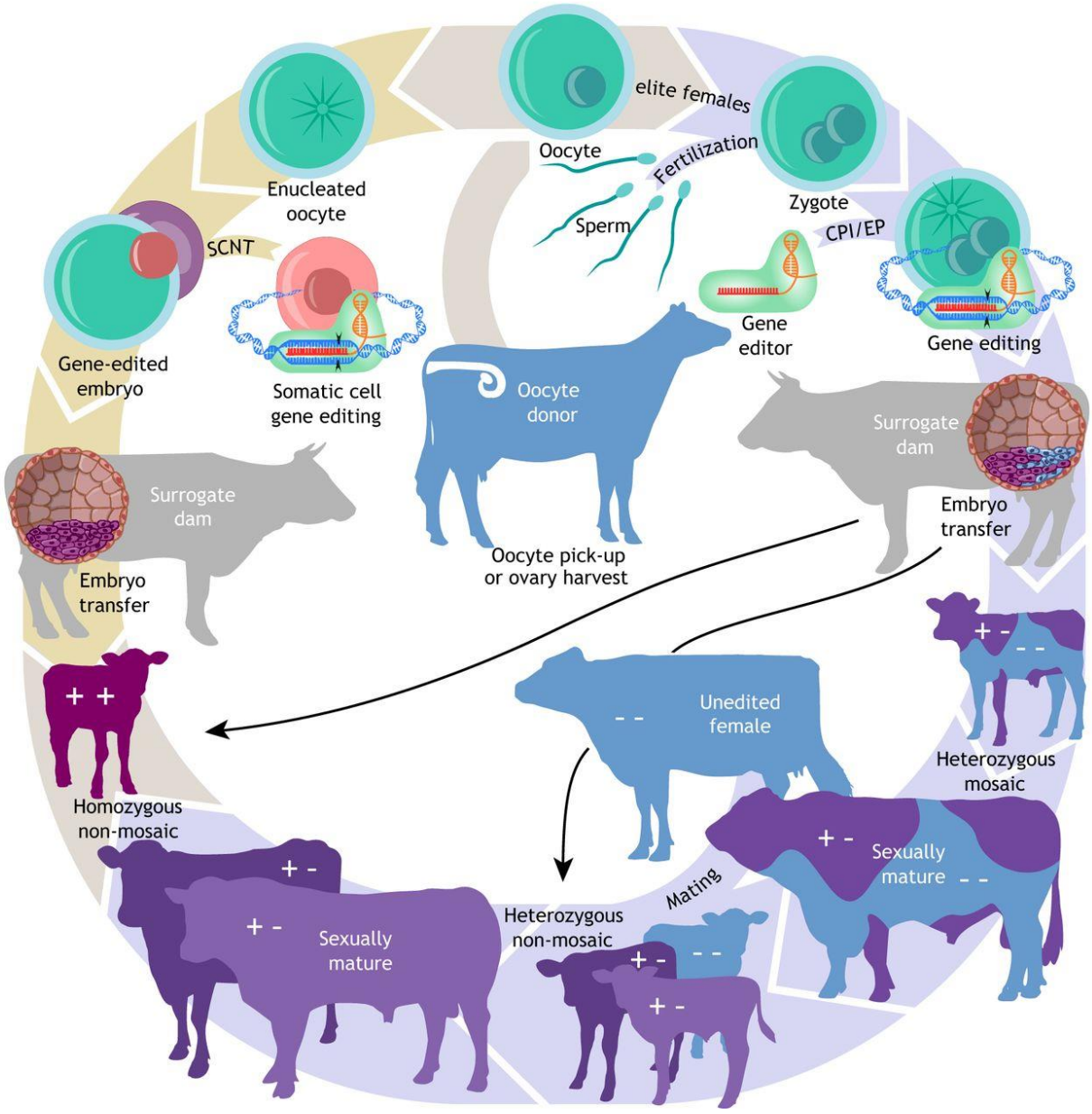

Figure 2.4. Schematic showing the number of steps required to produce live, homozygous, non-mosaic, GnEd livestock (maroon calf) through either somatic cell nuclear transfer (SCNT) cloning (tan arrows) or zygote microinjection (light purple arrows). Both methods include gamete collection and maturation, introduction of the gene-editing (GnEd) reagents, and transfer of embryos into synchronized recipients (surrogate dams). For the SCNT cloning approach (tan arrows) GnEd reagents are introduced into a somatic cell line and then SCNT cloning is used to

produce embryos for transfer. The GnEd cell line can be screened before cloning to ensure production of a homozygous, non-mosaic animal. For the zygote microinjection approach (light purple arrows) GnEd reagents are introduced directly into a zygote via cytoplasmic injection or electroporation. GnEd of zygotes can result in mosaic offspring, which requires subsequent breeding to produce first heterozygous and ultimately homozygous GnEd offspring. Therefore, gene editing of zygotes may require more steps to produce a homozygous, non-mosaic, GnEd animal, as indicated by the increased number of light purple arrows (7) compared to the number of tan arrows (3). Reproduced from (Bishop and Van Eenennaam 2020) under a CC-BY license

One option is to introduce the GnEd reagents (e.g., CRISPR/Cas9) into a somatic cell line and subsequently clone the cell line by SCNT to produce embryos. Thus far, SCNT has been the primary method for producing GnEd livestock because the clonal colony growth of cell lines provides large amounts of DNA that can be genomically sequenced to confirm and isolate cells with the desired edit such as to only produce animals with intended edits. However, as previously discussed, SCNT cloning often results in high rates of pregnancy loss and can also negatively affect the viability of live-born calves (Akagi et al. 2013; Keefer 2015). Additionally, unless a scheme similar to Kasinathan et al. (2015) is used, adult somatic cloning increases the generation interval by one generation (equivalent to two years in cattle), compared to ET of *in vivo* derived or IVP embryos.

Alternatively, GnEd reagents can be introduced directly into the cytoplasm of an IVP zygote, typically via microinjection or more recently, via electroporation (Lin and Van Eenennaam 2021; McLean et al. 2020). GnEd of zygotes is an attractive option because it avoids the inefficiencies associated with SCNT cloning, does not increase the generation interval because the GnEd process is occurring in the next generation of animals, and allows for the introduction of

GnEd reagents into a genetically diverse population of foundation animals as each zygote will produce a genetically distinct animal, as compared to animals derived from a clonal cell line. However, characterizing GnEd zygotes is difficult due to the challenges of GSE discussed above. Specifically, a major challenge associated with GnEd of zygotes is the production of mosaic animals (Bishop and Van Eenennaam 2020; Hennig et al. 2020; McLean et al. 2020). Mosaicism arises from mutations that occur after DNA replication (van Echten-Arends et al. 2011), resulting in one individual having two or more different genotypes. It is important to keep in mind that many livestock GnEd applications require homozygous modifications (i.e., two copies) to ensure inheritance of one copy in the F1 generation (Bishop and Van Eenennaam 2020). Therefore, mosaic GnEd animals will often require time-consuming and expensive subsequent crossbreeding to ultimately produce homozygous edited offspring (Fig. 2.4).

Regardless of the method used to generate GnEd bovine embryos, ET into synchronized recipient females is a crucial step in producing live GnEd offspring (Fig. 2.4). Therefore, GnEd in mammalian livestock species is currently reliant on the use of ART (i.e., IVP or SCNT to produce GnEd embryos, and ET to produce live, GnEd offspring).

6.2. Synergistic strategies for incorporating GnEd into livestock breeding programs: simulations

To be an effective tool for genetic improvement, GnEd must integrate smoothly into existing cattle breeding programs (Bishop and Van Eenennaam 2020). Thus far, GnEd has not yet been applied at commercial scale, and so strategies for incorporating GnEd into livestock breeding programs have primarily been modeled via computer simulation.

One of the first simulation studies to explore the potential of combining GnEd with GS in a livestock breeding program was by Jenko et al. (2015). Although, GnEd is currently being used to improve monogenic traits, Jenko et al. (2015) modeled a hypothetical breeding scheme of GS

supplemented with promotion of alleles by GnEd (PAGE) to improve a quantitative trait and compared the results to a baseline scenario of using GS alone. In the PAGE scheme, the top sires (5, 10, or 25) based on their true breeding values (i.e., GS with perfect accuracy) were selected and then GnEd for 1–100 loci. They found that using GS + PAGE for 20 loci using 25 sires doubled the rate of genetic gain as compared to using GS alone. It is important to note that this simulation assumed a quantitative trait that had 10,000 known quantitative trait nucleotides (QTN), but identifying such QTN is not a trivial exercise and to date relatively few QTN with large effects on quantitative traits have been identified (Georges et al. 2019).

Bastiaansen et al. (2018) modeled GnEd of a monogenic trait at the zygote stage in a generic livestock population combined with GS for a quantitative trait (i.e., index-based selection). In this simulation, zygotes from either 0, 10, or 100% of matings from genomically-selected elite parents were GnEd for the desired monogenic trait. Additionally, due to the low efficiencies of GnEd reported in the literature (Tan et al. 2016), they modeled various GnEd success and embryo survival rates. When they modeled 100% GnEd efficiency and embryo survival, they observed a strong favorable impact of GnEd on decreasing the time to fixation for the desired allele (four-fold faster), compared to GS alone. However, when they modeled a 4% GnEd efficiency, this had a major impact on the number of GnEd procedures needed (increased by 72%) and the selection response for the polygenic trait decreased by eight-fold, compared to the 100% efficiency model (Bastiaansen et al. 2018). As discussed previously, GnEd of zygotes is typically not 100% and mosaic animals are common (Hennig et al. 2020; McLean et al. 2020). Therefore, in a commercial setting GnEd embryos will likely need to be biopsied to confirm the desired change before ET to avoid transferring embryos without the desired edit(s). Moreover, the current technical limitations of embryo biopsying will need to be overcome to not only identify embryos with the intended

edit(s), but also to use GS to select embryos with superior genetic merit to increase rates of genetic gain.

Van Eenennaam (2017) proposed a scheme where GnEd could be incorporated as an added step to the Kasinathan et al. (2015) elite cattle production system (Fig. 2.5). This approach was modeled to introduce a beneficial, monogenic, dominant allele (i.e., the *POLLED* Celtic allele (P_C)) into the U.S. dairy cattle (Mueller et al. 2019) and northern Australian beef cattle populations (Mueller et al. 2021). In these simulations, fetal tissue from the next generation of yet-to-be-born bulls was genomically screened and selected, edited, and then successfully cloned such that this production system added 3–5 months to produce a homozygous GnEd bull (Fig. 2.5).

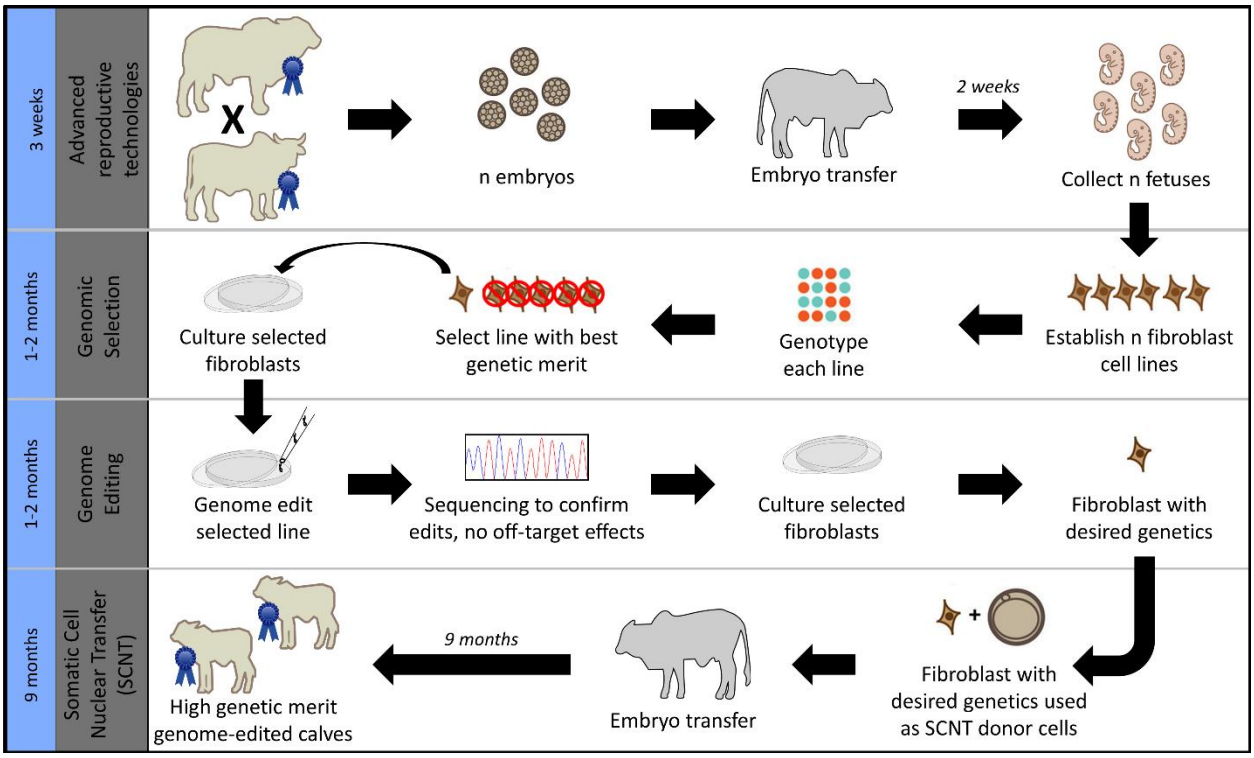

Figure 2.5. Production of high genetic merit calves using a range of biotechnologies and showing where gene editing might fit into the process. Blue ribbons represent elite genetics. Modified from Van Eenennaam (2017) and reproduced from (Mueller et al. 2021) under a CC-BY license

Mueller et al. (2019) modelled the U.S. dairy population and found that the use of GnEd was the most effective way to increase the frequency of the desired P_C allele while minimizing detrimental effects on inbreeding and the rate of genetic gain based on an economic selection index (NM$). They observed that GnEd only the top 1% of bull calves per year based on their index value while placing moderate selection pressure on the polled phenotype was sufficient to maintain the same or a better rate of genetic gain compared to conventional selection on genetic merit alone, while significantly increasing the P_C allele frequency to greater than 90% (Mueller et al. 2019). Additionally, both Bastiaansen et al. (2018) and Mueller et al. (2019) found that GnEd reduced long-term inbreeding levels in scenarios that placed moderate to strong selection emphasis on the monogenic trait of interest (e.g., polled) compared to conventional breeding alone. Importantly, Mueller et al. (2019) modeled conventional breeding to represent the widespread use of AI in the U.S. dairy population (i.e., maximum of 5000 (5%) matings/bull/year) (Capper and Cady 2019; Capper et al. 2009; García-Ruiz et al. 2016; VanRaden 2007), so a single dairy sire was able to have a large impact on the whole population. Therefore, only a small number of elite, GnEd polled, dairy sires were needed to see population-level results (Mueller et al. 2019).

In contrast, AI is rarely used in northern Australian beef cattle breeding herds (< 1%) (MLA 2015), thus Mueller et al. (2021) modeled all matings via natural service (i.e., maximum of 35 matings/bull/year). The natural mating limits prevented individual GnEd beef bulls from having an extensive impact on the whole population. Consequently, GnEd only the top 1% of seedstock beef bull calves per year in mating schemes that placed moderate to strong selection on polled resulted in significantly slower rates of genetic gain as compared to conventional selection based on genetic merit alone. However, they did find that if the proportion of GnEd animals was increased to the top 10% of seedstock beef bull calves per year then similar rates of genetic gain

could be achieved compared to conventional selection on genetic merit alone. In all scenarios, regardless of whether GnEd was applied, the population inbreeding level never exceeded 1%. This level of inbreeding has been found to have relatively minor effects on traits of economic or biological significance in tropical beef cattle (Burrow 1998). This simulation study modeled solely natural mating because currently ARTs are scarcely used in this beef cattle population (MLA 2015). However, the authors explain that, "this is unlikely to be the situation with valuable GnEd bulls. It is more probable that a high-genetic-merit homozygous polled sire would be used for AI or IVP followed by ET, in the seedstock sector. This system would amplify the reach of each GnEd bull using well-proven ART and enable these bulls to produce hundreds or even thousands of progeny, and thus have a greater impact on the whole population."

Although Mueller et al. (2021) modeled a northern Australian beef cattle population, many findings are also applicable to the global beef industry and the situation in many developing countries (Baruselli et al. 2019; MLA 2015; Ojango et al. 2016a, b; Setiana et al. 2020; USDA 2020). AI is logistically challenging to implement for both smallholder farms in developing countries (e.g., lack of AI technicians and difficulties transporting cryopreserved semen) and often for commercial-scale extensive beef operations in developed countries (e.g., additional labor required to identify females in estrus and constrain them to perform AI). Therefore, a large number of GnEd natural service bulls would currently be needed to broadly disseminate GnEd traits globally in systems that have limited adoption of ARTs.

6.3. Surrogate sires to disseminate GnEd traits

A potential alternative to AI that could be enabled through GnEd is a concept called surrogate sires. Surrogate sires would be host bulls that carry germ cells from more genetically elite donor sires, and they will be able to pass on these desirable donor genetics through natural mating to improve production efficiency (Gottardo et al. 2019). Additionally, surrogate sire

technology could potentially provide an efficient means for the distribution of traits that have been improved through GnEd (McFarlane et al. 2019).

It is anticipated that surrogate sire technology could be realized through germline complementation, which consists of using donor cells from one genetic background to complement or replace the germline of an otherwise sterile host of a different genetic background (Giassetti et al. 2019; Richardson et al. 2009). Germline complementation requires two components: (1) a host that lacks his own germline, but otherwise has normal gonadal development (e.g., intact reproductive tract), and (2) donor cells that are capable of becoming gametes (Fig. 2.6).

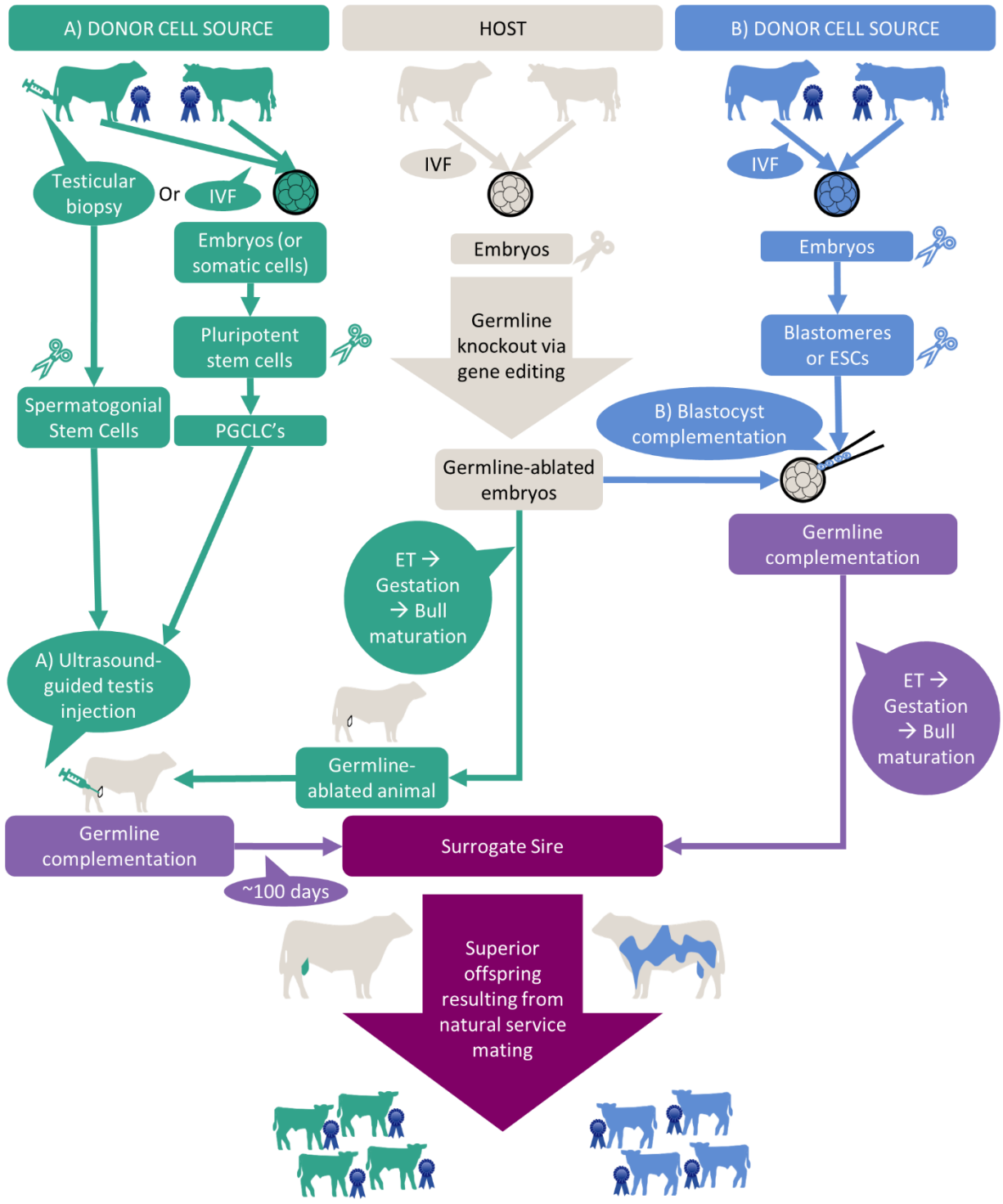

Figure 2.6. Schematic of potential surrogate sire production systems. Grey represents steps to generate the host animal. Green and blue represent potential alternative sources and steps for generating donor cells. Light purple represents the germline complementation steps and dark

purple/maroon represents the resulting final surrogate sire product. Key differences are that in the green (A) path, germline complementation would take place in a live, juvenile or adult, animal and the host would be non-mosaic. In contrast, in the blue path (B), germline complementation would take place at the embryo stage and the resulting host could be mosaic. Blue ribbons represent elite genetics and scissors represent steps that require (solid fill) gene editing or where gene editing could potentially be introduced (outline only). PGCLC: primordial germ cell-like cells, ESC: embryonic stem cell

One method to generate germline-deficient hosts is via treatment with chemotoxic drugs (e.g., busulfan) or local irradiation, but these methods are not efficient in livestock because they either fail to completely eliminate the endogenous germline, or the treatment has undesirable side effects on animal health (Giassetti et al. 2019). A promising alternative is to use GnEd to knockout a gene (e.g., *NANOS2* or *DAZL*) in a zygote that is necessary for that animal's own germ cell production (Ciccarelli et al. 2020; McLean et al. 2021; Miao et al. 2019; Park et al. 2017; Taylor et al. 2017).

Donor cells could be blastomeres (i.e., embryo cells) or stem cells, as reviewed by Bishop and Van Eenennaam (2020) and McLean et al. (2020). Potential sources of germline competent stem cells are ESCs, iPSCs, or spermatogonial stem cells (SSCs), which can be isolated from mature or juvenile testes (Ciccarelli et al. 2020; Giassetti et al. 2019). Additionally, ESCs or iPSCs could possibly be induced in culture to become PGCLCs (Hayashi et al. 2011). Stem cells provide several advantages over blastomeres, as an embryo has a limited number of blastomeres and therefore a limited amount of genomic screening and multiplication potential (McLean et al. 2020). In contrast, stem cells are self-replicating so they can provide a potentially unlimited supply of donor cells. Additionally, stem cells could be GnEd in culture, possibly multiple times

sequentially, and then DNA could be extracted without harming the viability of the remaining stem cells to both confirm the intended gene edit was made and to use GS to determine the genetic merit of each line. This scheme would be especially useful when applied to ESCs, which represent the next generation, to overcome the current challenges associated with GSE and to avoid the mosaicism issues currently associated with zygote GnEd.

The process of germline complementation (i.e., combining donor cells with a host) can occur at different stages of a host animal's development, depending on the donor cell source (Fig. 2.6). If the donor cells are SSCs or PGCLCs then they can be injected into a juvenile or adult host's germline-deficient gonad (Fig. 2.6A). SSCs transfer has been demonstrated in pigs and goats and represents germline cloning of the current generation of sires (Ciccarelli et al. 2020; Park et al. 2017). Whereas, PGCLCs derived from ESCs would represent germline cloning of the next generation since the donor cells would originate from an unborn 7-day old embryo. Alternatively, donor blastomeres or ESCs, which both represent the next generation, could be combined with the host at the developing embryo stage (Fig. 2.6B) (Ideta et al. 2016; McLean et al. 2020).

Irrespective of the production method, surrogate sires could unlock an opportunity to both accelerate rates of genetic gain and widely distribute traits improved via GnEd. The selection of only elite males for donor cells would increase selection intensity. Additionally, since the use of surrogate sires will not require any additional labor for commercial producers, there could be widespread adoption of this technology, which would dramatically reduce the lag in genetic merit that typically exists between the seedstock sector and the commercial sector. For example, Gottardo et al. (2019) performed simulations to develop and test a strategy for exploiting surrogate sire technology in a pig breeding programs. Their model projected that using surrogate sire technology in the swine industry would significantly raise the genetic merit of commercial sires

by closing the typical 4 year genetic lag (difference in genetic mean between the nucleus and commercial populations), resulting in as much as 6.5 to 9.2 years' worth of genetic gain as compared to a conventional pig breeding program (Gottardo et al. 2019; Visscher et al. 2000).

7. CONSIDERATIONS FOR INCORPORATION OF RECORDS FROM ANIMALS PRODUCED USING ADVANCED REPRODUCTIVE OR MOLECULAR BIOTECHNOLOGIES INTO NATIONAL CATTLE GENETIC EVALUATIONS

Currently, an important question is how to best accommodate animals produced using advanced reproductive and/or molecular biotechnology and their progeny into genetic evaluations. In the U.S., the majority of genetic evaluations for beef cattle are carried out by breed associations following the industry-standard Beef Improvement Federation (BIF) guidelines (BIF 2021d; Van Eenennaam 2019). U.S. dairy cattle genetic evaluations were previously performed by the U.S. Department of Agriculture-Agricultural Research Service-Animal Genomics and Improvement Laboratory (USDA-ARS-AGIL) and are currently performed by the Council of Dairy Cattle Breeding (CDCB). Additionally, the International Committee for Animal Recording (ICAR), which is an international Non-Governmental Organization (NGO), provides guidelines, standards, and certification for animal identification, animal recording, and animal evaluation.

7.1. Records from animals resulting from ART

For animals resulting from MOET, BIF recommends that all observations, or phenotypic information, for traits that do not have maternal effects be used in genetic evaluations and that observations "for traits that have maternal effects, be used in genetic evaluations as long as the recipient dams' ages (heifer, 1st parity, or multiparity) and approximate breed compositions are available" (BIF 2021b). Additionally, "BIF recommends that embryo stage (1–9) and grade (1–3) and whether frozen, split, sexed, or genotyped be recorded and submitted to breed association or other recording organization" and that, "when sufficient information becomes available, genetic

evaluation models for MOET calves include effects of fresh versus frozen and of biopsied (sexed and/or genotyped) or not" (BIF 2021b). However, due to historic concerns of large offspring syndrome, BIF does not recommend to use phenotypic observations from animals resulting from IVP in genetic evaluations (BIF 2021b; Thallman and Snider 2021). Although, BIF does recommend that observations on all ET calves (i.e., resulting from MOET or IVP) be recorded and submitted to breed association or other recording organizations, along with the form of technology used and other pertinent details related to producing the ET calves (BIF 2021b), so that this information could eventually be used in analyses that would enable the incorporation of records from IVP produced beef cattle to be included in future genetic evaluations (Thallman and Snider 2021). In contrast, phenotypic observations from animals resulting from both MOET and IVP are included in dairy cattle genetic evaluations. For dairy animals known to be produced by ET (both MOET and IVP), production records (e.g., lactation records) are included in genetic evaluations, but fertility and calving data (e.g., stillbirth records) are excluded from genetic evaluations of those traits because they don't represent "normal" expressions of fertility (personal communication, John B. Cole).

Regarding animals resulting from NT, due to concerns of large offspring syndrome and abnormal clone syndrome, BIF recommends to not use phenotypic observations from these animals in genetic evaluations (BIF 2021b; Thallman and Snider 2021), but also recognizes that "there are instances where genetically identical animals are in the pedigree (i.e. identical twins and clones)." In these cases where genetically identical animals exist in the pedigree, BIF recommends that, "for purposes of routine genetic evaluation, each set of genetically identical individuals is assigned a common identifier, so they have identical expected progeny differences (EPDs)," and recommends that, "they should also be assigned different permanent identification numbers"

(BIF 2021c). An EPD, which is the standard term used in the U.S. beef industry, is a predictor of the genetic merit of an animal's progeny and is equal to half of an animal's EBV. Data from clones is handled similarly for dairy genetic evaluations, where each clone receives a unique permanent identification number and an individual evaluation, but the same predicted transmitting ability (PTA) is distributed for all clones from the same donor (personal communication, John B. Cole). A PTA, which is the standard term used in the U.S. dairy industry, is a predictor of the genetic merit of an animal's progeny and is equal to half of an animal's EBV.

ICAR recommends that detailed data should be recorded at all steps of embryo production (e.g., embryo stage, embryo grade, and whether frozen, split, sexed, or genotyped) and this information should be submitted to breed association or other recording organizations. ICAR is working to develop standardized codes for identifying features of embryos (e.g., sex, NT, IVP, etc.). Additionally, ICAR advises having parentage verification for animals resulting from ET (ICAR, 2017, 2019).

7.2. Records from animals resulting from GnEd

Given that all GnEd animals are currently produced via SCNT or IVP the phenotypic observations of the resulting animals would be recommended to be excluded from beef genetic evaluations, but could potentially be included in dairy genetic evaluations (BIF 2021b; Thallman and Snider 2021). ICAR recommends that "breed Associations should check the rules of their countries with regard to allowing GnEd animals in the herd book," and "if an animal has been GnEd it should be recorded against the animal when registered and should appear on the Zootechnical Certificate" (ICAR 2019). Additionally, BIF has developed more detailed guidelines for what data should be required from GnEd animals for breed association registration (BIF 2021a). Recently, two major beef breed associations, the American Angus Association (AAA) and the Red Angus Association of America (RAAA) adopted bylaws regarding the

registration requirements for GnEd founders (GEF) and descendants (GED) (AAA 2021; RAAA 2021). Moreover, in September of 2021 the RAAA was the first breed association to announce that "they will provide herdbook registry of Red Angus animals carrying GnEd traits for heat tolerance and coat color" (RAAA 2021).

Moving forward, the GED will eventually enter genetic evaluations and the method for inclusion of these phenotypic records may differ depending on the type of trait affected by the GnEd (Thallman and Snider 2021). Most GnEd targeting qualitative traits (e.g., horned/polled or coat color), would have no influence on genetic evaluations. In contrast, GnEd targeting quantitative traits (e.g., muscle yield or disease resistance) could have a major impact on the genetic evaluations of close relatives. Thallman and Snider (2021) state that "gene editing directly violates fundamental assumptions of traditional (non-genomic) genetic evaluation." However, they also point out that fortunately, it will likely be easier to accommodate GnEd in genomic evaluation models (e.g., Single Step), and that research will be needed to determine the best way to include these records in different genomic models (Thallman and Snider 2021).

7.3. Records from surrogate sires

Based on the current proposed methods, surrogate sires will also be produced using IVP to generate the germline knockout host for germline complementation (Fig. 2.6). Therefore, based on current BIF guidelines, phenotypic observations on surrogate sires would also be excluded from beef genetic evaluations (BIF 2021b). However, phenotypes recorded on the somatic host are unrelated to the genetic merit of the donor germline, and therefore should not be included in the genetic merit estimate calculations associated with the donor. It should be noted that GnEd, homozygous *NANOS2* knockout females are expected to be fertile, so when crossed with a GnEd, heterozygous *NANOS2* knockout, fertile male this mating would be expected to produce 50% homozygous *NANOS2* knockout, infertile male offspring, even in the absence of IVP or other

ARTs (Park et al. 2017). Similar to animals resulting from ET, it will be useful to record as much information as possible on all contributing factors to the surrogate sire embryo (i.e., sire and dam of the host embryo, identification and genomic information of the germline donor source, ET recipient identification, and details on the production process). Regarding progeny of the surrogate sires, they should be genotyped to confirm inheritance of the germline donor's DNA. Once paternal inheritance is confirmed, then potentially these progeny could be handled similarly to those of clones (BIF 2021), where all offspring data is attributed to the original germline donor and the progeny would all share a common identifier, but also be assigned unique permanent identification.

8. CONSIDERATIONS FOR GENETIC IMPROVEMENT OF CATTLE IN DEVELOPING COUNTRIES

Cattle are raised in more than 200 countries around the world in almost all climatic zones, with the exception of high elevations, and they have been bred for adaptations to heat, cold, humidity, extreme diet, water scarcity, mountainous terrain, dry environments, and for general hardiness. In 2019, the Food and Agriculture Organization of the United Nations (FAO) estimated global cattle numbers at 1.511 billion head (FAOSTAT 2020). Across the globe and between individual producers, there is a wide gap in production efficiency, which results in considerable variation, even up to a 50-fold difference, of the environmental impact of producing the same product (Herrero et al. 2013; Poore and Nemecek 2018). This production efficiency gap is especially large between developed and developing, or Low-to-Middle-Income Countries (LMIC). For example, while global beef production is currently split evenly between developed (49%) and developing (51%) countries, the environmental impact of production is not (FAO 2021b). Presently, LMIC contribute the majority of global ruminant greenhouse gas emission emissions (75%) and house 76% of the global cattle herd (FAO 2021a; Herrero et al. 2013). It's important to

note, in the 1990's the African continent became the region of the world with the largest number of cattle and now collectively is home to 361 million cattle. This exceeds the 215 million cattle located in Brazil, the individual country with the largest cattle population (#3 beef producer), and is more than triple the number of cattle in the U.S. (94.8 million head; #1 beef producer). Ethiopia alone has 63 million cattle, the most of any African country, followed by Sudan and Chad at 31 million head each. In 2019, the African continent accounted for 24% of the global cattle population, but only 10% of the global beef production (FAO 2021a, b).

Considering that 81% of the additional beef production expected by 2029 is predicted to occur in the developing countries of Argentina, Brazil, China, Pakistan, and Sub-Saharan Africa, this production efficiency gap is a crucial challenge for global cattle production sustainability. For example, Chang et al. (2021) estimated that improving livestock production efficiencies in the 10 countries with the largest emission reduction potential (i.e., the current production efficiency is low, resulting in a high emission intensity per kg protein, and a large increase in livestock production is projected), could contribute 60%–65% of the global reduction in livestock emissions by 2050 (compared to a baseline where emissions intensities are held constant in the future). Chang et al. (2021) determined that the 10 countries with the largest emission reduction potential were in Africa (Madagascar, Morocco, Niger, South Africa, Tanzania), Asia (China, India, Iran, Turkey) and South America (Brazil).

It is important to keep in mind that beyond meat and milk, cattle also produce fibers, hides, skins, fertilizer, and fuel, are used for transportation and draft power, serve ecological roles, and particularly in Africa and parts of Asia, they also serve socio-economic (e.g., asset building in the form of stock accumulation) and cultural (e.g., religious worship in India and Lobola, or 'bride price' in parts of Africa) purposes. Therefore, careful consideration of livelihood concerns will be

required when implementing production efficiency improvements. Van Eenennaam and Werth (2021) explain, “any proposed strategies for boosting the efficiency of cattle production need to consider these broader concerns, and also the fact that access to technologies may more be limited in some settings, often because of factors such as inaccessibility, unaffordability, lack of relevant knowledge, and/or of organizational capacity.” Although some LMIC, like Brazil, have successfully implemented ART on a large commercial scale, not all genetic improvement tools or strategies have translated as easily to other developing countries.

In LMIC, genetic progress can be frustrated by poor infrastructure and ecological and financial challenges (Mapiye et al. 2018; Nyamushamba et al. 2017). For example, in South Africa, it is difficult to develop genetic tools such as EBVs for smallholder farmers due to small herds, incomplete data recording for most traits, a lack of parentage recording, insufficient contemporary groups, and lack of organizational capacity (van Marle-Köster and Visser 2018). In a survey of 62 market-oriented smallholder beef farmers in South Africa, 77% percent of the farmers reported that they were constrained by cattle breeding challenges including a shortage of breeding bulls (12%), lack of enclosed breeding pens (46%), and poor breeding management skills (29%) (Mapiye et al. 2018). Additionally, a number of non-scientific challenges also face emerging market-orientated cattle farmers including land access and ownership issues, and access to financial support and markets (Khapayi and Celliers 2016; Mapiye et al. 2018). These studies suggest that providing South African smallholder farmers with superior genetic material for genetic improvement of their livestock will require different approaches than have been used to implement traditional genetic evaluation programs (van Marle-Köster and Visser 2018). Community-based breeding programs have seen the most success, especially when they “are based on the breeding goals of smallholder farmers, there are strong market incentives for improved

animal productivity, and strong support services such as extension and veterinary services" (de Haas et al. 2016).

The Consultative Group on International Agricultural Research (CGIAR) implemented a collaborative research program to observe, survey, and compare the dairy value chains in Tanzania and Kenya (East Africa), India (South Asia) and Nicaragua (Latin America) (Ojango et al. 2016). In these countries a large number of smallholder farmers that operate mixed crop–livestock production systems play a significant role in dairy production. CGIAR chose to include countries in multiple regions in order to allow for comparisons and cross-system learning that would support development of lessons, methodologies, and technologies of wide applicability (ILRI et al. 2011). This analysis revealed significant productivity gaps especially between large and small-scale producers and identified genetic and reproductive biotechnologies that hold promise for the advancement of global development goals in countries (ILRI et al. 2011).

Among these four countries, Ojango et al. (2016) observed that Kenya was the only country that had a national animal recording system where pedigree and performance recording is conducted. Although open to all producers, the system is primarily used by the large-scale dairy producers in high-input systems where purebred cattle are common. At the time, only 2.5% of the national dairy herd was accounted for in the national animal recording program. This low participation rate is a major obstacle because, as discussed previously, the foundation of genetic improvement is a well-structured breeding program with a clear breeding objective.

Crossbreeding is a more common practice within the smaller-scale livestock production enterprises in both Kenya and Tanzania, where the majority of the smallholder farmers have less than five cows. However, indiscriminate or uncontrolled crossbreeding can lead to the demise of indigenous breeds (van Marle-Köster and Visser 2018). For instance, unstructured crossbreeding

programs in Africa have produced non-descript crossbred cattle that now constitute more than two thirds of the smallholder herd (Scholtz et al. 2008). It has been suggested that, structured breeding programs of African indigenous livestock should be developed (Mwai et al. 2015), informed by knowledge of the population structure and genetic diversity of these breeds (Nyamushamba et al. 2017). Such developments should include active farmer participation in the selection of superior indigenous sires based on the local breeding objectives using a community based breeding program model (Mapiye et al. 2019).

The CGIAR study found that AI was the most widely used reproductive biotechnology in all four countries, especially in large-scale dairy systems. However, it has proven more difficult to successfully implement in smallholder cattle production systems in developing countries due to logistical and institutional challenges (Ojango et al., 2016).

In other LMIC, crossbreeding via AI has been used to try to intensify the beef cattle sector with limited success. For example, in Indonesia in the 1980s, the government promoted the AI of the local Ongole cattle with Simmental and Limousin semen to produce more productive F1 animals. In this country with a population of 270 million people and 17 million cattle, 90% of cattle production is from smallholder farming systems with about 6.5 million farmers living in the rural areas. These crossbred animals were not supported with better feed and health services, which limited their potential and the cattle keeping systems did not become more efficient through crossbreeding (Agus and Mastuti Widi 2018). More recently, a program which translated into “a cow must be pregnant” was launched in 2016 and set a target of 4 million head of productive cows inseminated to produce 3 million calves, this time with the support of improved feed provided by planting improved pastures and legumes, and the provision of health services. A report on the success of this program details some of the problems encountered in getting frozen semen to

remote locations, difficulty in getting cattle in the right body condition score to be reproductively cycling, and lack of farming experience (Setiawan 2018). Additionally, in a survey conducted in another region of Indonesia, adoption of AI was found to be inversely correlated with farmer age and cost of AI (Setiana et al. 2020).

In recent years, genomics has started to be used to try to identify animals that have both enhanced productivity and adaptation to African conditions (Marshall et al. 2019; van Marle-Köster and Visser 2018). Crossbred animals that retain some of the resilience of indigenous breeds while being more productive can improve production efficiency. In a case study with dairy production in Senegal, crossbred indigenous zebu by *Bos taurus* dairy cattle, as identified by genomics, and kept under better management produced up to 7.5-fold higher milk-yields, eightfold higher household profit, and threefold lower greenhouse gas emission intensity, per cow per annum, in comparison to indigenous Zebu kept under poorer management, for a typical herd size of eight animals (Salmon et al. 2018). There are glaring disparities when it comes to the implementation of GS in LMICs, and even among small breeds in the developed world. GS is not a scale-neutral technology, advantaging large breeds and genetic providers over small ones. It is difficult to implement in the absence of structured breeding programs with sufficiently sized genotyped and phenotyped reference populations. Therefore, more investment in data recording and structured breeding programs, linked to multiplication and delivery systems that can be delivered at scale will be needed to enable genetics and genomic technologies to deliver sustained benefits in LMIC cattle production systems.

Additionally, genomics can provide information on important traits of indigenous breeds. For example, it is well known that African cattle have improved thermo-tolerance levels and an increased ability to regulate their body temperature (Kim et al. 2017a). It has been suggested that

the greatest benefit of genomics to smallholder farmers might be the characterization of the drought tolerant, resistance to ticks and tick-borne diseases, thermo tolerance and resistance to trypanosomosis traits present in adapted native breeds (Kim et al. 2017b; Nyamushamba et al. 2017). Other potential genotype-derived information includes the breed composition of the animal, which may be particularly useful in devising structured crossbreeding strategies (Kuehn et al. 2011; Marshall et al. 2019; Ojango et al. 2014).

GnEd could potentially be a useful tool for genetic improvement of cattle in LMIC because GnEd can be used to efficiently introduce useful Mendelian traits from other breeds into existing, locally adapted breeds, rather than having to introgress useful alleles via crossbreeding. Additionally, GnEd could be used to introduce novel beneficial traits (e.g., disease resistance), possibly from different species. In Africa, a particular focus has been placed on using GnEd to combat animal disease (Karavolias et al. 2021). One approach is to gene edit virulence genes of parasites, like *Theileria parva*, to weaken the pathogen so that it could be used in the development of a more effective vaccine against East Coast Fever, which is a disease that is estimated to kill one cow every 30 s across a dozen African countries (Enahoro et al. 2019; Karembu 2021). Alternatively, GnEd could be used to introduce disease resistance into indigenous breeds of cattle. For example, the Apolipoprotein L1 (ApoL 1) gene has been found to confer resistance to trypanosomiasis in primates (O'Toole et al. 2017), and African researchers are currently working to use the CRISPR/Cas9 system to knock-in Apol 1 into an indigenous goat breed (Karembu 2021). If successful, this GnEd scheme could also be used to combat the devastating disease of trypanosomiasis in cattle.

It is important to keep in mind that the effective and efficient use of GnEd will require infrastructure to perform ART to both facilitate the production of animals, and the dissemination

of improved traits. To accelerate rates of genetic gain, a structured breeding program, ideally including GS, should be used to ensure that the best (i.e., highest genetic merit) parents of and/or animals are put forward as selection candidates. This alone would improve production and accelerate genetic improvement, even in the absence of GnEd. Additionally, surrogate sires distributing elite, locally adapted genetics, with or without useful GnEd traits, could provide a workable approach for the more widespread dissemination of improved genetics via natural service.

9. REGULATORY CONSIDERATIONS FOR TOOLS FOR THE GENETIC IMPROVEMENT OF CATTLE

9.1. Regulation of GS

Animals produced from conventional breeding methods are routinely evaluated for changes in productivity, reproductive efficiency, reactions to disease, and quality characteristics by breeders. However, they are not subject to regulatory approval, other than it is illegal to sell unsafe food irrespective of the breeding method that was used to produce it. Regulatory agencies do not evaluate new conventionally bred varieties or breeds for health and environmental safety or approve their sale prior to commercial release; nor are they evaluated for unintended effects at the molecular level. There are more than 86.5 million known genetic variants between different breeds of cattle, including 2.5 million insertions and deletions of one, or more, base pairs of DNA, and 84 million single nucleotide variants (Hayes and Daetwyler 2019). Genetic variation per se does not pose a unique hazard as it relates to food safety (Van Eenennaam et al. 2019). The variations fuel genetic improvement programs and drive GS, which was rapidly adopted in livestock breeding programs globally, in the absence of any specific regulatory oversight or approvals or public controversy.

9.2. Regulation of cloning

In North America, South America, and New Zealand, cloning for agricultural purposes is not legally restricted (Table 2.2). Additionally, both the U.S. Food and Drug Administration (FDA) in 2008, and the European Food Safety Authority (EFSA) in 2012, concluded that products derived from animal clones are not different from those derived from non-cloned animals. However, in the European Union (EU), food derived from animal clones falls under the 'Novel Foods Regulation,' as food derived from animals obtained by non-traditional breeding practices. Current regulation in the EU has placed a ban on food products from animal clones due to, amongst others, ethical considerations regarding animal welfare. This ban does not cover products from their progeny, which are considered to be indistinguishable from traditionally bred livestock (van der Berg et al. 2019). Currently, no company in Europe is contemplating bringing products derived from animal clones, or their offspring, to market (Galli and Lazzari 2021). In contrast, several companies in other parts of the world now specialize in cloning farm animals (van der Berg et al. 2019). A Supply Chain Management Program to identify cloned livestock in the U.S. was set up by Viagen and Trans Ova in 2007. However, according to these companies, although the program was run from 2008 until 2012, no other cloning companies showed interest in participating in the program, and it was never accessed by industry (van der Berg et al. 2019).

Table 2.2 Regulation of animal cloning, transgenesis and gene editing in livestock in the main countries exporting beef to the European Union (EU)

Country	Animal cloning	Transgenic livestock	Gene edited livestock
EU member states	Prohibited, until specific regulations on animal cloning are in place	Requires approval according to EU Directive 2001/18/EC and Regulation (EC) No. 1829/2003, safety assessment performed by EFSA GMO Panel	Requires approval according to EU Directive 2001/18/EC and Regulation (EC) No. 1829/2003, safety assessment performed by EFSA GMO Panel
USA	Allowed, a risk management plan and guidance for industry have been issued by the FDA	Requires approval according to Federal FD&C Act, regulations for new animal drugs as stated in 2009 FDA Guidance for industry #187 (Draft guidance) and NEPA	Requires approval according to Federal FD&C Act, regulations for new animal drugs as stated in 2017 FDA Guidance for industry #187 (Draft guidance) and NEPA
Canada	Allowed, food products of cloned animals and clone progeny are considered “novel foods” and require pre-market safety assessments according to the regulations in Division 28, Part B, of the Food and Drug Regulations (Novel Foods)	Requires approval according to the Canadian Environmental Protection Act, 1999, the New Substances Notification Regulations (Organisms) and Food and Drugs Act	No specific policy on gene editing, may be considered “novel” and require case-by-case safety assessment by Health Canada
Argentina	Allowed	Requires approval according to animal biotechnology regulation, case-by-case assessment by CONABIA	Requires approval according to animal biotechnology regulation, case-by-case assessment by CONABIA
Brazil	Allowed, commercial animal cloning mostly in partnership with EMBRAPA, registration of cloned cattle at ABCZ	Requires approval according to animal biotechnology regulation, case-by-case assessment by CTNBio	Requires approval according to animal biotechnology regulation, case-by-case assessment by CTNBio, GnEd animals lacking recombinant DNA are

Country	Animal cloning	Transgenic livestock	Gene edited livestock
			regarded non-GM according to Normative Resolution #16
Australia	Allowed, generally in confined research environment	Requires approval according to Gene Technology Act 2000, by OGTR	Requires approval according to Gene Technology Act 2000, by OGTR, gene editing techniques that do not introduce new genetic material are not regulated as GMOs
Uruguay	No specific legislation on animal cloning performed in research institutes, such as Institute Pasteur in Montevideo and the Animal Reproduction Institute of Uruguay	No specific legislation on animal biotechnology. Environmental release of GMOs and biosecurity is subject to prior authorization by competent authorities, as stated in article 23 of law No. 17283 on the protection of the environment	No specific legislation on gene editing in animals, during a meeting of the CAS the minister of agriculture signed a declaration in favor of gene editing. GnEd animals may be subject to prior authorization according to law No. 17283

Modified from van der Berg et al. (2020)

EFSA, European Food Safety Authority; FD&C Act, Food, Drug and Cosmetic Act; NEPA, National Environmental Policy Act; FDA, Food and Drug Administration; CONABIA, National Advisory Commission on Agricultural Biotechnology; EMBRAPA, Brazilian Agriculture and Livestock Research Enterprise; ABCZ, Brazilian Zebu Cattle Association; CTNBio, National Technical Biosafety Commission; OGTR, Office of the Gene Technology Regulator; CAS, Southern Agricultural Council

9.3. Regulation of genetic engineering (Transgenesis)

Genetically engineered (GE) or transgenic cattle have been around since the 1990s, but none have ever been successfully commercialized for food or feed production. In 2008, the Codex Alimentarius Commission published guidelines for the safety assessment of foods derived from recombinant DNA (rDNA) animals (FAO/WHO 2008). A "rDNA Animal" is defined as "an animal in which the genetic material has been changed through *in vitro* nucleic acid techniques, including rDNA and direct injection of nucleic acid into cells or organelles." The guidelines recommend evaluations of product composition and animal health as essential steps in ensuring the safety of food derived from rDNA animals. Only a single GE food animal application has ever been sold for food consumption, the fast-growing AquAdvantage salmon, and even then, only in Canada and the U.S. The regulatory approval process for this product took over 20 years and several million dollars (Van Eenennaam et al. 2021). A second GE food animal application approval, for an Alpha-gal (galactose-α-1,3-galactose) knockout "GalSafe" pig, was announced by the FDA in 2020, for a line of pigs that was first reported in the literature in 2003 (Phelps et al. 2003). This pig was developed using a traditional gene knockout approach and carries a plasmid (pPL657) rDNA construct disrupting the Alpha-gal gene along with the neomycin phosphotransferase (nptII) selection marker gene in its genome. The approval was for a single swine farm to produce a maximum of 1000 GalSafe® pigs annually to be raised in the absence of aminoglycosides, such as neomycin, to produce meat that is safe for consumption for people with Alpha-gal syndrome and porcine-based materials to produce human medical products.

9.4. Regulation of GnEd

The regulatory picture for GnEd is currently mixed (Table 2.2). Argentina was the first country to publish their proposed approach to the regulation of GnEd organisms. The trigger for regulation is whether animals carry a "novel combination of genetic material" (i.e., transgenic). Those that do will be considered a "GMO" (Genetically Modified Organism) under Argentine law, and those that do not will not trigger additional regulatory oversight (Whelan and Lema 2015). The Argentine regulation calls for GnEd plants and animals to be presented to the biosafety commission in order to establish, on a case by case basis, whether it is a GMO. An interesting aspect of this regulation is that there is an opportunity to present projects at the "design stage," whereby a preliminary opinion based on the expected outcome of the project will be issued by the commission. Later, when the plants or animals have been obtained and fully characterized, applicants must present a follow up report that will be used to establish a final decision. That determination is mostly based on any changes present in the genome of the product intended to be sold commercially. Conversely, in the EU, New Zealand, and the U.S., GnEd is being treated as equivalent to GE, with implications for global trade.

The Department of Biotechnology in India published draft guidelines for GnEd regulation in 2020. These guidelines propose a tiered approach depending upon the characteristics of the end product, but include requirements for a quite extensive characterization of trait efficacy and phenotypic equivalence of GnEd organisms triggered solely by the use of GnEd, and which is not required for those plants and animals resulting from conventional breeding.

To date, no African nation has passed regulations for GnEd animals, but similar to India, proposed guidelines are being drafted in many countries. Kenya has begun drafting guidelines to regulate GnEd products, using the Argentinean approach as a model. The draft guidelines define what needs to be regulated, what is partially regulated and what is not regulated at all. Kenya's

National Biosafety Authority (NBA) has approved, at the research level, six applications for genome editing applications in agriculture, including one application focused on making pigs resistant to African swine fever. Other applications include improving banana and yam to resist two destructive plant viruses.

The decision by the FDA to regulate GnEd animals—or more correctly the intentional alterations in the genome of animals—as new animal drugs irrespective of product risk was done in the absence of public discourse. Similarly, the decision by the European Court of Justice that genome edited organisms would be subject to the full range of testing and regulation as if they were transgenic according to the EU Directive, was made without engagement with the public. Moreover, the decision by the European Court of Justice effectively side-stepped any processes of wider societal discussion (Bruce and Bruce 2019). In considering this decision, these authors wrote, "regulation sets bounds to what can be done, who can do it and under what conditions can things be done. But if there has been no discussion with the public, this could be argued to be a case where regulation has been socially premature, and not done on behalf of the society."

Interestingly, following the United Kingdom's (UK) departure from the EU, a public consultation was held in 2021 by the UK government's Department for Environment, Food and Rural Affairs (DEFRA) as to whether GnEd technology should be regulated in the same way as GE, if it yields a result that could have been produced by conventional breeding. Following this consultation, it was determined that UK plant researchers who planned to conduct field trials of GnEd plants no longer need to submit risk assessments to DEFRA, but UK research involving GnEd animals will continue to be regulated as before to ensure animal-welfare standards are met (Ledford 2021).

While a highly precautionary regulatory approach may be of little consequence in food-secure developed regions like North America and the EU, such an approach is likely to hinder the adoption of GnEd in some LMIC that could most benefit from targeted applications, such as disease-resistant livestock. For resource-poor Africa, responding to the promises and challenges of GnEd is likely to be complex, not least because most lack the capacity for regulatory oversight. Additionally, if GnEd livestock are not required to undergo unique regulatory approval in some parts of the world, they will not necessarily be segregated from conventionally bred animals and there will often be no way to uniquely detect the products derived from them, especially if the genetic alteration already exists in the target population. This is somewhat analogous to the situation for clones, where there is no molecular way to differentiate or track the products from a clone as compared to those of its progenitor.

9.5. Public perception of GnEd

In countries where food security is not a priority, consumer acceptance of GnEd animals is expected to be lower, especially for those applications offering economic advantages mainly to the livestock producer. Bruce and Bruce (2019) considered two examples of GnEd in livestock; hornless cattle and disease resistant pigs, from the perspective of Responsible Research and Innovation (RRI). They suggested that the public's knowledge gap of current practices in livestock agriculture, could lead to unexpected outcomes from public consultations. For example, if an argument is made regarding using GnEd to introduce the *POLLED* allele, the advantage of polled cattle might not be immediately obvious to those not versed in agricultural practice, and more generally "the need for dehorning may be considered shocking by some publics" (Bruce and Bruce 2019).

A 2017 public consultation performed by the UK Royal Society found that GnEd animal applications that targeted reducing antibiotic use, greenhouse gas emissions, and zoonotic disease

transmission were all deemed acceptable (van Mil et al. 2017). However, it should be noted that a major pre-occupation of these participants in this consultation was to ensure GnEd was used to address inequality. The participants were particularly concerned about who owns the technology, who gets rich from its use, and whether it could be used to unfairly obtain monopoly power (van Mil et al. 2017). This raises interesting questions regarding whether the GnEd regulatory approaches that have been proposed in the U.S. and EU are fit for purpose (Van Eenennaam et al. 2019), as they advantage large companies and incentivize intellectual property protection. The latter of which may prove to be disruptive to the cattle breeding industry (Bruce 2017).

Evidence from Mora et al. (2012) suggested that if geographic differences are considered, consumers' acceptance of GE animals would be higher in developing countries where the requirement for enhanced food production might be met by application of this technology (Van Eenennaam and Young 2018). Historically, the debates around GE crops in Africa have been dominated by a few elite scientists or largely international NGOs, leading to a polarization that bypassed the farmers most directly affected by decisions. Roughly 65% of Africa's population relies on smallholder farming, and these farms are not highly productive. To date, only eight African countries have commercialized GE crops; Burkina Faso, Eswatini, Ethiopia, Kenya, Malawi, Nigeria, Sudan and South Africa, mostly insect-resistant Bt cotton and recently Bt cowpea in Kenya. Kenya, Nigeria and Eswatini are leading the agricultural GnEd research as they see its potential to increase farmers' income in Africa. As of yet, there is little research specifically gauging the acceptability of the use of GnEd livestock in LMIC, especially among the smallholder farmers and livestock keepers who would be most affected by any decisions around the technology.

10. CONCLUSIONS

Genetic improvement of livestock around the globe has been, and will continue to be, an important driver of the sustainability of animal agriculture. Livestock genetic improvement programs, beginning with selective breeding using statistical prediction methods (e.g., EBVs) and more recently GS, in combination with ART have enabled more accurate selection and intense utilization of genetically superior parents for the next generation to accelerate rates of genetic gain. Most recently, the ability to use GnEd to inactivate targeted gene function (i.e., knockout genes), knock-in genes, or achieve allele introgression in the absence of undesired linkage drag, offers promising opportunities to introduce useful genetic variation into livestock breeding programs. GnEd experiments in cattle have primarily focused on three main areas of improvement (1) animal health and welfare, (2) product yield or quality, and (3) reproduction or novel breeding schemes, which are all areas that are highly aligned with the goals of conventional breeding programs. Presently, GnEd is well-suited for introgressing alleles affecting typically qualitative, Mendelian traits at a more rapid pace than is possible using conventional selection alone. However, most of the traits that animal breeders seek to improve are polygenic and quantitative. Additionally, GnEd in livestock is only possible through the use of ART. Therefore, in order for GnEd to be an effective tool for genetic change it will need to seamlessly integrate into a structured breeding program with a clear breeding objective and ideally be used in conjunction with ART and GS to accelerate genetic gain by simultaneously altering multiple components of the breeder's equation. To accomplish this, several GnEd schemes have been modeled for livestock populations. The most efficient schemes have relied heavily on widespread adoption of ART, especially commercial sector use of AI. Considering the currently limited adoption of AI around the world and specifically in the commercial beef industry, novel breeding schemes, such as GnEd applied to surrogate sire production, will likely be required to widely disseminate desired traits improved via GnEd. The

lack of global regulatory harmonization around GnEd animals and products from these animals, including semen and embryos, will pose challenges in relation to global trade, and aspects of traceability in both animal breeding and the food chain.

11. ABBREVIATIONS

AAA: American Angus Association

ABCZ: Brazilian Zebu Cattle Association

AI: Artificial insemination

Apol 1: Apolipoprotein L1

ART: Assisted reproductive technologies

BIF: Beef Improvement Federation

BLG: Beta-lactoglobulin

BRD: Bovine respiratory disease

BSE: Bovine spongiform encephalopathy

CAS: Southern Agricultural Council

CDCB: Council of Dairy Cattle Breeding

CGIAR: Consultative Group on International Agricultural Research

CONABIA: National Advisory Commission on Agricultural Biotechnology

CPI: Cytoplasmic injection

CRISPR/Cas9: Clustered regularly interspersed short palindromic repeats and associated protein 9

CSN2: Beta-casein

CTNBio: National Technical Biosafety Commission

DAZL: Deleted in AZoospermia Like

DEFRA: Department for Environment, Food and Rural Affairs

DSB: Double stranded breaks

FAO: Food and Agriculture Organization of the United Nations

FDA: Food and Drug Administration

FD&C Act: Food, Drug and Cosmetic Act

EBV: Estimated breeding value

ECNT: Embryonic cell nuclear transfer

EFSA: European Food Safety Authority

EMBRAPA: Brazilian Agriculture and Livestock Research Enterprise

EP: Electroporation

EPSC: Expanded potential stem cells

EPD: Expected progeny difference

ESC: Embryonic stem cells

ET: Embryo transfer

EU: European Union

JIVET: Juvenile *in vitro* ET

GE: Genetically engineered

GEBV: Genomic estimated breeding values

GED: Gene edited descendants

GEF: Gene edited founders

GMO: Genetically Modified Organism

GnEd: Gene editing

GS: Genomic selection

GSE: Genomic screening of embryos

HDR: Homology-directed repair

IARS: Isoleucyl-tRNA synthetase

ICAR: International Committee for Animal Recording

ICBF: Irish Cattle Breeding Federation

ICM: Inner cell mass

ILRI: International Livestock Research Institute

iPSC: Induced pluripotent stem cells

ITGB2: Integrin subunit beta 2

IVB: *In vitro* Breeding

IVP: *In vitro* Embryo production

IVC: *In vitro* Culture

IVF: *In vitro* Fertilization

IVM: *In vitro* Maturation

LacS: Sulfolobus solfataricus beta-glycosidase

LMIC: Low-to-Middle-Income Countries

MLA: Meat and Livestock Australia

MOET: Multiple ovulation embryo transfer

MSTN: Myostatin

NANOS2: Nanos C2HC-Type Zinc Finger 2

NASEM: National Academies of Sciences, Engineering, and Medicine

NEPA: National Environmental Policy Act

NGO: Non-Governmental Organization

NHEJ: Non-homologous end joining

NM$: Lifetime Net Merit selection index

NT: Nuclear transfer

OGTR: Office of the Gene Technology Regulator

OPU: Ovum-pick up

PAGE: Promotion of alleles by gene editing

P_C : POLLED, Celtic allele

PCR: Polymerase chain reaction

PGCLC: Primordial germ cell-like cells

PMEL: Premelanosomal protein gene

PRLR: Prolactin receptor

PRNP: Prion protein

PTA: Predicted transmitting ability

QTN: Quantitative trait nucleotides

RAAA: Red Angus Association of America

rDNA: Recombinant DNA

SNP: Single nucleotide polymorphisms

SCNT: Somatic cell nuclear transfer

SRY: Sex determining region Y protein

SSC: Spermatogonial stem cells

TAI: Timed artificial insemination

TET: Timed embryo transfer

TALEN: Transcription activator-like effector nucleases

UK: United Kingdom

U.S.: United States

USDA: United States Department of Agriculture

USDA-ARS-AGIL: United States Department of Agriculture-Agricultural Research Service-Animal Genomics and Improvement Laboratory

WHO: World Health Organization

ZFN: Zinc finger nucleases

12. ADDITIONAL INFORMATION

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12.4. Author information

MM performed the literature review and wrote the first draft of the manuscript, with input from AVE. Both authors read and approved the final manuscript. MM is an Animal Biology Ph.D. candidate working in the laboratory of AVE. AVE is a Professor of Cooperative Extension in Animal Biotechnology and Genomics in the Department of Animal Science at the University of California, Davis.

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CHAPTER 3

Germline-ablation achieved via CRISPR/Cas9 targeting of *NANOS3* in bovine zygotes

ABSTRACT

NANOS3 is expressed during early development in primordial germ cells (PGCs) and plays a crucial role in safeguarding migrating PGCs from apoptosis. Its expression is vital for germline development in both sexes across various organisms. However, to date live *NANOS3* knockout (KO) cattle have not been reported, and the specific role of *NANOS3* in male cattle, or bulls, remains unexplored. In this study, conditions for the direct cytoplasmic microinjection of the CRISPR/Cas9 gene editing (GnEd) components in *in vitro* produced bovine zygotes to KO *NANOS3* were optimized to achieve high editing efficiency and repeatability. This enabled the production of *NANOS3* KO cattle and subsequent evaluation of the effect of *NANOS3* elimination on bovine germline development, from fetal development through reproductive age. By employing a dual guide RNA (gRNA) approach, a high rate of *NANOS3* KO in developing embryos was accomplished through the co-injection of two selected guide RNA/Cas9 ribonucleoprotein complexes at 6 hours post fertilization. Subsequent embryo transfers into synchronized surrogate recipients realized a 31% (n = 8/26) pregnancy rate. The resulting pregnancies were collected at 4 different timepoints thereby allowing analyses of *NANOS3* KO gonads during fetal development, both during sexual differentiation (41 days of fetal age (d); n = 2 *NANOS3*-presumptively-edited and 2 genetically wildtype (WT) males) and after fetal sexual differentiation (90d; n = 2 *NANOS3*-presumptively-edited and 2 WT males), at the perinatal, or birth, stage (283d; n = 1 *NANOS3*-presumptively-edited and 1 WT males), and post-puberty (15 months of age (mo); n = 3 *NANOS3*-presumptively-edited (2 male and 1 female) and 1 WT male). The CRISPR/Cas9 *NANOS3* edited gonads (fetal and perinatal stages) and live cattle were thoroughly assessed at the DNA, RNA, protein, physiological levels. Based on long-read sequencing analysis of the *NANOS3* target site of these samples, a 75% (n = 6/8) total KO rate was achieved with the dual gRNA editing approach. Although a 71% mosaicism rate (n = 5/7) was observed in the *NANOS3*

edited bovine samples, it is noteworthy that all but one of the 23 edited alleles resulted in a predicted KO. A KO allele was defined as having either a frameshift-inducing indel or a moderate sized indel (> 21 bp) in the NANOS3 protein-coding region that was predicted to generate a complete loss-of-function mutation. Immunofluorescence analysis using known pluripotency and PGC markers indicated that in male *NANOS3* KO bovine gonads, PGCs were eliminated as early as 41d. Immunofluorescence analysis using a known late PGC/germ cell marker and single cell RNA-sequencing (scRNA-Seq) also demonstrated a complete loss of germ cells, but similar testis cord formation and the presence of all key somatic support cell populations in the *NANOS3* KO 90d fetal and perinatal testes compared to age-matched control testes. Ultimately, three live, healthy calves derived from *NANOS3*-presumptively-edited embryos were born without assistance, a heifer calf #854, and two bull calves, #838 and #3964. Heifer #854 and bull #838 were determined to be *NANOS3* mosaic KOs, whereas bull #3964 was found to be edited (i.e., no WT alleles present), but he carried an allele (30% of long-read sequences) with only small, in-frame deletions, and thus was determined to not be a KO allele. All three live *NANOS3* edited animals exhibited normal growth patterns and monthly blood samples were collected from birth to sexual maturity for reproductive hormone analyses. Reproductive exams were performed around 12 months of age (i.e., age of sexual maturity) and the cattle were harvested at 15mo to enable collection of meat samples and reproductive tracts. At sexual maturity, KO bull #838 had normal libido and an anatomically normal reproductive tract, although no spermatozoa was present in his ejaculate. Histological analysis of his testes confirmed the successful ablation of the germline while preserving the integrity of the somatic gonad, including the presence of Sertoli cells lining the seminiferous tubules. In contrast, the sexually mature *NANOS3* edited bull #3964 exhibited fertility as evidenced by the production of a satisfactory ejaculate for his age, and histological

analysis of his testes confirmed the occurrence of spermatogenesis. These findings suggest that bovine *NANOS3* is a haplosufficient gene. Serum hormone analyses indicated that both the *NANOS3* KO and edited bull had endocrinologically functional Leydig cells throughout development. Additionally, prior to puberty, both bulls showed normal function of Sertoli cells. However, in the *NANOS3* KO bull #838, Sertoli cell function appeared to have been impaired after puberty. The *NANOS3* KO heifer #854 was germline ablated as evidenced by the lack of oogenesis observed by histological analysis of her ovarian tissue. However, heifer #854 had an anatomically abnormal reproductive tract and irregular gonad development with a putative primitive streak in place of the right ovary. Moreover, hormone analyses showed no signs of reproductive cycling or functional granulosa cells, which aligns with the complete absence of germ cells and follicles in this animal. Germ cells and supporting somatic cells have a complex relationship in the gonad, where they interact and regulate each other to ensure the successful development and function of gametes. This interplay is essential for proper gametogenesis, so any disruption of germ cells can impair the coordinated processes and functions of both germ and somatic cells. Finally, the composition of meat from these cattle was determined to be within the range of normal variation documented in international meat compositional databases, as expected given the absence of germ cells would not be expected to have an impact of meat composition. Overall, this study demonstrated that the absence of *NANOS3* in cattle leads to the specific deficiency of both male and female germ cells. Importantly, it was evident that despite the absence of germ cells, seminiferous tubule development was not impaired in *NANOS3* KO bovine testes during fetal, perinatal, and adult stages and a live *NANOS3* KO bull exhibited normal somatic gonadal development and structure, and pre-pubertal hormone levels. Additionally, a live, *NANOS3* KO germline-ablated heifer was evaluated, and it was evident that the absence of germ cells in

NANOS3 KO cattle compromised the normalcy of ovarian development to a greater extent than it did testes development. Taken all together, these findings suggest the potential of *NANOS3* KO cattle to act as hosts for donor-derived exogenous germ cell production in both sexes. Germline complementation in cattle could provide an opportunity to expand the availability of gametes from both genetically desirable sires and dams, potentially enabling the efficient generation of absolute transmitters of homozygous GnEd gametes of both sexes. In conclusion, these findings contribute to the understanding of *NANOS3* function in cattle and have exciting implications for the development of valuable novel breeding technologies using germline complementation in *NANOS3* KO germline ablated hosts.

1. INTRODUCTION

The transmission of genetic information across generations relies on the integrity of the germline. During fetal development, primordial germ cells (PGCs) are specified as the embryonic precursors of mature germ cells, which ultimately give rise to spermatozoa or oocytes. These PGCs are initially established in extraembryonic regions and must undergo migration to the developing gonads. Following migration, a period of mitotic proliferation occurs, during which the PGCs undergo meiosis and differentiate into fully mature gametes. These gametes carry the genetic material necessary for the formation of a new individual upon their fusion after mating. New PGCs are produced within the developing embryo, thus perpetuating the germline cycle (Dechiara et al., 2009).

The *NANOS* gene family plays a crucial role in germ cell development across diverse organisms. Initially discovered in Drosophila embryos, the *nanos* gene encodes a protein necessary for the development of both male and female germlines (Wang and Lehmann, 1991). In mammals,

three homologs of *NANOS* genes have been identified, two of which exhibit specific expression in germ cells (Tsuda et al., 2003).

NANOS2 is predominantly expressed in male germ cells and is essential for maintaining the spermatogonial stem cell (SSC) population. However, it is not required for female germline development or fertility (Tsuda et al., 2003). Recently, *NANOS2* knockout (KO) pigs, sheep, and cattle were found to phenocopy *NANOS2* KO mice with male specific germline ablation and normal female germline development (Ciccarelli et al., 2020, Park et al., 2017).

Compared to *NANOS2, NANOS3* exhibits earlier expression in developing embryos, as it is predominantly found in migrating PGCs, where it plays a critical role in protecting them from apoptosis. The loss of *Nanos3* in mice leads to the complete absence of both male and female germ cells (Suzuki et al., 2007, Tsuda et al., 2003). Furthermore, decreased levels of *NANOS3* in human cells have been associated with a reduction in both germ cell numbers and expression of genes involved in germ cell regulation (Julaton and Reijo Pera, 2011). Additionally, studies involving *NANOS3* KO livestock have further demonstrated the conserved role of *NANOS3* in germline development. In pigs, both male and female *NANOS3* KO animals exhibited a complete loss of germ cells, while their gonadal development remained normal (Kogasaka et al., 2022, Park et al., 2023, Wang et al., 2023). Similarly, in cattle, researchers produced a *NANOS3* KO female fetus through somatic cell nuclear transfer (SCNT) cloning, which also showed a complete absence of germ cells but normal gonadal development (Ideta et al., 2016). However, in this study no live cattle were produced, and the impact of *NANOS3* elimination on male bovine germline development was not reported. Based on these findings, it is hypothesized that *NANOS3* KO bulls would also exhibit a complete loss of germ cells while maintaining normal gonadal development,

which would make them suitable candidates for germline complementation studies (Ledesma et al., 2023, Oback and Cossey, 2023).

Germline complementation is the concept of using donor cells from one genetic background to complement or replace the germline of an otherwise sterile host of a different genetic background. By introducing germ cells from high genetic merit donors into the gonads of infertile hosts, it becomes possible to expand the availability of gametes from genetically desirable dams and sires which could be of benefit to livestock breeding programs (Gottardo et al., 2019). This approach, often known as surrogate sires or surrogate hosts, offers several advantages. Firstly, in extensively managed systems such as beef cattle, surrogate sires could enable the transmission of desirable donor genetics through natural mating, facilitating the rapid dissemination of superior genetic traits and potentially decreasing the lag between the genetic merit of the elite seedstock sector and that of the commercial sector. Secondly, donor cells could additionally be gene edited (GnEd) to allow for the targeted introgression of beneficial traits (Bishop and Van Eenennaam, 2020, Gottardo et al., 2019, Ledesma et al., 2023, McLean et al., 2020, Mueller and Van Eenennaam, 2022, Oback and Cossey, 2023).

Successful germline complementation requires two components; donor cells that are capable of becoming gametes, and a germline-ablated host capable of supporting gametogenesis. Efficiently generating germline-ablated hosts that retain reproductive capabilities is a crucial step in enabling successful germline complementation technology in livestock. Traditional physicochemical approaches, such as toxic drug treatment, irradiation, and heat shock, are inefficient and impractical for livestock due to their inability to completely eliminate the endogenous germline or their undesirable side effects on animal health (Giassetti et al., 2019). An alternative approach involves utilizing genetic tools, such as GnEd, to inactivate essential genes

for germline production at the embryo stage, creating a germline developmental niche within the host. This niche provides the donor cells with unfettered access to the germline cell lineage, paving the way for a chimera whose germ cells are exclusively derived from a single donor genotype, sometimes referred to as “absolute transmitters” (Ledesma et al., 2023, Oback and Cossey, 2023). This genetic approach holds promise for improving the efficiency and practicality of germline complementation technology in livestock breeding.

GnEd in zygotes provides an efficient method for targeted gene disruption, avoiding the use of cell lines and SCNT cloning of reconstructed embryos that were historically employed for the targeted disruption of specific genes in livestock species (McFarlane et al., 2019, Mueller and Van Eenennaam, 2022). GnEd refers to the use of site-directed nucleases to precisely introduce double-stranded breaks (DSBs) at predetermined locations in the genome (Gaj et al., 2013). Cells possess repair pathways for these DSBs, such as non-homologous end joining (NHEJ) and homology-directed repair (HDR). The NHEJ pathway often introduces errors, resulting in gene disruption or KO. Currently, the most efficient, versatile, and cost-effective GnEd tool is the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system, which uses a guide RNA (gRNA) to direct the reprogrammed Cas9 nuclease to the desired target site (Ferreira and Choupina, 2022, Sander and Joung, 2014).

The objectives of this study were to optimize a gene KO approach of *NANOS3* using the CRISPR/Cas9 system in bovine zygotes, generate pregnancies using *NANOS3* KO embryos, and evaluate the effect of disrupting *NANOS3* on bovine germline development from fetal development through reproductive age. By investigating the consequences of *NANOS3* disruption, this study aims to advance our understanding of bovine germline development and inform the development of improved livestock breeding strategies.

2. MATERIAL & METHODS

2.1. Animal care

All the experiments carried out with the use of animals were approved and performed in accordance with the University of California (UC), Davis, Institutional Animal Care and Use Committee (IACUC) approved protocol # 21513. Cattle were housed and managed at the UC Davis Beef Barn and Feedlot.

2.2. *NANOS3* gRNA Design

Single gRNAs (sgRNA) targeting bovine *NANOS3* exon 1 were designed using sgRNA Scorer 2.0 (Chari et al., 2017) and Cas-OFFinder (Bae et al., 2014). Based on a systematic analysis of CRISPR/Cas9 mismatch tolerance (Anderson et al., 2015) and testing in bovine zygotes (Hennig et al., 2020), only sgRNAs that met specific mismatch parameters were selected for testing. A mismatch was defined as a discrepancy between a base of the sgRNA and a predicted off-target site. sgRNA selection was undertaken with the requirements of 1) at least 3 total mismatches in the sgRNA sequence and 2) at least 1 mismatch in the seed region (8-11 bp upstream of the PAM site). Selected sgRNAs (Table 3.1) were ordered from Synthego (Menlo Park, CA). *In vitro* cleavage assays were performed to test cleavage efficiency by incubating 80 ng of PCR amplified target sequence, 100 ng of sgRNA, 150 ng of Cas9 protein (PNA Bio, Inc., Newbury Park, CA), in 1× Buffer 3.1 (New England Biolabs, Ipswich, MA) at 37 °C for 1 h. The resulting cleavage products were electrophoresed and imaged using a 2% agarose gel.

Table 3.1. List of guide RNA (sgRNA) sequences tested for targeting bovine *NANOS3*

Guide name	Location (exon 1)	Sequence (20 nt sgRNA + PAM)
1 (sgRNA1)	5'	GTGGACAGACTACTTGGGTTTGG
2 (sgRNA2)	5'	CTACTTGGGTTTGGCACGCCTGG
3 (sgRNA3)	5'	GTTCGGGCACTGCTTCTGGCTGG
4 (sgRNA4)	5'	AGAAGCAGTGCCCGAACCGGGGG
5 (sgRNA5)	Center	CGCTTCATCCTTGAGCACGTGGG
6 (sgRNA6)	3'	TGGTCCGCTCGGACAAGGCGAGG
7 (sgRNA7)	3'	CTCGGACAAGGCGAGGACGCAGG

2.3. Bovine embryo production

Bovine ovaries were obtained from an abattoir and transported to the laboratory in 35–37 °C sterile saline. Collection of cumulus-oocyte complexes (COCs) was performed via aspiration of follicles. Groups of 50 COCs were matured in 4-well dishes containing 500 μL of maturation media (BO-IVM, IVF Bioscience, Falmouth, United Kingdom). COC maturation was performed in a humidified 5% CO2 incubator at 38.5 °C for 18–22 h. Oocytes were fertilized in groups of 25 per drop (60 μL) of SOF-IVF (Bakhtari and Ross, 2014) covered with OVOIL (Vitrolife, Sweden). A concentration of 2 x 10^6 sperm per mL was used for an incubation period of 6 h at 38.5 °C in a humidified 5% CO2 incubator. Six hours post fertilization (hpf), presumptive zygotes were denuded of cumulus cells by light vortexing in SOF-HEPES medium (Bakhtari and Ross, 2014) for 5 min. No more than 100 zygotes per well were incubated in 400 μL of culture media (BO-IVC, IVF Bioscience) covered with 300 μL of OVOIL (Vitrolife) at 38.5 °C in a humidified atmosphere of 5% CO2, 5% O2 and 90% N2 for 7–8 days.

2.4. *NANOS3* gRNA testing - *in vivo*

To determine sgRNA mutation rates, laser-assisted cytoplasmic microinjection (Bogliotti et al., 2016) of presumptive zygotes was performed using 6 pL of a mixture of 67 ng/μL of a sgRNA (Synthego) and 167 ng/μL of Cas9 protein (PNA Bio) incubated at room temperature in Tris-low (0.1mM) ethylenediaminetetraacetic acid (EDTA) buffer for 30 min prior to injection to

form ribonucleoprotein (RNP) complexes. Embryos were incubated for 7–8 days and those that reached the blastocyst stage were individually collected and lysed in 10 µL of Epicenter DNA extraction buffer (Lucigen, Palo Alto, CA) at 65 °C for 6 min and then 98 °C for 2 min.

The target region was amplified by two rounds of nested polymerase chain reaction (PCR) using primers (Eurofins Genomics, Louisville, KY) designed with Primer3 (Untergasser et al., 2012). The first round of nested PCR contained 10 µL GoTaq ® Green Master Mix (Promega, San Luis Obispo, CA), 0.4 µL of each primer at 10 µM (*NANOS3*_F1 and *NANOS3*_R1; Table 3.2), and 9.2 µL of DNA in lysis buffer for 3 min at 95 °C, 35 cycles of 30 sec at 95 °C, 30 sec at 62 °C (annealing), and 1 min 72 °C (extension), followed by 5 min at 72 °C. The second round of nested PCR was run on 1 µL of first round PCR reaction using 10 µL of GoTaq ® Green Master Mix (Promega), 8.2 µL of water, and 0.4 µL of each primer at 10 µM (*NANOS3*_F2 and *NANOS3*_R2; Table 3.2) for 3 min at 95 °C, 35 cycles of 30 sec each at 95 °C, 60 °C (annealing), and 72 °C (extension), followed by 5 min at 72 °C. Products were electrophoresed and visualized on 1% agarose gels, and then excised and purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Purified PCR products were Sanger sequenced (GENEWIZ, San Francisco, CA), and alignments to the target region were visualized with SnapGene (Dotmatics, San Diego, CA) and analyzed using Synthego's Inference of CRISPR Edits (ICE) tool (Conant et al., 2022).

Mutation rates for dual gRNA combinations were determined using the same methods described above using 67 ng/µL of each sgRNA (Synthego) alongside 167 ng/µL of Cas9 protein (PNA Bio). Both sgRNAs (Synthego) were incubated together with Cas9 protein (PNA Bio) at room temperature in Tris-low (0.1mM) EDTA buffer for 30 min prior to microinjection using 6 pL of the RNP mixture.

Table 3.2. List of PCR primer sequences. Forward (fwd); Reverse (rev)

Name	Target	Type	Expected amplicon (bp)	Sequence	Notes
NANOS3_F1	NANOS3, exon 1	Fwd	770	GAACTGACAGCCCAGACTCC	
NANOS3_R1	NANOS3, exon 1	Rev		GCTTACCCACTAGGGCAACA	1st round of nested PCR
NANOS3_F2	NANOS3, exon 1	Fwd	610	GCGTTTCTCCTGTCTTCTGC	2nd round of nested PCR
NANOS3_R2	NANOS3, exon 1	Rev		AACCCTCTGAAGTGGGTCAG	
NANOS3_6kb_2F	NANOS3, long-range	Fwd	6,274	CCTCAACTGACGGGGAAGTC	
NANOS3_6kb_2R	NANOS3, long-range	Rev		TTGTTGTCGGTGGGTTGTGA	
DDX3_F	DDX3	Fwd	X = 184; Y = 208	AGGAAGCCAGGAAAGTAA	
DDX3_R	DDX3	Rev		CATCCACGTTCTAAGTCTC	

2.5. <u>Embryo transfers (ET)</u>

Recipient cattle estrus synchronization began 16 days preceding the ET. On day 0, recipients received an intravaginal progesterone (1.38 g) releasing device (EAZI-BREED™ CIDR® (controlled internal drug release); Zoetis, Parsippany, NJ) and gonadorelin (100 μg; Factrel; Zoetis). On day 7, CIDRs were removed and prostaglandin (25 mg; Lutalyse; Zoetis) was administered. Recipients were monitored for signs of estrus using heat patches and visual observation. A second dose of gonadorelin (100 μg; Factrel; Zoetis) was given on day 9. On day 9 of synchronization, presumptive zygotes were microinjected with dual gRNA_4+7 (sgRNA_4 and sgRNA_7 combined) RNP complexes as described above. Embryos were microinjected in groups of 50–60, and fresh RNP complexes were prepared between each group. Recipient synchronization was confirmed on day 15 via detection of a corpus luteum using a transrectal ultrasound (5.0 MHz linear probe; EVO Ibex, E.I. Medical Imaging, Loveland, CO). ETs were

performed on day 16 of recipient synchronization. A caudal epidural of 100 mg 2% lidocaine (Xylocaine; Fresenius, Germany) was administered to recipients prior to ET. Straws (0.25 cc) were loaded with one to two blastocysts each and transferred into the uterine horn ipsilateral to the corpus luteum using a non-surgical transcervical technique. Any remaining blastocysts that were not transferred were analyzed via PCR and Sanger sequencing as described previously to get an editing profile of embryos produced on the same day as those that were transferred to recipients. On day 28 of embryonic development, blood was drawn from the recipients to diagnose pregnancy via PAG (pregnancy-associated glycoprotein) detection. Transrectal ultrasonography was used to confirm pregnancies on day 35 of gestation, to determine fetal sex between 50-70 days of gestation, and periodically thereafter to monitor pregnancies until delivery.

2.6. Sample collection

Bovine gonadal samples resulting from transferred blastocysts that were presumptively edited were collected at four different stages of development: 41 days of fetal age (41d fetuses), 90 days of fetal age (90d fetuses), 283 days of fetal age (283d perinate or birth), and 15-months-of-age (15mo cattle). To collect 41d and 90d fetuses, recipient cattle were slaughtered via penetrating captive bolt and subsequent exsanguination. The reproductive tracts were collected, and fetuses were recovered from the uterine horns. Fetuses were phenotyped for crown rump length (CRL) and sex, and tail tissue samples were collected for DNA extraction. Fetal gonadal ridges (41d) and gonads (90d) were identified based on their location within the abdominal cavity, anatomy, and relationship with neighboring organs (mesonephros and/or kidneys) and were collected and preserved for analysis (described below). Age-matched control wildtype (WT) samples, produced via artificial insemination, were also collected in the same manner. In this study, WT refers to the genetically WT form of *NANOS3* (i.e., *NANOS3*+/+), which represents the

natural, non-mutated state of the gene. For the 283d perinate sample collection, blood and gonads were collected during the necropsy of a full-term, stillborn calf. Around 15-months of age, live cattle were slaughtered via penetrating captive bolt and meat samples and reproductive tracts were collected. Meat samples were analyzed by proximate analysis and for minerals. Reproductive tracts were analyzed for abnormalities and gonads were isolated and preserved for analysis (described below). Age-matched (283d perinate and 15mo cattle) control WT gonads were collected as part of separate ongoing departmental experiments. DNA extraction was performed using Qiagen's DNeasy Blood and Tissue Kit according to the manufacturer's protocol for tissue (41d and 90d fetuses) and blood (283d perinate and live calves).

2.7. Genotypic analysis of bovine samples

2.7.1. *Fetal sex genotype determination*

Fetuses were sexed by PCR targeting the DEAD box helicase 3 gene (DDX3X/DDX3Y), as described by Gokulakrishnan et al. (2012). PCR amplification was accomplished using 12.5 µL GoTaq® Green Master Mix (Promega), 100 ng of DNA, 9.5 µL of water, 0.5 µL of each primer at 10 µM (DDX3_F and DDDX3_R; Table 3.2) for 3 min at 95 °C, 35 cycles of 30 sec each at 95 °C, 55 °C (annealing), and 72 °C (extension), followed by 5 min at 72 °C. Products were electrophoresed and visualized on 2% agarose gels. Amplicon size allowed discrimination between X and Y chromosomes (184 bp versus 208 bp, respectively). Genomic DNA from adult testes and ovaries were used as controls.

2.7.2. *Short-range PCR of bovine NANOS3*

The *NANOS3* exon 1 target region was amplified by PCR using 12.5 µL GoTaq® Green Master Mix (Promega), 100 ng of DNA, 9.5 µL of water, 0.5 µL of each primer at 10 µM (*NANOS3*_F2 and *NANOS3*_R2; Table 3.2) for 3 min at 95 °C, 35 cycles of 30 sec each at 95 °C,

60 °C (annealing), and 72 °C (extension), followed by 5 min at 72 °C. Products were electrophoresed and visualized on 1% agarose gels, and then excised and purified using the QIAquick Gel Extraction Kit (Qiagen). Purified PCR products were Sanger sequenced (GENEWIZ), and alignments to the target region were visualized with SnapGene (Dotmatics) and analyzed using ICE (Synthego) (Conant et al., 2022).

2.7.3. *Long-range PCR of bovine NANOS3*

A 6,274 bp region centered around the *NANOS3* dual gRNA_4+7 target location was amplified by long-range PCR using primers (Integrated DNA Technologies, Coralville, IA) designed with Primer-BLAST (Ye et al., 2012) and Primer3 (Untergasser et al., 2012). Long-range PCR was performed using 12.5 µL of Phusion® High-Fidelity PCR Master Mix (Thermo Fisher Scientific Inc., Waltham, MA), 50 ng of DNA, 10 µL of water, 0.5 µL of each primer at 10 µM (*NANOS3*_6kb_2F and *NANOS3*_6kb_2R; Table 3.2), and 0.5 µL of dimethyl sulfoxide (DMSO) for 3 min at 98 °C, 35 cycles of 15 sec at 98 °C, 30 sec at 65 °C (annealing), and 6 min 72 °C (extension), followed by 6 min at 72 °C. Long-range PCR products were visualized on a 1% agarose gel.

2.7.4. *NANOS3 long-amplicon library preparation, sequencing, and evaluation*

Long-range PCR products were purified using an AMPure PB Kit (Pacific Biosciences of California, Inc, ("PacBio") Menlo Park, CA) following the manufacturer's protocol. SMRTbell libraries were prepared with PacBio barcoded overhang adapters, which allowed for pooling of the samples (SMRTbell® Express Template Prep Kit 2.0 and Barcoded overhang adapter kit 8A, PacBio). Sequencing was performed on a PacBio Sequel II system by the UC Davis DNA Technologies & Expression Analysis Core. HiFi reads (reads generated with Circular Consensus Sequencing (CCS) analysis whose quality value is equal to or greater than 20) were sorted by

barcode and BAM files were converted to individual FASTQ files for each sample using SMRT Link v11.0.0.146107. HiFi reads were aligned to a reference FASTA file corresponding to the 6,274 bp target region of bovine *NANOS3* (ARS-UCD1.2-Ensembl version 108: Chr7:11,805,072-11,811,345) using the "MEM" algorithm implemented in the BWA MEM2 v2.2.1 software (Vasimuddin et al., 2019). SAM files were converted to BAM files and sorted and indexed using SAMtools v1.15 (Danecek et al., 2021). The resulting BAM files were used as input for the variant determination algorithm (.batch) implemented in AlleleProfileR (Bruyneel et al., 2019) to define and count alleles present in each sample.

2.8. Phenotypic analysis of bovine samples

At 41d (n = 2 *NANOS3*-presumptively-edited and 1 WT control), the whole urogenital ridge was isolated and preserved for histology. Whole fetal testes were isolated from the 90d fetuses (n = 2 *NANOS3*-presumptively-edited and 2 WT control). One testis from each fetus was preserved for histology while the other testis was preserved for single-cell RNA-sequencing (scRNA-Seq) analysis. Testicular cross-sections from the 283d perinates (n = 1 *NANOS3*-presumptively-edited and 1 WT control) were collected and preserved for both histology and scRNA-Seq analysis. Testicular cross-sections from the 15mo bulls (n = 2 *NANOS3*-presumptively-edited and 1 WT control) and heifer (n = 1 *NANOS3*-presumptively-edited) were also collected and preserved for histology as described below.

2.8.1. *scRNA-Seq*

2.8.1.1. Gonad preservation & dissociation for scRNA-Seq

Single cells were isolated from whole 90d fetal testes and cross-sections of equal weight from 283d perinatal testes. Gonadal samples collected for scRNA-Seq were washed in ice-cold phosphate-buffered saline (PBS) and slow frozen in DMEM containing 20% fetal bovine serum

(FBS) and 10% DMSO using a freezing device (Mr. Frosty™, Thermo Fisher Scientific) (Soto and Ross, 2021). Cryovials of slow-frozen gonadal samples were removed from liquid nitrogen storage and thawed at 37 °C in a water bath until the tissue could be removed and for no longer than 3 min. Gonads were then rinsed in room temperature Hank's Balanced Salt Solution (HBSS) with calcium and magnesium (+/+)(and minced into ~0.1 mm pieces.

Perinatal samples were subject to a two-step digestion procedure, as described by Guo et al. (2017) and Guo et al. (2018), with modifications optimized for bovine samples. Gonads were first digested in a pre-warmed mixture of HBSS+/+ containing collagenase type IV (1 mg/mL) (#11088858001, Sigma-Aldrich Roche®, Burlington, MA) and DNase I (1 kU/mL) (#10104159001, Sigma-Aldrich Roche®) for 5 min at 37 °C with gentle agitation (250 rpm), then shaken vigorously for 1 min and incubated for another 3-5 min with gentle agitation. The dissociated tubules were sedimented by centrifugation at 600× g for 5 min at 4 °C and washed with HBSS without calcium or magnesium (-/-). The pellet was resuspended in a second pre-warmed digestion media of 5 mL of 0.25% trypsin/ EDTA supplemented with DNase I (1 kU/mL). The suspension was pipetted vigorously three to five times with a wide-bore pipette and incubated at 37 °C for 5 min. The process was repeated in 5 min increments for up to 15 min total. The digestion was stopped by adding 10% FBS. Single testicular cells were obtained by filtering through strainers with mesh size 100 μm and 30 μm. The cells were pelleted by centrifugation at 600× g for 15 min at 4 °C and washed with ice-cold HBSS-/-. Cells were then re-suspended in ice-cold HBSS-/- supplemented with 0.4% BSA.

Fetal samples were digested in a pre-warmed mixture of HBSS+/+ containing collagenase type IV (1 mg/mL) and DNase I (5 kU/mL). The suspension was triturated vigorously three to five times with a wide-bore pipette and incubated at 37 °C for 5 min with gentle agitation (150 rpm).

The process was repeated 3-5 times in 5 min increments for up to 25 min total. The digestion was stopped by adding 10% FBS. Single cells were obtained by filtering through strainers with mesh size 100 μm and 30 μm. The cells were pelleted by centrifugation at 600× g for 15 min at 4 °C. Cells were then re-suspended in ice-cold HBSS-/- supplemented with 0.4% BSA.

2.8.1.2. scRNA-Seq library preparation, sequencing, and analysis

Single cell samples were processed using the cell fixation (SB1001) and single-cell whole-transcriptome (SB2001) kits from Parse Biosciences (Seattle, WA), according to the manufacturer's instructions. This scRNA-Seq approach is based on combinatorial barcoding, which enables multiplexing of samples. The resulting sub-libraries (n = 8) were sequenced on an Illumina NovaSeq 6000 instrument (150 base paired-end). For data processing, the Parse Bioscience's processing pipeline (v0.9.6p) was used with default settings to demultiplex samples and align reads to the bovine reference genome (ARS-UCD1.2-Ensembl version 105). Downstream analysis was performed using the R package Seurat (v4.1.0) at default settings unless otherwise noted (Stuart et al., 2019).

Individual analyses were performed for each sample timepoint. For the 90d fetal testes (n = 4), to be included in the analysis cells had to have between 1,000 to 100,000 reads from at least 700 genes, and the genes had to be expressed in more than 10 cells. To be included in the analysis for the 283d perinatal testes (n = 2), cells had to have between 500 to 50,000 reads in at least 200 genes and, and similarly the genes had to be expressed in more than 10 cells. For both timepoints, the resulting gene–cell matrices were normalized and scaled using Seurat's NormalizeData and ScaleData functions, and principal component analysis was performed with Seurat's RunPCA function. Cells were clustered using the FindNeighbors and FindClusters functions. For visualizing clusters, dimensionality reduction was performed by uniform manifold approximation and

projection (UMAP). The identities of cell clusters were determined by plotting (VlnPlot & FeaturePlot functions) well-known mammalian germ cell and testicular somatic support cell markers. Once clusters were identified, the FindConservedMarkers and FindMarkers functions (Wilcoxon rank-sum test, minimal fraction of 10%, and log-transformed fold-change threshold of 0.25) were both run on each cluster subset to find genes that were conserved and/or differentially expressed, respectively, between treatments (i.e., KO versus control) for each cell type.

2.8.2. *Histology*

Gonadal samples collected for hematoxylin and eosin (H&E) staining were fixed in 4% paraformaldehyde for 24 hours at 4 °C. Fixed tissues were rinsed in PBS and then dehydrated through a stepwise ethanol gradient of PBS, 30% ethanol, 50% ethanol, and 70% ethanol. Each step was for 24 hours at 4 °C. Tissues were stored in 70% ethanol at 4 °C until being processed in a Tissue-Tek VIP® processor (Sakura Finetek USA, Inc., Torrance, CA). Tissue was then embedded in paraffin, sectioned at 5 μm thickness and stained with H&E.

Gonadal samples collected for immunostaining analyses were fixed in 4% paraformaldehyde for 6 hours at 4 °C. Fixed tissues were rinsed in PBS and then washed through a stepwise sucrose gradient of 15% sucrose for 24 hours and then stored in 30% sucrose, all at 4 °C, until being embedded in Tissue-Tek optimal cutting temperature (OCT) compound (Sakura Finetek). Cryoblocks were stored at -80 °C prior to sectioning. Cryoblocks were sectioned at 10 μm thickness and tissue sections were stored at -20 °C until staining. For immunostaining, cryosections were washed to remove the OCT compound prior to antigen retrieval. Antigen retrieval was performed in a steamer for 5 min in 10 mM citrate-based antigen unmasking solution (pH 6.0, H-3300, Vector Laboratories, Newark, CA) for cytoplasmic targets or 20 min in 10 mM tris-based antigen unmasking solution (pH 9.0, H-3301, Vector Laboratories) for nuclear targets.

Additionally, for nuclear targets, antibody permeability was increased by incubation with 0.1% Triton X-100 in tris-buffered saline (TBS) for 10 min. Non-specific binding of immunoglobulins was blocked by incubation with 0.3M Glycine and 10% normal donkey serum for 1 h, at room temperature. Tissue sections were then incubated overnight at 4 °C with the following primary antibodies: anti-PRDM1 (1:50;14-5963-80, Invitrogen, Waltham, MA), anti-OCT4 (1:100; AF1759, Novus Biologicals, Centennial, CO), and anti-DDX4 (1:500; ab13840, Abcam, Fremont, CA). Also, sections of tissues were incubated with a rabbit isotype control antibody (02-6102, Invitrogen) as the primary antibody to serve as negative control sections. All sections were incubated with appropriate secondary antibodies, anti-rabbit IgG - Alexa Fluor™ 488 (1:500; A21206, Invitrogen) or anti-goat IgG - Alexa Fluor™ 568 (1:500, Invitrogen), for 1 h at room temperature. Hoechst 33342 was used for counterstaining to detect nuclei. Slides were mounted using ProLong Gold Antifade (Invitrogen) and imaged using an Echo Revolve microscope (Discover Echo Inc., San Diego, CA). Images were processed using ImageJ (v2.3.0/1.53t).

2.8.3. *Live animal evaluation and reproductive examinations*

Monthly weights were recorded for the live *NANOS3* edited animals (n = 3) and starting at 5-months-old scrotal circumference was measured monthly for males only (n = 2). Starting around 12-months of age, at which time bulls typically reach reproductive maturity, breeding soundness exams (BSE) were conducted by UC Davis veterinarians on the live bulls. If a bull failed the first BSE, then two more BSEs were performed at least one month apart. BSEs followed the standards set forth by the Society for Theriogenology and included a general physical examination, inspection of reproductive organs, and semen collection via electroejaculation (Chenoweth, 2015,

Chenoweth et al., 1993). Around 14-months of age, UC Davis veterinarians performed a reproductive exam, including rectal palpation and transrectal ultrasound, on the live heifer.

2.8.4. *Hormone analysis*

For hormone analyses, blood samples were collected monthly on the live *NANOS3* edited animals (n = 3) and a WT bull (n = 1). Samples were centrifuged to separate serum and plasma and the serum was stored at −80 °C before processing by the Clinical Endocrinology Laboratory at the UC Davis, School of Veterinary Medicine in the Department of Population Health and Reproduction. Radioimmunoassays (RIA) were used to measure serum testosterone (Anti-testosterone, C. Munro, UC Davis) and estradiol (Ultra-Sensitive Estradiol RIA kit #DSL-4800, Beckman Coulter, Brea, CA) levels. Enzyme immunoassays (EIA) were used to measure Anti-Müllerian Hormone (AMH) (Bovine AMH assay #AL-114, Ansh Labs, Webster, TX), Inhibin B (Equine/Canine/Rodent Inhbin B assay #AL-163, Ansh Labs), and progesterone (Anti-Progesterone-R4859, C.Munro, UC Davis) levels.

2.8.5. *Meat analysis*

A sample of both sirloin cap and chuck arm was dissected from the carcasses of the *NANOS3* KO 15-month-old heifer #854 and bull #838, trimmed of excess fat, and frozen at -80^{o}C. The muscle was analyzed by Midwest Laboratories (Omaha, NE) by proximate analysis and for minerals (Fe, Zn, and P), using AOAC International methods (Rockville, MD) and internally established protocols (Midwest Laboratories; MWL FO 014 & MWL FO 022). Reference nutrient data for beef was taken from Trott et al. (2022).

3. RESULTS

3.1. CRISPR/Cas9 mediated KO of *NANOS3* in bovine embryos

Bovine *NANOS3* is a 2,633 bp gene with two exons (Figure 3.1). Guides were designed targeting exon 1, as this exon contains the highly conserved coding regions for the N-terminal and zinc finger binding domains (Beer and Draper, 2013, Suzuki et al., 2014). Only sgRNAs that met specific mismatch (i.e., discrepancy between a base of the sgRNA and a predicted off-target site) criteria were selected for testing. sgRNA selection criteria were 1) at least 3 total mismatches in the sgRNA sequence, and 2) at least 1 mismatch in the seed region (8-11 bp upstream of the PAM site) (Anderson et al., 2015, Hennig et al., 2020). Seven sgRNAs were selected for testing based on these criteria (Table 3.1).

sgRNA cutting efficiency was first tested by *in vitro* cleavage assays. Guides that successfully cut the target region *in vitro* (sgRNA #1, #4, #5, #7), were then tested *in vivo* via embryo microinjection. Each sgRNA was incubated with Cas9 protein to form a RNP complex and independently microinjected into zygotes (n = 30 embryos/sgRNA) 6 hpf, following a previously established protocol (Hennig et al., 2020). Uninjected embryos were cultured to the blastocyst stage as a within experiment, contemporary developmental control. All microinjected groups had acceptable blastocyst development rates (≥ 20%), and three sgRNAs (#4, #5 and #7) had an over 60% mutation rate, defined as the end product being different than the starting, wildtype (WT) genome (Table 3.3). However, none of the sgRNAs achieved over a 75% KO rate, with KO being defined as no WT alleles remaining in the blastocyst sample. Therefore, we next tested a dual gRNA system (i.e., co-injection of two sgRNAs simultaneously), which has been shown to be an efficient method for complete gene disruption, or KO, in livestock species (Vilarino et al., 2017, Wu et al., 2017). Guides #4 and #7 (dual gRNA_4+7) were selected based

on their mutation efficiencies and genomic locations to target both the 5' and 3' regions of exon 1 (297 bp between sgRNA cut sites; Figure 3.1). Using dual gRNA_4+7 we achieved an over >75% KO rate and an acceptable blastocyst development rate (19%) (n = 22/28, 4 replicates; Table 3.4).

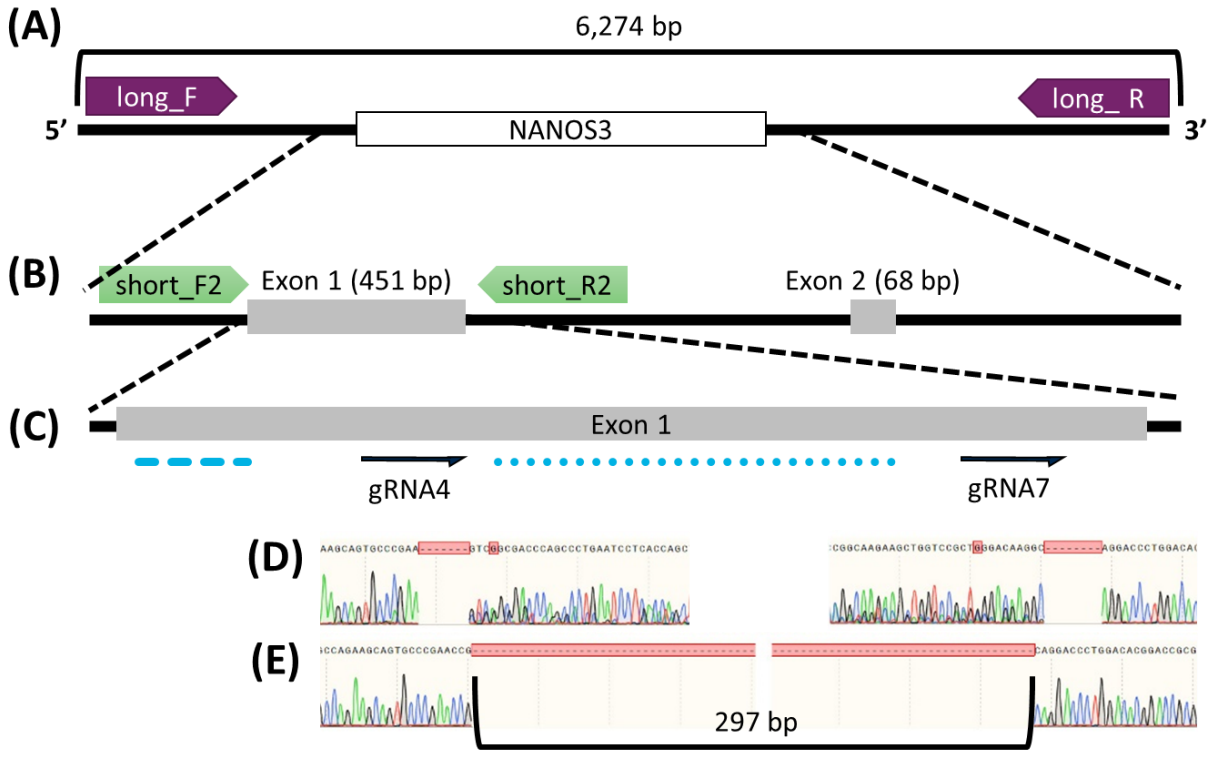


Figure 3.1. Bovine *NANOS3* targeting and PCR analysis strategy. Diagram of bovine *NANOS3* showing the genomic locations of A) long-range PCR primers (NANOS3_6kb_2F, NANOS3_6kb_2R), B) short-range PCR primers (NANOS3_F2, NANOS3_R2), and C) selected dual guides, sgRNA4 and sgRNA7 (dual gRNA_4+7) in relation to the highly-conserved N-terminal (blue dashed line) and zinc finger (blue dotted line) domains. D) Sanger sequencing results showing representative frameshift mutations from sgRNA4 (left) and sgRNA7 (right), and E) Sanger sequencing results showing a targeted dual gRNA_4+7 297 bp deletion.

Table 3.3. Comparison of blastocyst development rates and mutation efficiencies of *NANOS3* sgRNAs.

Guide	Location (Exon 1)	Blastocyst Rate	Mutation rate[1]	KO rate[2]
Control	-	42/120 (35%)	0/10 (0%)	-
sgRNA1	5'	7/30 (23%)	1/7 (14%)	0/7 (0%)
sgRNA4	5'	7/30 (23%)	5/6 (83%)	4/6 (67%)
sgRNA5	Center	6/30 (20%)	5/6 (83%)	2/6 (33%)
sgRNA7	3'	8/30 (27%)	5/8 (63%)	2/8 (25%)

[1] Mutation: End product being different than the starting, wildtype (WT) genomic sequence

[2] Knockout (KO): No WT alleles were present in the sample

Table 3.4. Blastocyst development rates and mutation efficiencies of *NANOS3* dual gRNA_4+7

Replicate	Blastocyst Rate	Mutation rate[1]	KO rate[2]
1	6/30 (20%)	6/6 (100%)	4/6 (67%)
2	6/30 (20%)	4/6 (67%)	3/6 (50%)
3	5/40 (13%)	5/5 (100%)	5/5 (100%)
4	11/50 (22%)	11/11 (100%)	10/11 (91%)
	28/150 (19%)	**26/28 (93%)**	**22/28 (79%)**

[1] Mutation: End product being different than the starting, wildtype (WT) genomic sequence

[2] Knockout (KO): No WT alleles were present in the sample

3.2. Generation of *NANOS3* KO cattle

Dual gRNA_4+7 *NANOS3* targeted bovine embryos were produced, as described previously, and 26 resulting embryos were transferred by collaborating veterinarians into 26

synchronized recipients (Table 3.5). We achieved a 31% pregnancy rate (n = 8/26) as confirmed by transrectal ultrasound on day 35 of embryonic development.

Table 3.5. Pregnancy results from microinjected bovine ETs (ET)

Rep	ET Date	KO rate[1]	Pregnancies				
			28-day	35-day	70-day	Male (#)	Female (#)
1	2/27/20	26/26 (100%)	3/8 (38%)	2/8 (25%)	2/8 (25%)	1	1
2	5/27/20	n/a[2]	0/6 (0%)	0/6 (0%)	0/6 (0%)	0	0
3	6/17/20	n/a[2]	2/5 (40%)	1/5 (20%)	1/5 (20%)	1	0
4C[3]	12/16/20	10/12 (83%)	3/4 (75%)	3/4 (75%)	3/4 (75%)	3	0
4Y[4]	12/16/20	2/4 (50%)	2/3 (67%)	2/3 (67%)	n/a	2	0
		38/42 (90%)	**10/26 (38%)**	**8/26 (31%)**	**6/24 (25%)**	**7**	**1**

[1]Knockout (KO) was defined as no wildtype (WT) alleles being present in the sample. KO rate was determined by analyzing the remaining blastocysts that were not used for ET.

[2]Due to low development, all blastocysts from these replicates were used for ET.

[3]4C: Embryos in replicate 4C were *in vitro* fertilized using conventional semen but all resulting pregnancies were male.

[4]4Y: Embryos in replicate 4Y were *in vitro* fertilized using male-sex-sorted semen and the resulting fetuses were collected on day 42 of gestation (41d).

A total of 8 pregnancies with *NANOS3* targeted embryos were established. To evaluate *NANOS3* KO bovine fetal gonad development, fetuses were collected at different developmental stages, including during sexual differentiation (41d; n = 2 *NANOS3*-presumptively-edited and 2 WT) and post sexual differentiation (90d; n = 2 *NANOS3*-presumptively-edited and 2 WT). Additionally, one full-term male pregnancy was stillborn, so gonadal samples from the perinatal stage (i.e., during and immediately after birth) were evaluated (283d; n = 1 *NANOS3*-presumptively-edited and 1 WT). Ultimately, three live, healthy calves derived from *NANOS3*-

presumptively-edited embryos were born without assistance at the UC Davis Beef Barn, a heifer calf, #854 (“FunBun”) and two bull calves, #838 (“Fauci”) and #3964 (“Frodo”). These calves were grown and developed for analysis at reproductive age (~12 mo) and finally were harvested at 15mo to examine their reproductive tracts (Figure 3.2 and 9). Additionally, meat samples were collected from the NANOS3 KO cattle, heifer #854 and bull #838, at harvest for compositional analysis.

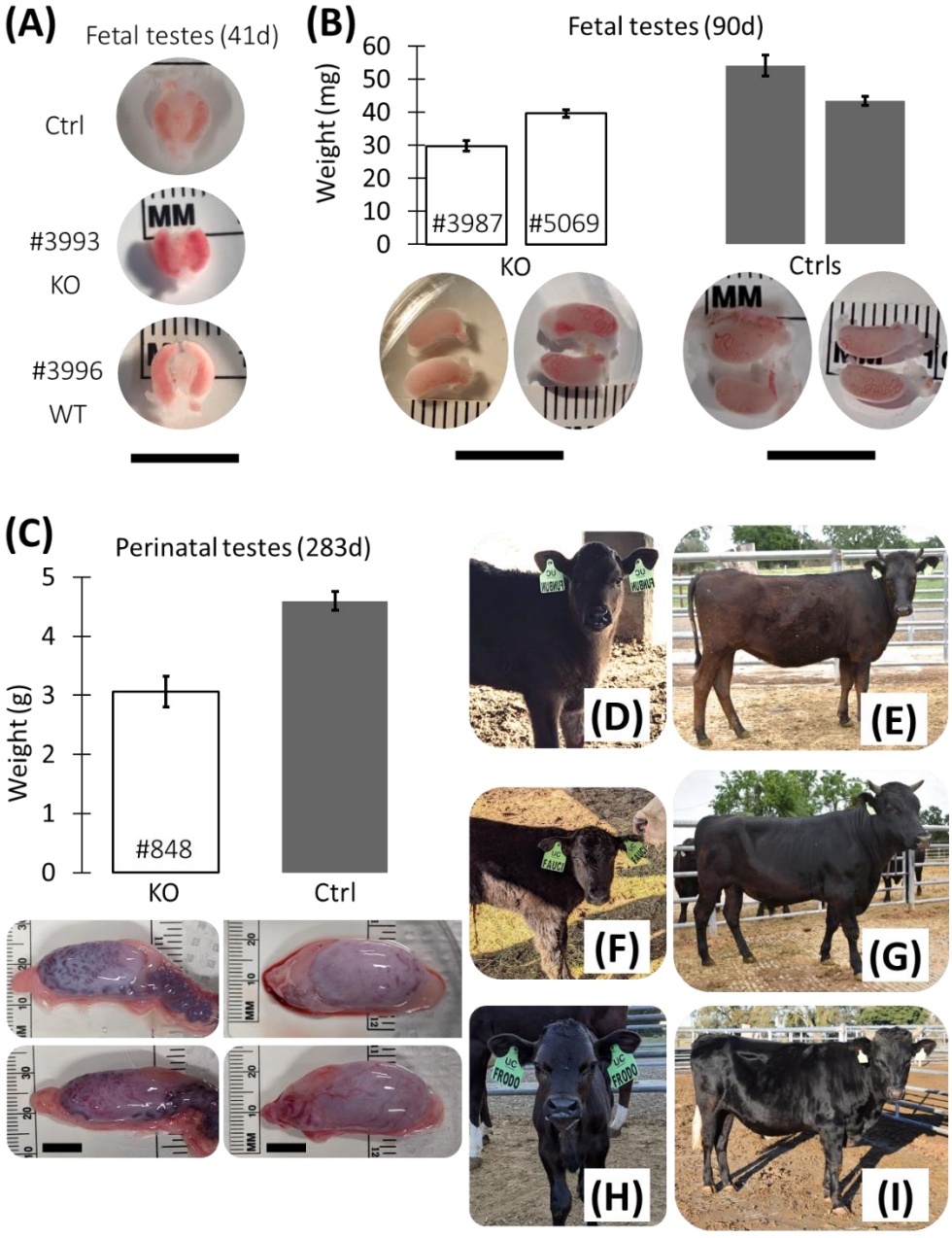


Figure 3.2. Collection of *NANOS3* targeted bovine samples. A) Images of 41d fetal urogenital ridges. B-C) Comparison of size and average weight of *NANOS3* KO versus control testis pairs at 90d (B) and 283d (C) of fetal age. D-I) Images of live *NANOS3* edited cattle at 1-week-old (D, F, H) and 15-months-old (E, G, I). The top panel is heifer #854, middle row is bull #838, and bottom row is bull #3964. Scale bars are 1 cm and error bars are SEM.

3.3. Genotypic analysis of CRISPR/Cas9 *NANOS3* targeted bovine samples

DNA was extracted from tail tissue (41d and 90d fetuses) or blood (283d perinate and live calves). All fetal samples were determined to be male by PCR of DDX3X/DDX3Y. Initial *NANOS3* genotypes were determined by short-range PCR amplification (Figure 3.3A) and Sanger sequencing of the *NANOS3* exon 1 target region. This analysis showed that seven of the eight *NANOS3* targeted bovine samples (87.5%) were successfully edited (0% WT alleles remained). One *NANOS3* targeted bovine sample, 41d_3996, was not edited (100% WT). Out of the seven edited *NANOS3* targeted bovine samples, four (57%) appeared to be mosaic (carried > 2 alleles). However, all of the alleles present in the four mosaic samples were predicted to be KO alleles. A KO allele was defined as having either a frameshift-inducing indel (i.e., small indels that are not multiples of three) or a moderate sized indel (> 21 bp) in a protein-coding region that are predicted to generate a complete loss-of-function mutation. Two samples, 283d_848 and 15mo_854, appeared to be bi-allelic KO (i.e., ≤ 2 KO alleles and 0% WT alleles) with a homozygous targeted dual gRNA_4+7 deletion KO allele, or two KO alleles each with targeted dual gRNA_4+7 indels (i.e., compound heterozygote), respectively. Finally, one *NANOS3* edited bovine sample, 15mo_3964, appeared to carry only one allele and that allele had only small, in-frame deletions, and thus was determined to not be a KO allele (Figure 3.3C). This allele resulted in one amino acid substitution and a deletion of three total amino acids at the target sites (Figure 3.3D). These

mutations were all outside of the highly conserved coding regions for the N-terminal and zinc finger binding domains and it was unknown whether the deleted amino acids were necessary for *NANOS3* protein function. The exact amino acid sequence that was predicted to result from the mutated allele was not found in any other species when a protein BLAST (Basic Local Alignment Search Tool) analysis was conducted. Overall, six of the seven *NANOS3* edited bovine samples (85.7%) were observed to be successful *NANOS3* KOs, and four (67%) had at least one allele with a targeted dual gRNA_4+7 indel.

In order to identify and measure the proportion of alleles present in the mosaic samples and confirm other genotypes, we completed further genotype analysis using long-range PCR amplification and next generation sequencing on all eight *NANOS3* targeted bovine samples. The long-range PCR was designed to amplify a 6,274 bp region centered around the *NANOS3* dgRNA_4+7 target location, and it enabled us to detect large (> 500 bp) indels. Three of the samples, 90d_5069, 15mo_838, and 15mo_3964, were observed to carry potentially large (> 500 bp) deletions, as indicated by the presence of bands smaller than the WT control sample (6,274 bp; Figure 3.3B).

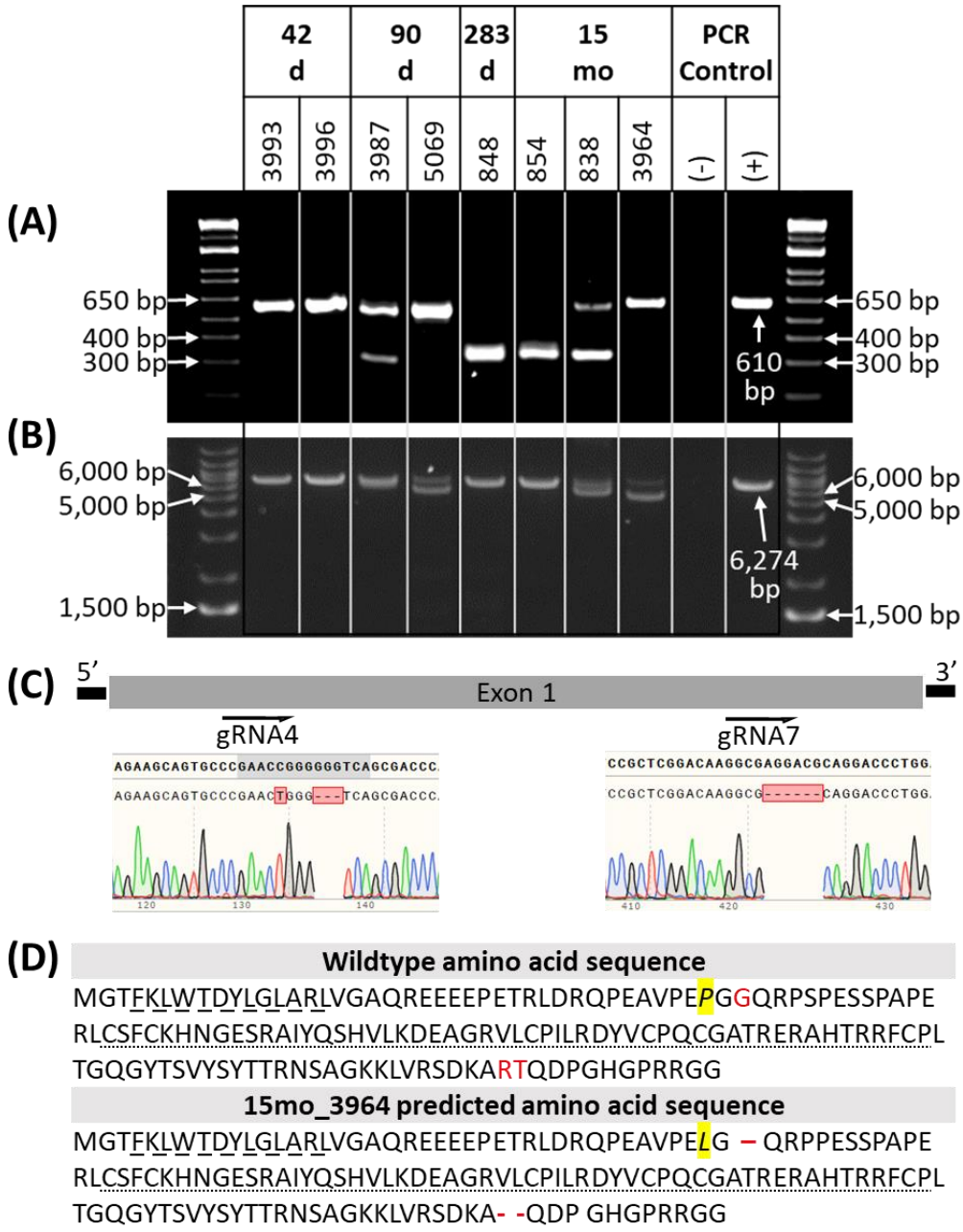


Figure 3.3. Genotypic analysis of CRISPR/Cas9 *NANOS3*-presumptively-edited bovine samples (n = 8). A) *NANOS3* PCR results using short-range primers (NANOS3_2F, NANOS3_2R). B) *NANOS3* PCR results using long-range primers (NANOS3_6kb_2F, NANOS3_6kb_2R). Genetic wildtype (WT; +) band sizes are 610 bp (A) and 6,274 bp (B). C**)** Diagram showing the Sanger sequencing results of small, in-frame mutations present in one of the edited *NANOS3* alleles carried by 15mo_3964. There was a single bp substitution (C to T) and a 3 bp deletion near the sgRNA4

cut site and a 6 bp deletion near the sgRNA7 cut site. D) Comparison of the WT bovine NANOS3 exon 1 amino acid sequence to 15mo_3964's predicted amino acid sequence. The amino acid substitution is highlighted in yellow and italicized (P to L). The three deleted amino acids are represented by red font (WT) and dashes (15mo_3964). The highly conserved N-terminal (dashed underline) and zinc finger binding (dotted underline) domains are underlined.

The long-range PCR products were submitted for PacBio long-read sequencing, and this data revealed a variety of alleles present in the *NANOS3* targeted bovine samples, with indels ranging from 1 bp up to 1.5 kb (Table 3.6). We confirmed that seven of the eight *NANOS3* targeted bovine samples (87.5%) were successfully edited (i.e., 0% WT alleles remained), and that one *NANOS3* targeted bovine sample, 41d_3996, remained unedited (100% WT). Additionally, we confirmed that six of the seven *NANOS3* targeted bovine samples (85.7%) carried only KO allele(s). Bovine sample 41d_3993 was found to be a bi-allelic, homozygous KO, carrying only 1 KO allele with small indels at both sgRNA4 and sgRNA7 cut sites. Bovine sample 283d_848 was confirmed to be a bi-allelic KO. However, he was found to be carrying two KO alleles, making him a compound heterozygote. One of his KO alleles had the targeted dual gRNA_4+7 deletion (36% of reads) and the other had an intermediate-sized deletion (-273 bp) near the sgRNA4 and a small deletion (-8 bp) near the sgRNA 7 cut sites.

In contrast, heifer, 15mo_854, which originally appeared to be a bi-allelic, compound heterozygote KO, was actually a mosaic KO and was found to carry five alleles each with a targeted dual gRNA_4+7 indels, but varying by the number of bp insertions (+ 0 to 6 bp). Bull, 15mo_838, was confirmed to be a mosaic KO with 5 mutated alleles and no WT alleles. The majority (59%) of his alleles had a large deletion (-979 bp) at the sgRNA4 cut site and a intermediate-sized deletion (-32 bp) at the sgRNA7 cut site. Two of the alleles (total of 23%) had

the targeted dual gRNA_4+7 deletion and the other 2 alleles (18%) had a combination of small, intermediate, and large indels at both sgRNA cut sites. In total, five of the seven (71%) *NANOS3* edited bovine samples were mosaic. However, all but one of the alleles present in the mosaic samples were predicted to be KO alleles.

Moreover, the sequencing data confirmed the results that were observed when visualizing the long-range PCR productions, with three of the *NANOS3* edited bovine samples (43%), 90d_5069, 15mo_838, and 15mo_3964, carrying large (> 500 bp) deletions. Interestingly, the *NANOS3* edited bull, 15mo_3964 that originally appeared to carry only one allele with small, in-frame deletions was found to additionally carry 2 large deletion alleles (total of 70% of the reads). Bovine sample 15mo_838 was confirmed to be a mosaic KO. However, we found that the majority of his alleles (59%) had a previously undetected large indel (-979 bp) at the sgRNA4 and an intermediate-sized deletion (-32 bp) at the sgRNA7 cut sites. Additionally, two of his alleles (total of 23%) had the targeted dual gRNA_4+7 deletion and his other 2 alleles (18%) had a combination of small, intermediate, and large-sized indels at both sgRNA cut sites.

Overall, the *NANOS3* long-read sequencing data confirmed many of the initial genotyping results, but importantly it also enabled identification and measurement of the proportion of alleles present in the mosaic samples and revealed large deletion alleles (> 500 bp) that ablated the short-range PCR primer sites. Ultimately, this analysis showed that a 75% (n = 6/8) total KO rate was achieved with our dgRNA_4+7 editing approach, and 50% (n = 4/8) of the samples had at least one allele with the targeted dual gRNA_4+7 indel (Table 3.6).

Table 3.6. Proportion and types of alleles present in the CRISPR/Cas9 *NANOS3*-presumptively-edited bovine samples. Alleles are ordered by the proportion of reads (largest to smallest).

			sgRNA4[1]		sgRNA7[2]		Dual (d) gRNA_4+7		
Sample	**Allele #**	**Allele description (indel size category)[3]**	**Indel start[4]**	**Indel size (bp)**	**Indel start[4]**	**Indel size (bp)**	**Indel start[4]**	**Indel size (bp)**	**Proportion of reads**
41d_ 3993	1	sgRNA 4 & 7: small	118	-1	409	-8			100%
41d_ 3996	1	wildtype							100%
90d_ 3987	1	sgRNA4: intermediate & sgRNA7: small	83	-51	409	-8			37%
	2	sgRNA 4 & 7: intermediate	115	-27	410	-32			36%
	3	dgRNA_4+7: targeted					112	-298	28%
90d_ 5069	1	dgRNA_4+7: large					-331	-960	36%
	2	dgRNA_4+7: large					-662	-1122	32%
	3	sgRNA4: small & sgRNA7: intermediate	107	-20	393	-27			26%
	4	sgRNA 4 & 7: intermediate	115	-27	367	-55			5%
280d_ 848	1	sgRNA4: intermediate & sgRNA7: small	116	-273	409	-8			64%
	2	dgRNA_4+7: targeted					117	-297 (-298, +1)	36%
15mo_ 854	1	dgRNA_4+7: targeted					119	-297	61%
	2	dgRNA_4+7: targeted					119	-291 (-297, +6)	26%
	3	dgRNA_4+7: targeted					117	-298	9%
	4	dgRNA_4+7: targeted					117	-293 (-298, +5)	2%
	5	dgRNA_4+7: targeted					119	-294 (-297, +3)	2%

			sgRNA4[1]		sgRNA7[2]		Dual (d) gRNA_4+7		
Sample	**Allele #**	**Allele description (indel size category)[3]**	**Indel start[4]**	**Indel size (bp)**	**Indel start[4]**	**Indel size (bp)**	**Indel start[4]**	**Indel size (bp)**	**Proportion of reads**
15mo_838	1	sgRNA4: large & sgRNA7: intermediate	-714	-979 (-1000, +21)	410	-32			59%
	2	dgRNA_4+7: targeted					117	-298	15%
	3	sgRNA4: large & sgRNA7: intermediate	-715	-1000	410	-32			10%
	4	sgRNA4: moderate & sgRNA7: small	97	-30	412	-7			8%
	5	dgRNA_4+7: targeted					114	-298 (-301, +3)	8%
15mo_3964	1	dgRNA_4+7: large					107	-1326	64%
	2	sgRNA 4 & 7: small[5]	119	-3	410	-6			30%
	3	dgRNA_4+7: large					59	-1502	6%

[1] The sgRNA4 cut site is position 118, relative to the start of exon 1.

[2] The sgRNA7 cut site is position 415, relative to the start of exon 1.

[3] Description of the indel(s) present in the allele based on indel size and start location. All indels are predicted to KO bovine *NANOS3* (i.e., either a frameshift-inducing indel or an intermediate-sized indel in a protein-coding region that are predicted to generate a complete loss-of-function mutation), unless otherwise noted. Size categories: small < 21 bp, intermediate = 21-500 bp, targeted = 297 ± 5 bp, large > 500 bp.

[4] Starting position of the indel, relative to the start of exon 1.

[5] Allele #2 present in 15mo_3964 has only in-frame deletions (i.e., small deletions that are multiples of 3), which results in an amino acid substitution and a deletion of 3 amino acids total.

3.4. Phenotypic analysis of CRISPR/Cas9 *NANOS3* KO bovine fetal & perinatal samples

Two fetuses each were collected during the stage of sexual differentiation (41d) and post sexual differentiation (90d). Additionally, gonadal samples were collected from a full-term (283d), stillborn male calf. Age-matched, male, WT gonadal samples were also collected for comparisons. On average, the *NANOS3* KO fetal and perinatal testis pairs weighed less than age-matched control testis pairs, although this difference did not reach statistical significance (Figure 3.2B and C).

3.4.1. *Immunofluorescence shows germline-ablation as early as 41d in CRISPR/Cas9 NANOS3 KO bovine fetal gonads*

The 41d genital ridges were co-stained for pluripotency and early PGC markers, OCT4 (also known as POU5F1) and PRDM1 (also known as Blimp1), respectively (Planells et al., 2019, Soto and Ross, 2021, Stukenborg et al., 2014). The 90d and 283d testes samples were stained for a known germ cell marker, DDX4 (also known as "Vasa"), which is expressed in differentiated germ cells from spermatogonia to round spermatids (Bartholomew and Parks, 2007, Caires et al., 2009, Pennetier et al., 2004, Planells et al., 2019, Raz, 2000, Stukenborg et al., 2014).

In this study, *NANOS3* targeted, and control fetuses were first collected at 41 days of fetal age, corresponding to the stage of sexual differentiation, which is approximately two weeks after PGCs would first be expected to reach the genital ridge and two-three days after peak *SRY* expression. Genital ridges from both the 41d control samples and the unedited (100% WT) 41d_3996 sample, stained positive for pluripotency and early PGC markers, OCT4 and PRDM1, respectively (Figure 3.4A) (Planells et al., 2019, Soto and Ross, 2021, Stukenborg et al., 2014). OCT4 and PRDM1 detection was confined to the nuclei of PGCs, which agrees with their roles as transcriptional regulators. In contrast, the genital ridge of sample 42d_3993, which was determined

to be a *NANOS3* KO, stained negatively for both OCT4 and PRDM1, showing germline-ablation at this stage (Figure 3.4A).

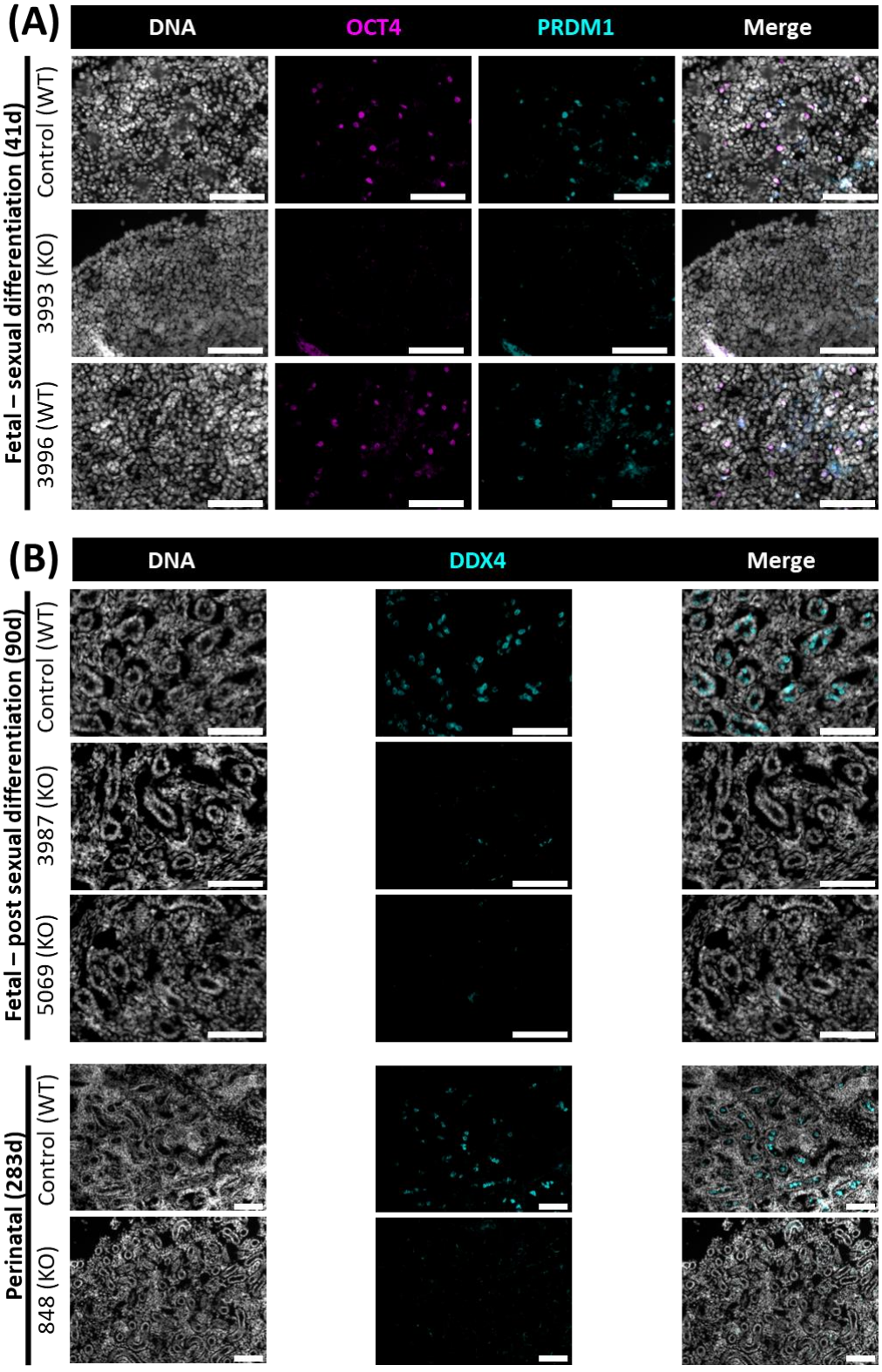


Figure 3.4. Germ cell-deficient phenotype in *NANOS3* KO fetal and perinatal testes. Representative images of immunostaining for well-conserved germ cell markers. A) Immunostaining for OCT4 (magenta) and PRDM1 (cyan) in the genital ridges of samples at 41 days of fetal age. B) Immunostaining for DDX4 (cyan) in the testes of samples at 90 days and 283 days of fetal age. All sections were co-stained for DNA (Hoechst 33342; gray). Scale bars are 100 μM. *NANOS3* genotypes are noted in parentheses next to the sample name. Wildtype (WT): 100% WT, or non-mutated, genome. Knockout (KO): all alleles present in the sample were predicted to KO, or inactivate, *NANOS3* (see Figure 3.3 and Table 3.6).

After sexual differentiation, testis cord formation was observed in both the control and *NANOS3* KO fetal testes at 90d (Figure 3.4B). However, staining for the germ cell marker, DDX4, was only positive in the developing testis cords of control samples. Additionally, seminiferous cord development was observed in both the control and *NANOS3* KO perinatal (283d) testes (Figure 3.4B). In the perinatal control testes, DDX4 positive cells were observed in the center of many of the seminiferous cords. In contrast, no DDX4 positive cells were observed in the *NANOS3* KO sample, 283d_848, even though the seminiferous cord structures were present. It should be noted, DDX4 detection in the control samples was primarily observed in the cytoplasm of cells, which agrees with its role as RNA-binding protein. Overall, the *NANOS3* KO testes had similar testis cord formation patterns compared to age-matched control testes, indicating that the somatic support cells remained intact through fetal development in the *NANOS3* KO testes, even in the absence of germ cells (Figure 3.4).

3.4.2. *scRNA-Seq analysis shows germline ablation with intact somatic support cell populations in CRISPR/Cas9 NANOS3 KO bovine fetal and perinatal testes*

scRNA-Seq analysis was employed to confirm immunostaining results and fully characterize the germ and somatic cell populations of the 90d and 283d *NANOS3* KO, compared to WT, bovine testes (Figure 3.5-7). Each timepoint was analyzed individually.

For the 90d timepoint, a total of 45,630 and 40,237 cells passed quality filtering and were analyzed for control (n = 2) and KO (n = 2) treatments, respectively. On average, approximately 21,000 cells were analyzed for each sample (control_1 = 25,909, control_2 = 19,721, KO_3987 = 15,799, and KO_5069 = 24,438). Clusters were identified as cell types based on differential gene expression of well conserved marker genes. The 90d PGC cluster was identified by high expression of late PGC/gonocyte markers, *DDX4* and *DAZL* (Figure 3.5D). At 90d, pluripotency (e.g., *OCT4* and *NANOG*) and early PGC (e.g., *NANOS3*) markers were only expressed in a small number of PGCs, indicating that at 90d the majority of PGCs are in the late PGC/gonocyte stage. The early PGC marker, *KIT*, was expressed highly in the 90d PGC cluster, but also highly expressed in the endothelial and blood cells cluster, which agrees with several studies showing that *KIT* is not a specific method to identify germ cells (Kritzenberger and Wrobel, 2004, Lavoir et al., 1994, Ohno and Gropp, 1965, Soto and Ross, 2021). Only 90d control cells were present in the PGC cluster. Additionally, there was no expression of *NANOS3* or late PGC markers, *DDX4* or *DAZL*, in the *NANOS3* KO testicular cells (Figure 3.5E). In contrast, *NANOS3* was expressed in 2% of the control PGCs, and the PGC cluster represented 9% of the total control cells analyzed. Key somatic support cell populations, including Sertoli, Leydig, and Peritubular Myoid (PTM) cells, were identified in all four samples (Figure 3.5A and B). Additionally, the 90 KO somatic cell populations were present in similar proportions to the WT control samples, and the majority of

marker genes for the somatic cell populations were conserved across treatments (Figure 3.7). Conserved genes were defined as those genes that were differentially expressed (log-transformed fold-change ≥ 0.5) in a specific cell type of both treatments when compared to all other cell types at the same timepoint.

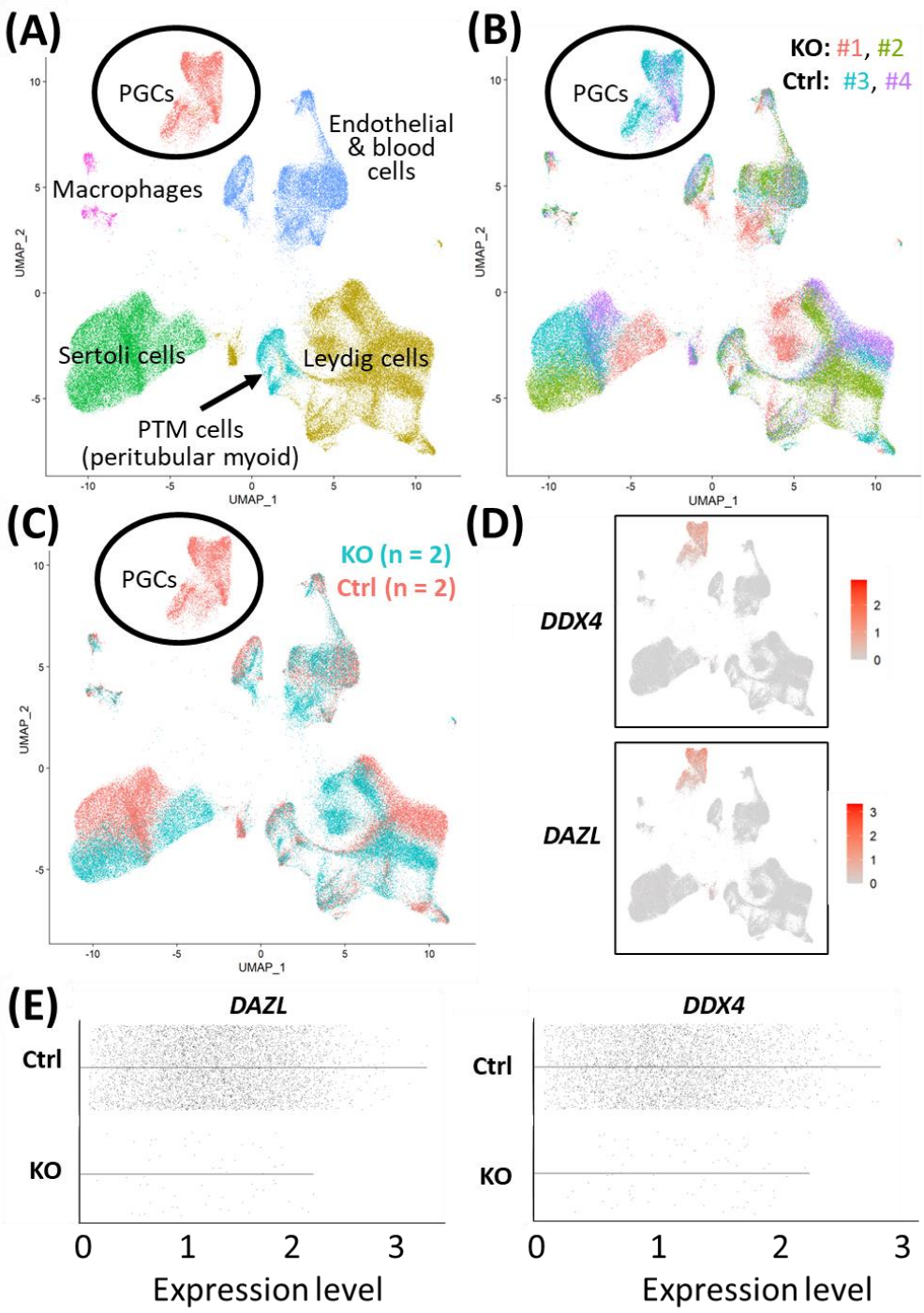


Figure 3.5. scRNA-Seq analysis of 90d fetal testes, comparing *NANOS3* KO samples to control (ctrl) samples. A) UMAP plot of different cell populations of the fetal testis. Clusters were identified based on expression of known marker genes. B) UMAP plot colored by individual samples (n = 4). C) UMAP plot colored by treatment showing that only control samples are present in the primordial germ cell (PGC) cluster. D) UMAPs showing differential expression of known late PGC/gonocyte markers, *DAZL* and *DDX4*. E) Violin expression plots of late PGC/gonocyte markers (*DAZL* and *DDX4*) showing the lack of germ cell marker expression in *NANOS3* KO samples (n = 2) compared to the ctrl samples (n = 2).

For the 283d timepoint, a total of 25,733 and 8,828 cells passed quality filtering and were analyzed for the control and KO samples, respectively. Clusters were identified as cell types based on differential gene expression of well-known marker genes. Similar to the 90d samples, the 283d germ cell cluster was identified by high expression of gonocyte markers, *DDX4* and *DAZL* (Figure 3.6B). There was no *NANOS3* expression observed in the *NANOS3* KO sample. Only control cells were present in the germ cell cluster and less than 1% of the control germ cells expressed *NANOS3* (Figure 3.6C). At 283d, the germ cells represented a much smaller proportion (1%) of the total control cells analyzed than at 90d. Key somatic support cell populations, including Sertoli, Leydig, and Peritubular Myoid (PTM) cells, were identified in both samples (Figure 3.6A), but there was smaller proportion of Leydig cells present in the KO (28%) compared to the control (51%) (Figure 3.7). Several key marker genes for the somatic cell populations were conserved across treatments. However, many of these marker genes also had significantly ($p \leq 0.05$) different expression (log-transformed fold-change ≥ 0.5) between treatments (Figure 3.7). Ultimately, the scRNA-seq analysis showed a complete loss of PGCs and germ cells in *NANOS3* KO fetal and perinatal testis, while maintaining the development of somatic support cells.

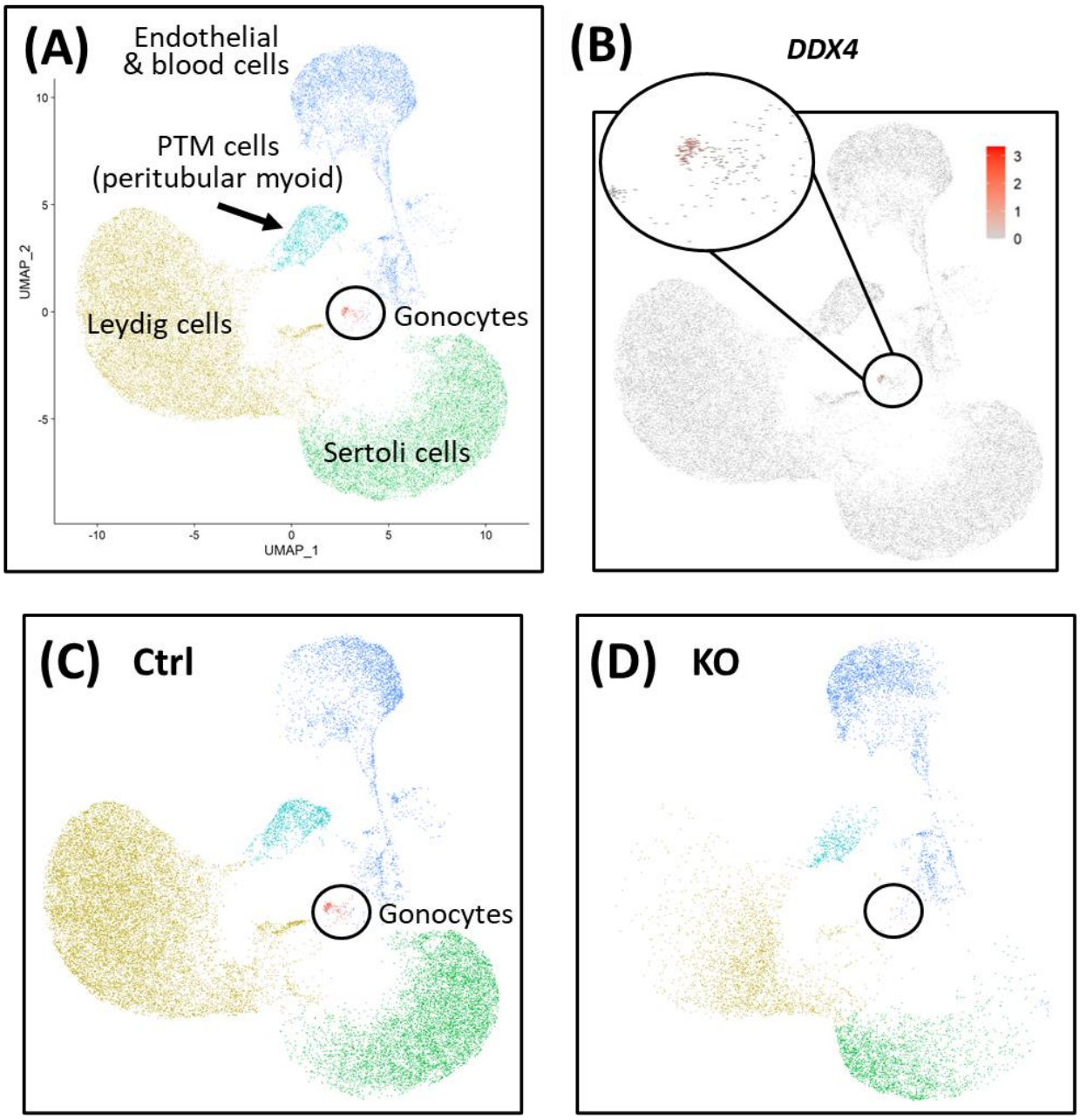
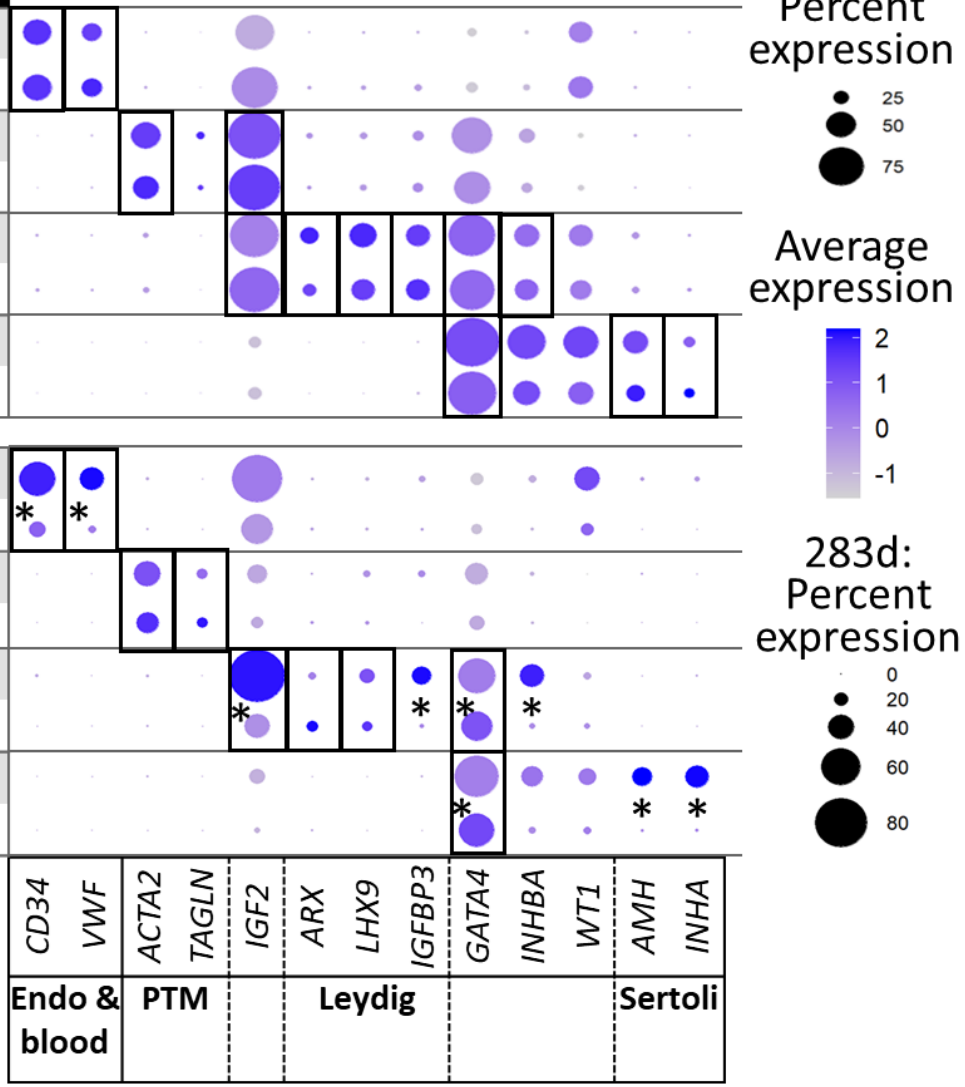


Figure 3.6. scRNA-Seq analysis of 283d perinatal testes, comparing a *NANOS3* KO sample to a control (ctrl) sample. A) UMAP plot of different cell populations of the perinatal testis. Clusters were identified based on expression of known marker genes. B) UMAP showing differential expression of known gonocyte marker *DDX4*. C) UMAP plots colored by cell type and split by control (left) and KO (right) cell, showing the lack of germ cell cells in the *NANOS3* KO sample.

	Cell type	Trt	% of sample	# of cells ± SEM
90d	Endo. & blood	CT	7%	3,082 ± 654
		KO	10%	3,907 ± 481
	PTM	CT	2%	706 ± 127
		KO	2%	804 ± 290
	Leydig	CT	20%	8,912 ± 1,644
		KO	25%	10,080 ± 2,252
	Sertoli	CT	13%	6,097 ± 2,539
		KO	11%	4,624 ± 1,136
283d	Endo. & blood	CT	10%	2,544
		KO	33%	2,900
	PTM	CT	4%	939
		KO	5%	431
	Leydig	CT	51%	13,024
		KO	28%	2,476
	Sertoli	CT	26%	6,683
		KO	26%	2,326



Key

Conserved genes

∗ Differentially expressed genes (DEG)

Figure 3.7. scRNA-Seq differential gene expression analysis of *NANOS3* KO and WT control (CT) testes at 90d (top panel) and 283d (bottom panel) of fetal age. The x-axis is known-marker genes for testicular somatic cell populations. The dashed lines indicate that the gene is shared maker for two cell populations. The dot size represents the percent of cells present in a particular cell type cluster expressing a particular gene (larger dots indicate a greater proportion of cells). The color of the dot represents the average scaled expression level of a particular gene in a particular cell type cluster (darker indicates higher average expression). Conserved genes (black boxes) were defined as those genes that were more highly expressed (log-transformed fold-change ≥ 0.5) in a specific cell type of both treatments when compared to all other cell types at that timepoint. Differentially expressed genes (DEG; black asterisks) had significantly ($p \leq 0.05$) different expression (log-transformed fold-change ≥ 0.5) between treatments. Some genes were both conserved and DEG. Endothelial (Endo.), Peritubular Myoid (PTM), treatment (Trt)

3.5. Phenotypic analysis of CRISPR/Cas9 *NANOS3* edited cattle

In order to evaluate the potential for *NANOS3* KO cattle to serve as hosts for donor-derived gamete production, the reproductive development and capabilities of the live *NANOS3* edited cattle (n = 3; 2 males and 1 female) were characterized through post-pubertal age (~15-months-old). Monthly, body weight and scrotal circumference (males only) were measured, and blood was collected for steroid hormone analysis. Body weights followed a normal linear growth pattern (Figure 3.8, top left panel). When the cattle reached reproductive age, around 12-months, reproductive exams were performed by UC Davis veterinarians. Lastly, the three *NANOS3* edited animals were slaughtered at 15-months-old to enable comprehensive analysis of their reproductive tracts, with specific focus on their gonads. Additionally, meat samples were collected from the *NANOS3* KO cattle, heifer #854 and bull #838, at harvest for compositional analysis.

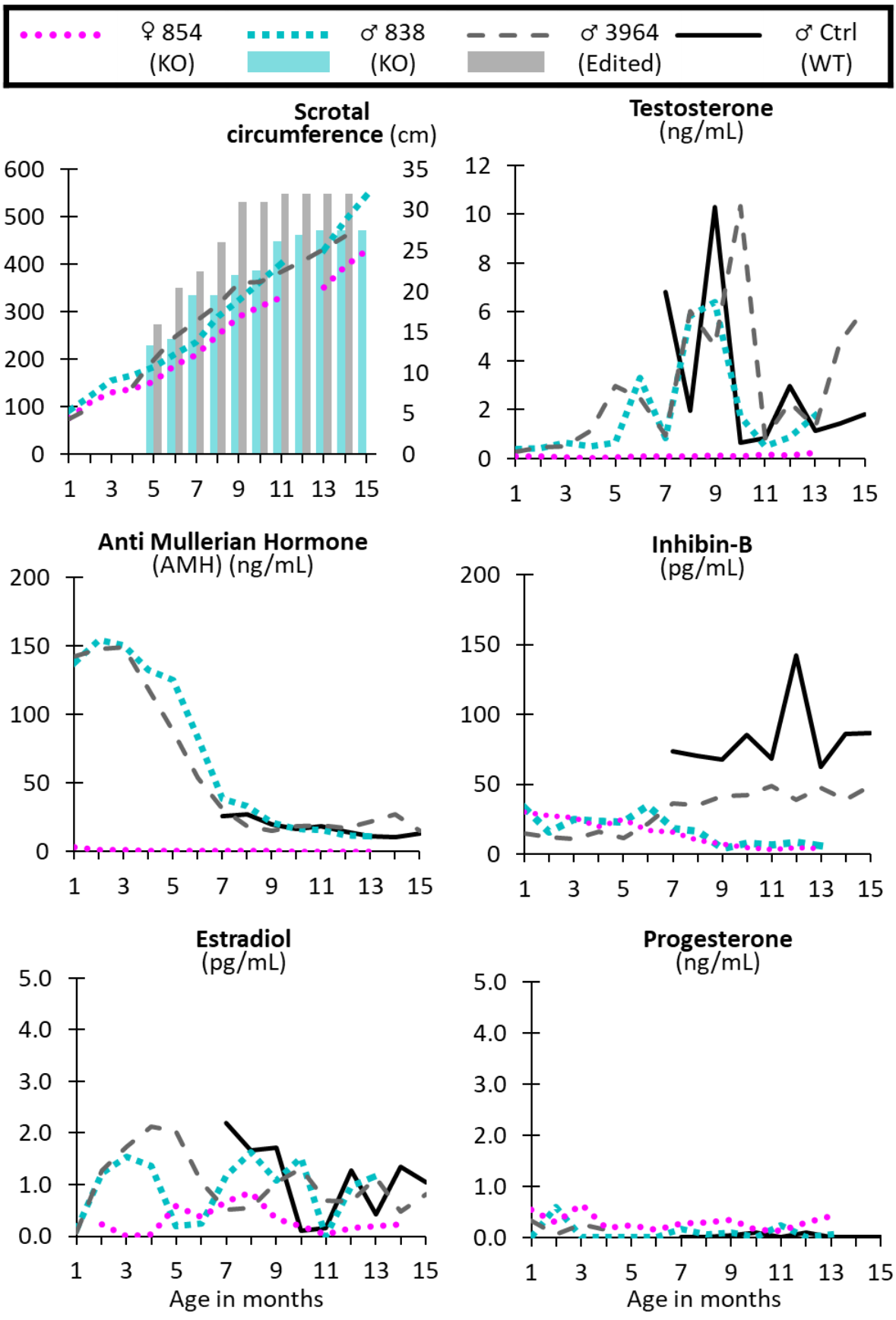

♀ 854 (KO)
♂ 838 (KO)
♂ 3964 (Edited)
♂ Ctrl (WT)
Scrotal circumference (cm)
Testosterone (ng/mL)
Anti Mullerian Hormone (AMH) (ng/mL)
Inhibin-B (pg/mL)
Estradiol (pg/mL)
Progesterone (ng/mL)
Age in months
Age in months

Figure 3.8. Monthly body weight, scrotal circumference (SC), and reproductive hormone levels of CRISPR/Cas9 *NANOS3* edited cattle from 1-15 months old. Monthly serum hormone levels for male primary hormones, testosterone (ng/mL), AMH (ng/mL), and Inhibin-B (pg/mL), and female primary hormones, estradiol (pg/mL) and progesterone (ng/mL). Sample key: *NANOS3* KO heifer #854 (pink round dots), *NANOS3* KO bull #838 (blue square dots and blue bar for SC), *NANOS3* edited bull #3964 (grey dashes and grey bar for SC), genetic wildtype (WT) control (ctrl) bull (black solid line).

3.5.1. *Reproductive hormone levels of CRISPR/Cas9 NANOS3 edited cattle*

For hormone analyses, blood samples were collected monthly on the live *NANOS3* edited animals (n = 3) and a WT bull (n = 1). Serum samples were processed by the Clinical Endocrinology Laboratory at the UC Davis, School of Veterinary Medicine in the Department of Population Health and Reproduction.

3.5.1.1. Male primary hormones

Both the *NANOS3* KO #838 and edited #3964 bulls showed an increasing testosterone pattern, exceeding 1 ng/ml just after approximately four months of age, and then reaching peak levels (6 ng/mL and 10 ng/mL, respectively) during puberty (Figure 3.8). The control bull also reached a peak testosterone level of 10 ng/mL during puberty. In contrast, testosterone was present at low levels (average 0.1 ng/mL) in the *NANOS3* KO heifer (#854) and never increased above 0.3 ng/mL.

In the *NANOS3* KO #838 and edited #3964 bull samples, a pattern of peak AMH levels during the first few months of life (reaching 150 ng/mL) followed by a decrease to stable levels during and post-puberty was observed. As for the control bull, since sampling began at 7 months, his peak level was unknown, but his post-pubertal AMH levels were similar to those of both

NANOS3 targeted bulls, ranging from 10-30 ng/mL. The *NANOS3* KO heifer #854 started at 3 ng/mL at one-month-old and then gradually decreased to undetectable levels (< 0.01 ng/mL) by ten-months-old.

Both the *NANOS3* KO #838 and edited #3964 bulls had low pre-puberty levels of inhibin-B (10-35 pg/mL). When puberty started, around seven-months-old, inhibin-B levels in the *NANOS3* KO #838 bull decreased to less than 10 pg/mL by 9-months. In contrast, after 7-months, the *NANOS3* edited #3964 bull's inhibin-B levels increased to be 40-50 pg/mL during puberty and post-puberty. During the same time (7-15 months), the average inhibin-B level for the control bull was 82 pg/mL. The *NANOS3* KO heifer #854, had a similar pattern to the *NANOS3* KO bull, with low pre-pubertal levels (15-30 pg/mL) and then decreasing to below 10 pg/mL after 7-months-old.

3.5.1.2. Female primary hormones

The *NANOS3* KO heifer #854 had an average estradiol level of 0.5 pg/mL, with levels even during post-puberty never exceeding 0.9 ng/mL. Both the *NANOS3* KO #838 and edited #3964 bulls had similar average estradiol levels from 2-4 months-old, 1.4 pg/mL and 1.7 pg/mL, respectively. Additionally, during puberty and post-puberty all three bulls, including the control bull, had similar estradiol levels of approximately 1 ng/mL. Post-pubertal progesterone levels in the *NANOS3* KO heifer (#854) never exceeded 0.4 ng/mL. Progesterone was detected at low levels in all of the bulls in this study.

3.5.2. *Phenotypic analysis of CRISPR/Cas9 NANOS3 KO bull #838*

Bull #838 was a *NANOS3* mosaic KO (Table 3.6) and therefore it was hypothesized that there would be a complete loss of germ cells in bull #838, but otherwise normal gonadal development (Tsuda et al., 2003). At 12-months of age, bull #838 had a masculine appearance, demonstrated normal libido, and a BSE found that he had an anatomically normal reproductive

tract (i.e., accessory sex glands and penis) and normal testicular development, although the scrotal circumference (27 cm) was smaller than expected for age and breed matched controls. However, microscopic evaluation of an ejaculate obtained via electroejaculation revealed seminal plasma only with no spermatozoa present. These results were repeated and confirmed with BSE at 13- and 15-months-old. At 15-months-old, bull #838 was harvested and his reproductive tract was collected. Bull #838's reproductive tract was anatomically normal with all accessory sex glands present (Figure 3.9A). Additionally, cross-sections of bull #838's testis were stained with H&E and immunostained for the germ-cell marker, DDX4 (Figure 3.10). Compared to an age matched, WT (*NANOS3*+/+) bull, bull #838 lacked any spermatogenesis, but importantly had Sertoli cells lining the seminiferous tubules.

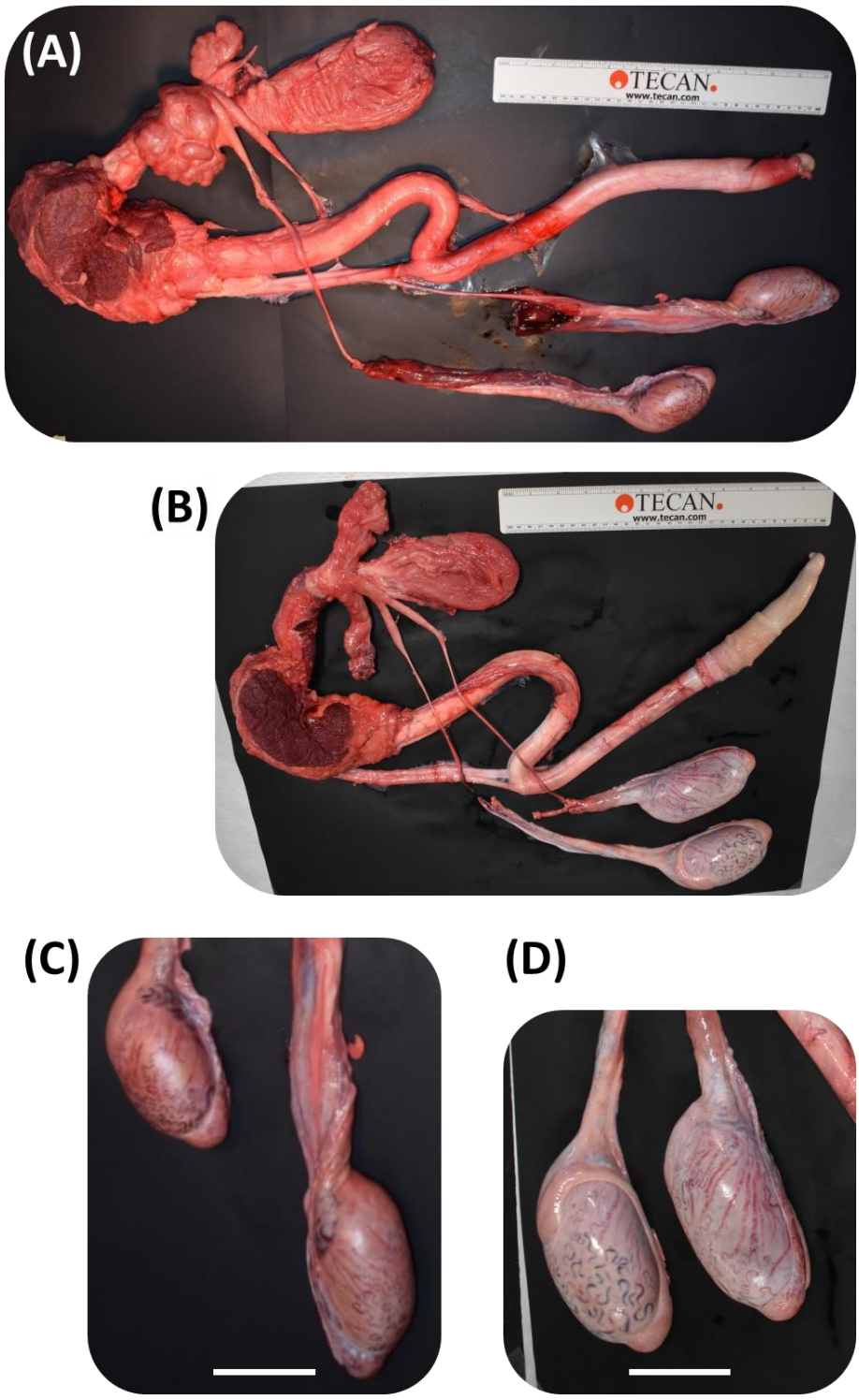
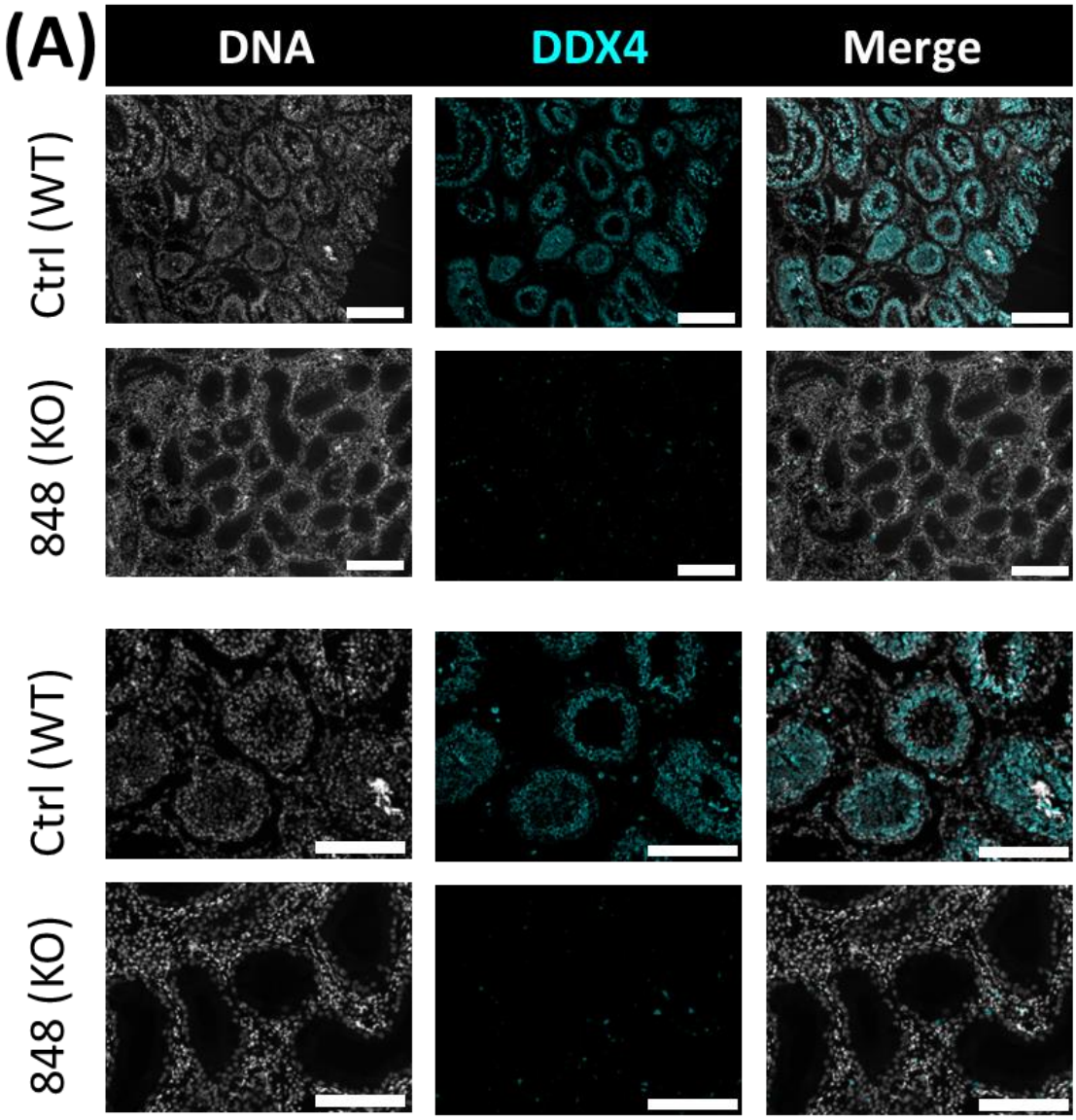


Figure 3.9. Comparison of CRISPR/Cas9 *NANOS3* KO bull #838 (A and C) and edited bull #3964's (B and D) reproductive tracts (A-B) and testes size (C-D). A) Image of bull #838 at 15-months-old. B) Image of bull #838's reproductive tract. Scale bars are 5 cm.


(A)
DNA
DDX4
Merge
Ctrl (WT)
848 (KO)
Ctrl (WT)
848 (KO)

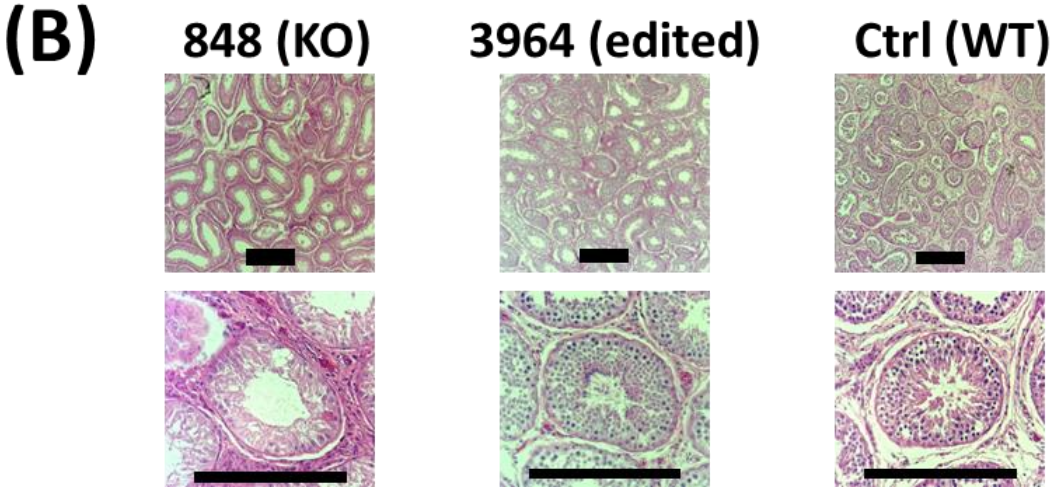

(B)
848 (KO)
3964 (edited)
Ctrl (WT)

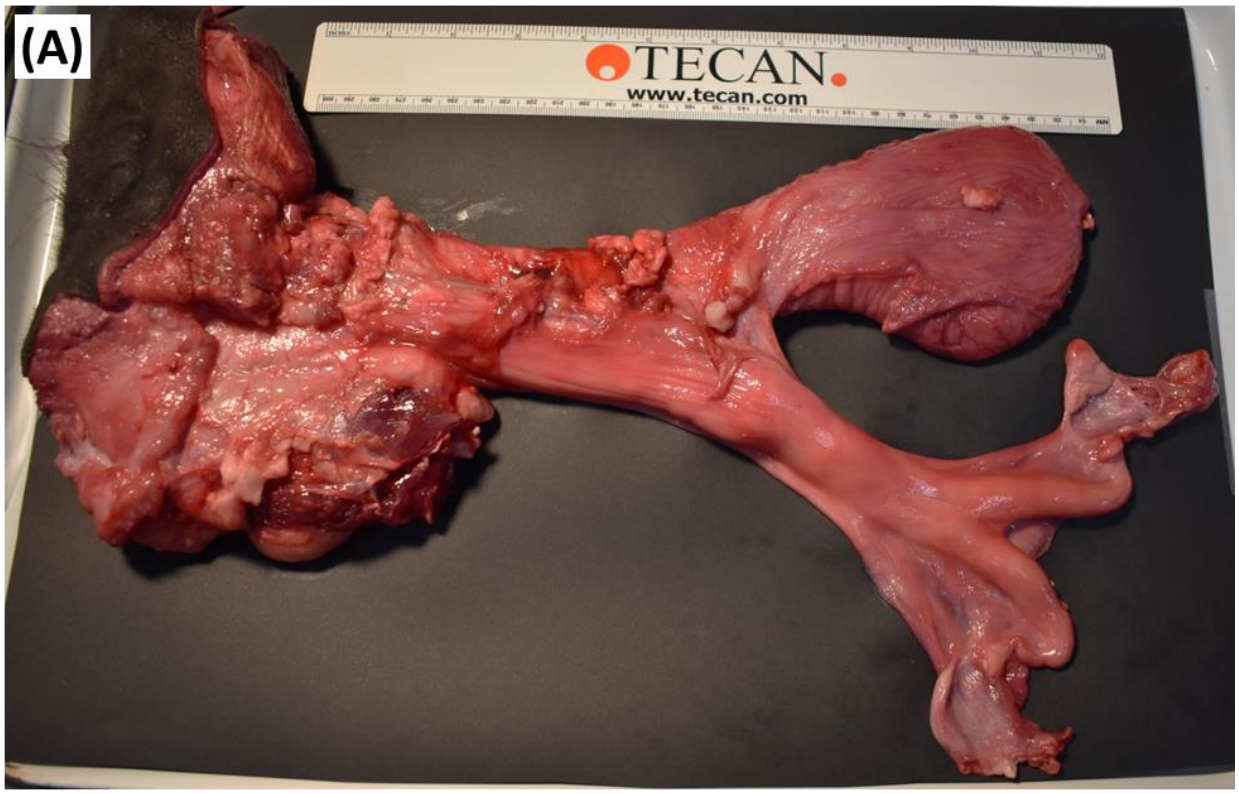
Figure 3.10. Histological analysis of CRISPR/Cas9 *NANOS3* KO bull #838 and edited bull #3964's testes. A) Representative images of DDX4 immunostained (cyan) testis cross-sections from KO bull #838 compared to an age matched, wildtype (WT) bull. All immunostained sections were co-stained for DNA (Hoechst 33342; gray). B) Representative images of H&E stained testis cross-sections from KO bull #838, edited bull #3964, and an age matched, WT bull. Scale bars are 100 μM. Histology indicates that all samples have Sertoli cells lining the seminiferous tubules, but KO bull #838 lacks any spermatogenesis.

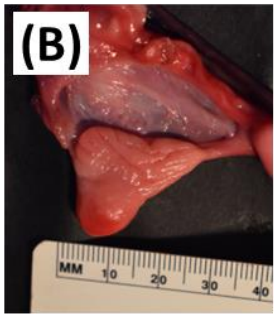
3.5.3. *Phenotypic analysis of CRISPR/Cas9 NANOS3 edited bull #3964*

Bull #3964 was carrying 3 mutated alleles and no WT alleles (Table 3.6). However, one allele (30% of reads) had only small, in-frame deletions that were all outside of the highly conserved zinc finger binding domain and resulted in only a few amino acid changes (Table 3.6; Figure 3.3). Therefore, it was hypothesized that these in-frame deletions could result in a functional *NANOS3* protein and thus an intact germline. At 12-months of age, bull #3964 had a masculine appearance, demonstrated normal libido, and passed a BSE. Bull #3964 was found to have an anatomically normal reproductive tract, normal testicular development with adequate scrotal circumference (32 cm), and produced a satisfactory ejaculate for his age (30% motility, 78% normal cells, 11% head abnormalities, 11% tail abnormalities, 0% tail abnormalities). At 15-months-old, bull #3964 was harvested and his reproductive tract was collected. Bull #3964's reproductive tract was anatomically normal with all accessory sex glands present and an adequate scrotal circumference of 32 cm (Figure 3.9B and D). Additionally, spermatogenesis was evident in bull #3964's testes via H&E staining (Figure 3.10B).

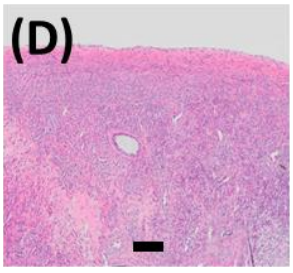
3.5.4. *Phenotypic analysis of CRISPR/Cas9 NANOS3 KO Heifer #854*

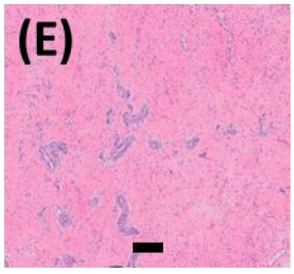
Heifer #854 was a mosaic KO, with 5 mutated alleles that all had targeted dual gRNA_4+7 indels (291-298 bp; Table 3.6) and no WT alleles. Due to these KO mutations, it was hypothesized that there would be a complete loss of germ cells in heifer #854, but otherwise normal gonadal development (Ideta et al., 2016, Tsuda et al., 2003). Heifer #854 had a feminine appearance and her behavior was observed through puberty until 15-months of age, but she never showed signs of estrus. UC Davis veterinarians performed a reproductive exam on heifer #854, around 14 months of age. A small, involuted, and hypoplastic reproductive tract, with a small cervix and flaccid uterine horns, was observed during palpation, which are similar characteristics of a juvenile or freemartin female. The right ovary was unable to be imaged with ultrasound and no structures could be identified. The left ovary was small (< 1 cm) and no structures or follicular development were observed with ultrasound. At 15-months-old, heifer #854 was harvested and her reproductive tract was collected. Heifer #854's reproductive tract was observed to be anatomically abnormal, with a small clitoris, long anterior vagina, and a putative primitive streak in place of the right ovary (Figure 3.11). Additionally, cross-sections of the left ovary and right primitive streak were processed for H&E analysis, which showed a complete lack of oogenesis (Figure 3.11).

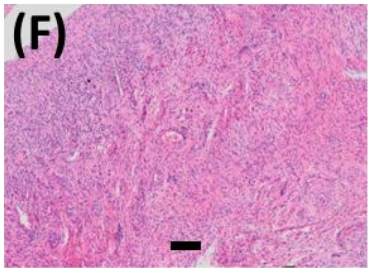

(A)
TECAN.
www.tecan.com

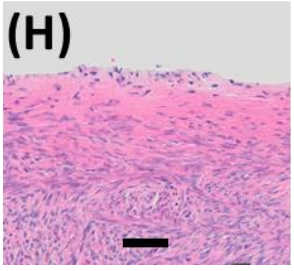

(B)
MM 10 20 30 40

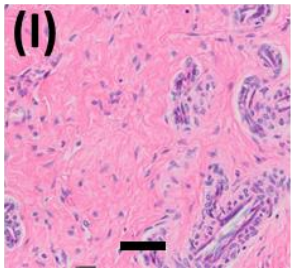

(C)
MM 10 20 30 40 50 60


(D)


(E)


(F)


(H)


(I)

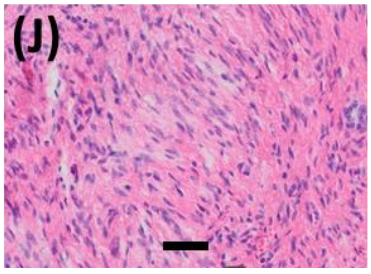

(J)

Figure 3.11. Phenotypic analysis of CRISPR/Cas9 *NANOS3* KO Heifer #854. A) Image of heifer #854's reproductive tract. B-C) Images of heifer #854's left ovary (B) and right putative primitive streak (C). D-J) Representative images of H&E-stained ovary cross-sections from heifer #854 showing a complete lack of oogenesis.

3.5.5. *Meat analysis of the three CRISPR/Cas9 NANOS3* edited *cattle*

The average meat composition values for proximate analysis and minerals of the *NANOS3* KO heifer #854 and bull #838 were within the normal variation seen in international databases (Table 3.7) (Trott et al., 2022).

Table 3.7. Meat compositions analysis of sirloin cap and chuck arm from CRISPR/Cas9 *NANOS3* KO cattle (n = 2) and values from Trott et al. (2022) analysis of international nutrient databases.

	NANOS3 KO cattle average (SD)	Literature mean[1]	Literature range[1]	Within literature range?
Proximate analysis				
Ash (%)	0.85 (0.06)	1.02	0.1–2.62	Yes
Protein (%)	22.175 (1.53)	20.5	11.0–29.8	Yes
Crude fat (%)	1.75 (1.22)	8.64	0.5–42.5	Yes
Minerals				
Iron (%)	0.002 (0.0005)	0.0021	0.0007–0.005	Yes
Phosphorus (%)	0.1965 (0.01)	0.185	0.09–0.37	Yes
Zinc (ppm)	45.25 (9.12)	47.1	10–98.5	Yes

[1] Values from Trott et al. (2022) analysis of international nutrient databases

4. DISCUSSION

In this study, we optimized direct cytoplasmic microinjection of the CRISPR/Cas9 system in *in vitro* produced bovine embryos to KO *NANOS3* with high efficiency and repeatability,

enabling us to produce *NANOS3* KO cattle and thereby characterize the effect of eliminating *NANOS3* on bovine germline development from fetal development through reproductive age.

Our gene KO approach using co-injection of two selected sgRNA/Cas9 RNP complexes into bovine zygotes (6 hours post insemination) achieved a high *NANOS3* KO rate in developing embryos (79%, n = 22/28, 4 replicates), while maintaining an acceptable blastocyst development rate (19%, n = 28/150, 4 replicates). During blastocyst testing, KO was defined as 0% WT, or non-mutated, *NANOS3* alleles, based on PCR and Sanger Sequence analysis of the target region. Given our high *NANOS3* KO rate in bovine embryos (> 75%), we proceeded with ET of CRISPR/Cas9 *NANOS3*-presumptively edited blastocysts. Through these ETs, we successfully generated 8 bovine pregnancies (31% 35-day pregnancy rate, n = 8/26) with CRISPR/Cas9 *NANOS3* -presumptively edited blastocysts. To evaluate the effect of disrupting *NANOS3* on bovine germline development, gonadal samples were collected at 4 different timepoints (41d, 90d, 283d, and 15mo).

Bovine *NANOS3* is a 2,633 bp gene with two exons (Figure 3.1). Exon 1 (451 bp) of bovine *NANOS3* contains the highly conserved coding regions for the N-terminal and zinc finger binding domains (Beer and Draper, 2013, Suzuki et al., 2014), thus we chose to target exon 1. A dual gRNA approach was used and sgRNAs #4 and #7 (dual gRNA_4+7) were selected based on their mutation efficiencies and genomic locations to target both the 5’ and 3’ regions of exon 1, which in combination would completely remove the critical zinc finger binding domain (297 bp between sgRNA cut sites; Figure 3.1).

Based on previous studies that have shown higher KO rates when using multiple adjacent sgRNAs targeting a key exon, spaced 20-300 base pairs apart (Joberty et al., 2020, Zuo et al., 2017), we chose to employ a dual gRNA approach to KO bovine *NANOS3*. Additionally, the dual

gRNA system, has been shown to be an efficient method for complete gene disruption in livestock species and this system allows initial evaluation of mutation efficiency by gel electrophoresis of the PCR products without the need for Sanger sequencing (Mark Cigan and Knap, 2022, Vilarino et al., 2017, Wu et al., 2017). Consistent with these findings, we achieved a 75% (n = 6/8) total KO rate with our dual gRNA_4+7 editing approach (Table 3.6).

This *NANOS3* KO efficiency in bovine zygotes enabled us to generate multiple *NANOS3* targeted bovine pregnancies which allowed evaluation of the effect of disrupting *NANOS3* on bovine germline development during key developmental stages. The four collection timepoints allowed us to analyze *NANOS3* deficient gonads during fetal development, both during sexual differentiation (41d; n = 2 *NANOS3*-presumptively-edited and 2 WT) and after fetal sexual differentiation (90d; n = 2 *NANOS3*-presumptively-edited and 2 WT), at the perinatal, or birth, stage (283d; n = 1 *NANOS3*-presumptively-edited and 1 WT), and post-puberty (15mo; n = 3 *NANOS3*-presumptively-edited and 1 WT).

For the eight bovine samples, initial *NANOS3* genotype analysis via short-range PCR amplification and Sanger sequencing of the *NANOS3* exon 1 target region, confirmed the high mutation efficiency of our dual gRNA_4+7 approach. This analysis showed that seven of the eight *NANOS3* targeted bovine samples (87.5%) were successfully edited (0% WT alleles remained). Additionally, four of the edited samples (57%) had at least one allele with a targeted dual gRNA_4+7 indel. Six of the seven alleles present in the seven edited embryos or calves were predicted to be KO alleles. For the bovine samples, a KO allele was defined as having either a frameshift-inducing indel or a moderate sized indel (> 21 bp) in a protein-coding region that are predicted to generate a complete loss-of-function mutation.

The one allele that was predicted not to be a KO was carried by the *NANOS3* edited bovine sample, 15mo_3964, and had two small, in-frame deletions. The allele resulted in one amino acid substitution and a deletion of three total amino acids, but each of the mutations were outside of the highly conserved N-terminal and Zinc-finger domains (Figure 3.3). The evolutionarily conserved zinc finger domain, which consists of two consecutive CCHC-type zinc finger motifs, is indispensable for *in vivo* functions in Drosophila where the *nanos* gene was first identified (Wang and Lehmann, 1991). Additionally, studies in mice have shown that both an intact N-terminal region and zinc finger domain are essential for murine *nanos2* functions *in vivo*, but other regions maybe dispensable (Suzuki et al., 2014, Suzuki et al., 2012). The exact amino acid sequence that was predicted to result from the mutated allele was not found in any other species when a protein BLAST (Basic Local Alignment Search Tool) analysis was conducted, and it was unknown if the deleted amino acids were necessary for *NANOS3* protein function. Therefore, we had no a priori information as to whether this in-frame allele could be functional.

In this initial genotype analysis, we observed that many of samples appeared to be mosaic but given the limited depth of Sanger sequencing, we could not discern the proportion of different alleles present in each sample. Additionally, several studies have reported that large deletions of up to several thousand bases occur with high frequencies (up to 15%) at the Cas9 on-target cut sites. Therefore, we undertook further sequence analysis for all eight CRISPR/CAS9 *NANOS3*-presumptively-edited bovine samples to 1) identify and measure the proportion of alleles present in the mosaic samples, and 2) detect potential large deletions present in our samples. Using long-range PCR (6,274 bp), we observed that three of the samples (43%), had previously undetected alleles with large (960-1,502 bp) deletions (Figure 3.3B and Table 3.6). These deletions eliminated the short-range PCR primer binding sites commonly used for on-target analysis. Consequently,

these alleles would have remained undetected using traditional screening methods. These findings align with studies that highlight the occurrence of both small indels and large deletions following Cas9 cleavage, emphasizing the importance of evaluating the presence of large deletions in genome editing experiments (Park et al., 2022). Interestingly, in the *NANOS3* edited bull 15mo_3964, the long-read sequencing revealed that the in-frame allele represented only 30% of the reads, while the remaining 70% of the reads from this sample were comprised of two large deletions alleles, each predicted to be non-functional. Overall, we observed a 71% mosaicism rate (n = 5/7) in our *NANOS3* edited bovine samples. However, it is noteworthy that all but one of the 23 edited alleles resulted in a predicted KO or loss of function.

Murine studies have shown that *nanos3* mutations do not affect PGC specification but rather impair the subsequent survival of PGCs during their migration to the developing gonad. In *Nanos3* KO murine fetuses a small proportion of PGCs (approximately 1/5 of the original population) can reach the genital ridge, but these cells are quickly lost to apoptosis and by murine day 10 no PGCs remain in the bipotential gonad (Suzuki et al., 2007, Tsuda et al., 2003). These studies were foundational to demonstrate that mammalian *nanos3* maintains the germ cell lineage by suppressing apoptotic pathways (Suzuki et al., 2008). *NANOS3* KO pig fetuses were also found to have significantly fewer migrating PGCs on day 18 of fetal age and subsequently a complete loss of PGCs in both fetal ovaries and testis after gonadal sex differentiation, day 35 (Park et al., 2023).

In cattle, putative migrating PGCs can first be identified at 18 days of fetal age and the first PGCs reach the developing genital ridge around 27 days of fetal age, with the majority of PGCs arriving by 31 days of fetal age (Wrobel and Süß, 1998). In male cattle, *SRY* is first expressed at 35 days of fetal age and sexual determination occurs from day 38-39 of fetal age when SRY

expression peaks (Planells et al., 2019, Ross et al., 2009, Wrobel and Süß, 1998). Testis cords become distinguishable during early gonad differentiation around 42-44 days of fetal age (Planells et al., 2019, Ross et al., 2009). Given the different developmental timing of bovine development compared murine and porcine, it was previously unknown if or how early bovine PGCs would be eliminated in *NANOS3* KO gonads. The previous *NANOS3* KO study in cattle, provided valuable evidence that *NANOS3* plays a similar role in female in cattle development (Ideta et al., 2016). However, this study only produced one *NANOS3* KO female fetus that was collected at 190 days of development, which is past the PGC stage.

In this current study, we examined *NANOS3*-targeted and control fetuses at 41 days of fetal age, which corresponds to the stage of sexual differentiation. At this point, it is expected that PGCs would have reached the genital ridge (Planells et al., 2019, Soto and Ross, 2021, Stukenborg et al., 2014, Wrobel and Süß, 1998). Consistent with this, the 41d control sample and the unedited (100% WT) 41d_3996 sample showed the presence of PGCs in the genital ridge, as observed through immunofluorescence analysis. However, in the *NANOS3* KO sample (42d_3993), no PGCs were observed in the genital ridge. This indicates that in male *NANOS3* KO gonads, bovine PGCs were eliminated as early as 41 days of fetal age. Although it is possible that as seen in mice and pigs, bovine PGCs could have reached the genital ridge in *NANOS3* KO cattle, importantly they were eliminated by the bipotential gonad stage. Following gonad sexual differentiation in bovine fetal development (approximately 44 days of fetal age), late PGCs, known as gonocytes, begin to cluster together with developing Sertoli cells surrounding them. This clustering leads to the formation of individual cords, and as fetal development progresses, distinct seminiferous cords are formed with fully enclosed populations of gonocytes by Sertoli cells. At birth, these gonocytes are separated from the basement membrane of the seminiferous cords by immature Sertoli cells (Culty, 2013,

Planells et al., 2019, Ross et al., 2009, Skinner and Anway, 2005). While this pattern of testis cord formation was observed in both the control and *NANOS3* KO fetal and perinatal testes, gonocytes were only observed in the developing testis cords of control samples. This finding is consistent with the absence of PGCs in the 41d *NANOS3* KO gonads. Importantly, despite the lack of germ cells in the *NANOS3* KO testes, the somatic support cells, such as Sertoli cells, remained intact. This finding is crucial for potential germline complementation strategies, as intact somatic support cells provide a favorable environment for the introduction of exogenous germ cells.

Additionally, the scRNA-Seq analysis of the 90d and 283d *NANOS3* KO testes confirmed the immunofluorescence results, by showing the presence of key somatic support cell populations (e.g., Sertoli cells, Leydig cells, and PTM cells), but a complete loss of PGCs & germ cells. Importantly, no *NANOS3* expression was observed in the *NANOS3* KO samples at either timepoint, although *NANOS3* was only expressed by a small proportion of 90d PGCs (2%) and 283d gonocytes (0.7%) in the control samples. In mice, *Nanos3* expression is first detected in newly induced PGCs as early as 7.25 days post conception (dpc) (Tsuda et al., 2003, Yamaji et al., 2010). In females, murine *Nanos3* expression persists until approximately E14.5, coinciding with the onset of meiosis, and then is no longer detectable in fetal or postnatal female germ cells. Conversely, in male germ cells, murine *Nanos3* expression continues throughout the fetal period, albeit at declining levels after E16.5, which corresponds to the time when male germ cells typically enter mitotic arrest (Yamaji et al., 2010). Additionally, in postnatal male mice, *NANOS3* is robustly expressed in all newborn gonocytes (1-day-old) and then by 2-weeks-old its expression is restricted to a subset of undifferentiated spermatogonia, specifically spermatogonial progenitors, and it is not observed in spermatocytes undergoing meiotic divisions (Suzuki et al., 2009, Yamaji et al., 2010). In cattle gonadal samples, *NANOS3* expression was observed in both males and females

from 35 to 43 days of fetal age via RNA-Sequencing (Planells et al., 2019). Additionally, scRNA-Seq analysis of bovine ovaries around 50 days of fetal age found that the majority of PGCs were in the early stage of differentiation, as the majority of cells expressed at least one pluripotency and one early PGC marker, including 80% expressing *NANOS3* (Soto and Ross, 2021). A smaller proportion of 50 day female fetal PGCs were also expressing late PGC markers, so it appeared that a subset of cells were already transitioning toward a more advanced stage (Soto and Ross, 2021). Our result of only a few PGCs/gonocytes expressing *NANOS3* in the control samples at 90d and 283d align with *NANOS3* being a known-marker of early stage PGCs. Although, robust *Nanos3* expression is observed in murine newborn gonocytes, this is likely due to the extremely short prepubertal period in mice of only days compared to over 6 months in cattle. From 90d to 283d of fetal age, the proportion of germ cells present in the WT control testes decreased from 9% to 1%, which is similar to findings in human testicular development (Sohni et al., 2019). The 283d *NANOS3* KO sample had significantly fewer cells analyzed (8,000) compared to all other samples at both timepoints (average of approximately 20,000 per sample), so it is possible that the lack of germ cells observed in this sample was due to the limited number of cells analyzed. However, immunofluorescence analysis of testicular cross-sections from this same 283d *NANOS3* KO sample also showed a complete lack of germ cells, thus supporting the scRNA-Seq analysis finding.

In the *NANOS3* KO testes, even in the absence of germ cells, many of the known marker genes for the somatic cell populations were conserved across treatments at both timepoints. In the 90d KO testes, the somatic cell populations were also present in similar proportions to the WT control samples. In contrast, in the 283d KO testis there were 23% fewer Leydig cells compared to the WT control sample and many of the known marker genes for somatic cell populations had

significantly ($p \leq 0.05$) different expression levels (log-transformed fold-change ≥ 0.5) between treatments. Given the strong relationship and constant communication between germ and somatic cells during gonad development, these differences found at 283d could be due to the lack of germ cells impairing the somatic gonad development. However, in the 90d samples there was large variation in cell numbers per cell type between biological replicates of both treatments. Since we did not have a biological replicate for the 283d timepoint, it is unknown if the differences in somatic cell populations were due to the *NANOS3* KO or individual sample variation. Importantly, histological analysis of testicular cross-sections from this same 283d *NANOS3* KO sample showed similar testis cord formation patterns compared to an age-matched control testis. Taken together, these findings indicate that the somatic support cell structures remain intact through bovine fetal development, but these cells may have impaired communication and endocrinological functions later in development due to the lack of germ cells in *NANOS3* KO gonads.

To the best of our knowledge, this is the first study to produce live *NANOS3* KO cattle, which allowed us to evaluate the effect of disrupting *NANOS3* on reproductive development of cattle from birth through puberty and into reproductive age. All three calves were born healthy and without assistance and had normal growth rates. The levels of primary male hormones, including testosterone, AMH, and Inhibin-B, as well as primary female hormones, such as estradiol and progesterone, were evaluated. These hormone levels were assessed monthly from birth to sexual maturity to evaluate potential disruptions in the endocrine function of somatic support cells in the absence of germ cells in *NANOS3* KO animals.

Testosterone is a steroid hormone that is produced primarily by Leydig cells in the testes in males and to a lesser extent by the ovaries in females. Testosterone regulates a range of physiological processes related to sexual development and function, including the development of

secondary sexual characteristics, sperm production, and libido. During fetal development of cattle, males have five to tenfold more serum testosterone than female fetuses at each trimester of gestation (Kim et al., 1972, Mongkonpunya et al., 1975). In bulls, plasma testosterone concentrations show an increasing pattern, exceeding 1 ng/ml after approximately four months of age, and then rising over 3 ng/ml between 6-10 months. Testosterone levels are highest during puberty (3-9 ng/mL) and then settle to maintenance levels (2-4 ng/mL) post-puberty (Berger, 2019, Kawate et al., 2011, Matsuzaki et al., 2000, Rota et al., 2002, Sakase et al., 2018). In this study, both the *NANOS3* KO #838 and edited #3964 bulls showed a typical increasing testosterone pattern with peak levels reached during puberty before settling to baseline adult levels. It should be noted that testosterone is known to be secreted in a pulsatile manner, and previous studies have shown that testosterone levels can vary within a short time period. For instance, when blood samples were taken at 15-minute intervals for 8 hours from pubertal bulls, the testosterone levels ranged from <1 ng/mL to 8 ng/mL in individual bulls (Hannan et al., 2015). Since the samples in our study were collected only once per month, it is possible that peak levels of testosterone could be even higher than those observed. In the *NANOS3* KO heifer #854, consistent with observations in normal heifers (Rota et al., 2002), testosterone was present at low levels (average 0.1 ng/mL) and never increased above 0.3 ng/mL.

AMH is a protein hormone produced by the Sertoli cells of the testes in males and by the granulosa cells of the ovary in females. AMH plays a crucial role in mammalian reproductive development as it is involved in regression of the Müllerian ducts of the male fetus. Additionally, it regulates the growth and maturation of the ovarian follicles in females. In males, AMH levels levels undergo dynamic changes during development. In cattle, they start to increase around 50 days of fetal age and continue to rise after birth, reaching peak levels (300-1,000 ng/mL) before 6

months of age (Coen et al., 2021, Scarlet et al., 2017). Prior to puberty, AMH levels drastically decrease and then remain at a low stable level (20-200 ng/mL) post-puberty (Copping et al., 2018). This typical pattern of AMH levels was observed in both the *NANOS3* KO #838 and edited #3964 bull samples, with peak levels during the first few months of life (around 150 ng/mL) and subsequent decrease to stable levels during and after puberty. In females, AMH levels also increase after birth and reach their peak before 6 months of age, but at much lower levels compared to males. Peak AMH levels in female calves have been reported between 0.1-5 ng/mL (Baruselli et al., 2018, Kelly et al., 2020, Monniaux et al., 2012, Mossa et al., 2017). In cycling cattle, AMH levels are constant throughout estrous, and they are positively correlated with the number of follicles present (Baruselli et al., 2018, Batista et al., 2014, Ireland et al., 2011, Succu et al., 2020). In the case of *NANOS3* KO heifer #854, by the age of ten months, her AMH levels had become undetectable, measuring less than 0.01 ng/mL. This suggests a significant reduction or absence of AMH production in the *NANOS3* KO heifer, which is consistent with the disruption of germ cell development and lack of follicle formation observed in the study.

Inhibins are glycoproteins produced primarily by Sertoli cells in the testis and granulosa cells. They consist of two distinct chains (α and β) linked by disulfide bridges, with the α subunit pairing with βA or βB subunits to form inhibin-A or inhibin-B, respectively. Inhibin-B has been identified as the predominant isoform in adult males of various mammalian species, including livestock (Illingworth et al., 1996, Jin et al., 2001, Kaneko et al., 2001, Kondo et al., 2000, Tanaka et al., 2002, Woodruff et al., 1996). Additionally, studies in humans have shown that inhibin-B expression and secretion are positively correlated with Sertoli cell function, sperm number, and spermatogenic status (Luisi et al., 2005, Phillips, 2005, Stewart and Turner, 2005). Similarly, in stallions, inhibin-B concentrations were strongly associated with testis volume (Ball et al., 2019).

Bovine studies have demonstrated that the testes produce both inhibin-A and inhibin-B, with high concentrations being secreted into the circulation during postnatal development (Kaneko and Hasegawa, 2007, Kaneko et al., 2006, Kaneko et al., 2003). Total inhibin concentrations in the testis and plasma inhibin concentrations were found to be highest around one month of age and gradually decreased as the bulls aged (Kaneko et al., 2006, Matsuzaki et al., 2001). However, immunoreactivity for inhibin-B was not detected in the peripheral circulation, likely due to the low sensitivity of the assay used (Kaneko et al., 2006). Bovine Sertoli cells have been shown to produce both the precursor and mature forms of inhibin, and the mature forms increase while the immature precursor forms decrease during pre-puberty (Kaneko et al., 2003). It has been hypothesized that the decrease in total inhibin concentrations during pre-puberty reflects mainly a change in the immature precursor form of inhibin at that stage (Kaneko and Hasegawa, 2007). Furthermore, it was observed that the mature forms of inhibin-A and inhibin-B increased in the testis of bulls during postnatal development (Kaneko et al., 2006). Consistent with previous findings, in this current study both the *NANOS3* KO #838 and edited #3964 bulls exhibited low levels of inhibin-B during pre-puberty (10-35 pg/mL). However, during puberty, the inhibin-B levels in the edited bull began to increase, while the KO bull's levels decreased to levels even lower than pre-puberty. In females, inhibin-B is associated with maturation of follicles in the ovaries. Interestingly, the *NANOS3* KO heifer #854, had a similar pattern to the *NANOS3* KO bull, with low pre-pubertal levels that decreased further during puberty likely due to the absence of follicles. It is hypothesized that Sertoli cell proliferation and germ cell complement is likely to contribute to the overall production of inhibin-B (Stewart and Turner, 2005). For example, low serum inhibin-B levels have been reported in men with azoospermia, a condition characterized by the absence of sperm in the semen (Brugo-Olmedo et al., 2001). In the case of bull #838, which lacks germ cells, it is then

logical to observe low levels of inhibin-B during post-puberty, as the contribution of germ cells to inhibin-B production would be absent.

Estradiol is predominantly expressed in females, where it is produced by the ovaries. However, in males, estradiol production also occurs, primarily in the testes by Leydig cells. Additionally, a smaller amount of estradiol in males is generated through the peripheral conversion of testosterone by the enzyme aromatase. A study in prepubertal heifers (4-5 months old) reported that during an 8-hour window, with sampling every 15-minutues, the average estradiol level was 0.4 pg/mL, with a minimum of 0.3 pg/mL and maximum of 0.7 pg/mL (Kelly et al., 2020). In post-pubertal dairy heifers and late lactating dairy cows, estradiol levels ranged from 1-8 pg/mL depending on the estrous stage, with peak levels occurring approximately 2 days before estrus (Cooperative Regional Research Project, 1996). In the case of the *NANOS3* KO heifer #854 during pre-puberty, her average estradiol level (0.5 pg/mL) falls within the reported range for heifers. However, post-puberty estradiol levels in the *NANOS3* KO heifer #854 never exceeded 0.9 ng/mL. It is important to note that the blood samples were collected on a monthly basis, which could have potentially missed the peak levels associated with an estrous cycle. In intact bull calves, a study has shown higher average estradiol levels (average 1.5 pg/mL) compared to castrated calves (average 0.6 pg/mL), indicating the contribution of testicular production to estradiol levels (Deaver et al., 1988). In line with these findings, both the *NANOS3* KO #838 and edited #3964 bulls in this study exhibited similar average estradiol levels ranging from 1.4 pg/mL to 1.7 pg/mL during the 2-4 months age range. Additionally, all three bulls, including the control bull, showed comparable estradiol levels of approximately 1 ng/mL during puberty and post-puberty.

Progesterone is a steroid hormone primarily produced by the corpus luteum in the ovary during the luteal phase of the menstrual cycle and by the placenta during pregnancy. In males,

progesterone is produced in small amounts by the adrenal glands and testes, although its specific functions are not well understood. In post-pubertal heifers (dairy and beef) and late lactating dairy cows (3-5 years-old), progesterone levels ranged were reported to range from 0.05 to 6 ng/mL depending on the estrous stage, with peak levels occurring approximately 16 days after estrus (Cooperative Regional Research Project, 1996, Jimenez-Krassel et al., 2009). However, in the *NANOS3* KO heifer (#854) in this study, post-pubertal progesterone levels never exceeded 0.4 ng/mL. This was likely due to the absence of follicle formation and agrees with the observation that she never displayed estrus behavior. However, it is important to note that the blood samples in this study were collected once monthly, which could have missed the peak of an estrous cycle. A study reported that post-pubertal bulls ranged from 0.09-0.2 ng/mL of progesterone (Whitlock et al., 2012) and the average levels for all of the bulls in this study fall within that reported range.

Collectively, the hormone assays revealed that the CRISPR/Cas9 KO bull exhibited normal hormonal activity. These results indicate that the KO bull had an intact hypothalamic-pituitary-gonadal (HPG) axis, underwent puberty, had endocrinologically functional Leydig cells, and, at least prior to puberty, also endocrinologically functional Sertoli cells. On the other hand, the results from the CRISPR/Cas9 KO heifer showed no signs of reproductive cycling or functional granulosa cells, which aligns with the complete absence of germ cells and follicles in this animal. Importantly, despite the absence of germ cells, neither the KO bull nor the heifer showed any indication of sex reversal.

Finally, we examined the meat composition from the two *NANOS3* KO animals (heifer #854 and bull #838) that were slaughtered at 15-months-old. This was done in part due to the regulatory requirements around food use of GnEd animals, which require a demonstration of low food safety risk to allow products to enter the food supply under enforcement discretion or

investigational food use authorization. The results showed the meat composition from these cattle by proximate analysis and mineral content was within the normal variation seen in international databases (Trott et al., 2022). This is not surprising as a *NANOS3* KO would not be expected to alter meat composition.

Overall, these results demonstrated that bovine *NANOS3* is necessary for male bovine germline development. *Nanos3* KO male mice display atrophic testes, yet have intact seminiferous tubules, with no detectable spermatozoa (Miura et al., 2021, Suzuki et al., 2008, Tsuda et al., 2003). Additionally, *NANOS3* KO boars at 3-months (pre-puberty) and at 6-months (during puberty) had no detectable germ cells but had intact seminiferous tubules (Kogasaka et al., 2022). As expected, the *NANOS3* mosaic KO bull #838 was germline ablated as evidenced by the lack of spermatozoa in his ejaculate and his germ cell deficient testis (Figure 3.10), and he also had an anatomically normal reproductive tract, with all accessory sex glands present and his testis had intact seminiferous tubules. However, his 15mo scrotal circumference (27.5 cm) was below the industry BSE benchmark of a minimum scrotal circumference of 32 cm for bulls 15-18 months-old. Therefore, the *NANOS3* KO bull was found to phenocopy *Nanos3* KO male mice, although the adult size of the *NANOS3* KO testes was less affected in bulls (86% of WT size) than in mice (20-30% of WT size) (Miura et al., 2021, Tsuda et al., 2003). Moreover, bull #838 demonstrated normal libido and his serum hormone levels for key reproductive hormones were within the normal ranges throughout development, with the exception of his post-puberty Inhibin-B levels. This indicates that bull #838 had an intact and activated HPG axis, went through puberty, had functional testicular interstitial tissue, and at least prior to puberty, also had endocrinologically functional Sertoli cells. Overall, the data collected in this study support the hypothesis that inactivation of *NANOS3* in male cattle will result in complete germline ablation (i.e., functionally sterile).

Furthermore, this study demonstrates that *NANOS3* KO bulls have a phenotype (i.e., normal libido, adequate reproductive hormone levels, and an intact reproductive tract with intact testicular seminiferous tubules) that would be well suited to serve as hosts for germline complementation.

The fertile CRISPR/Cas9 *NANOS3* edited bull #3964 indicated that bovine *NANOS3* is a haplosufficient gene. Although bull #3964 was carrying 3 mutated alleles, including 2 alleles with large (>500 bp) deletions, and no WT alleles, the one in-frame allele (30% of reads) resulted in a functional *NANOS3* protein that was sufficient for male germline development (Figure 3.3C-D and Table 3.6). This finding aligns with the observation that both male and female heterozygous *Nanos3*+/- mice are fertile with morphologically and functionally normal gonads (Tsuda et al., 2003).

The CRISPR/Cas9 *NANOS3* KO Heifer #854 confirmed that *NANOS3* is necessary for female bovine germline development. The ovaries of *Nanos3* KO female mice were greatly reduced in size and had no observable germ cells (Suzuki et al., 2008, Tsuda et al., 2003). Additionally, a *NANOS3* KO heifer at 190 days of fetal age showed a complete loss of germ cells, although there did appear to be a single layer of follicular epithelium-like cells present (Ideta et al., 2016). The authors also reported that the length of the major axis of the *NANOS3* KO ovaries was similar to that of the age-matched, control, WT ovaries (Ideta et al., 2016). Similarly, 1-day-old *NANOS3* KO pig ovaries were reported to have no notable differences in appearance or size compared to age-matched, control, WT ovaries (Kogasaka et al., 2022). Also, at 3-months-old *NANOS3* KO pig ovaries were found to have no primary and secondary follicles, or corpus luteum structures (Wang et al.). At 4-months-old the *NANOS3* KO pig ovaries were noticeably smaller than WT and in contrast to the WT ovaries, displayed no antral follicles and no oocyte-like or DDX4-positive cells were found (Kogasaka et al., 2022). Intriguingly, Kogasaka et al. (2022)

observed follicle-like circular structures but no oocyte-like structures or DDX4-positive cells in 4-month-old *NANOS3* KO pig ovaries, whereas Wang et al. (2023) did not observe any follicle structures in 6-month-old *NANOS3* KO pig ovaries. As expected, the *NANOS3* mosaic KO heifer #854 was germline ablated as evidenced by the lack of oogenesis observed in her H&E-stained ovarian tissue sections (Figure 3.11). However, heifer #854 had an anatomically abnormal reproductive tract (i.e., small clitoris and long anterior vagina), and abnormal gonad development with a putative primitive streak in place of the right ovary (Figure 3.11). Similar to murine and porcine studies, the *NANOS3* KO bovine ovary was much smaller in size than expected for her age. Additionally, no follicle-like circular structures were observed in either the left ovary or right putative primitive streak. Post-pubertal monthly estradiol and progesterone levels in the *NANOS3* KO heifer (#854) were much lower than expected for her age and indicated no active secretion or luteal activity. Although the blood samples were collected monthly and therefore could have missed the peak of an estrous cycle, the lack of observed estrus behavior supports these findings.

During mammalian fetal development, female germ cells start meiosis I and develop primordial follicles with primary oocytes arrested in prophase I at birth. Whereas male germ cells do not start meiosis I until after birth and puberty. Due to the advanced progression of mammalian female germline development during fetal development, it is reasonable to expect that the absence of germ cells in the *NANOS3* KO heifer would lead to more pronounced changes in the reproductive phenotype compared to the *NANOS3* KO bull. This reproductive phenotype would not be suitable for germline complementation unless the germline-competent donor cells can rescue the phenotype early in fetal development. Ideta et al. (2016) successfully generated exogenous primary oocytes and primordial follicles in sterile *NANOS3* KO fetal (141 days) bovine ovaries via blastocyst complementation involving the microinjection of WT donor blastomeres

into a *NANOS3* KO host, providing evidence that this KO phenotype can be rescued. Therefore, it is likely that *NANOS3* KO heifers could serve as hosts for germline complementation strategies. Future germline complementation studies will be required to determine if *NANOS3* KO heifers complemented with germline-competent donor cells can produce follicles and functional oocytes.

Ideal hosts for germline complementation are animals that lack an endogenous germline but provide an intact gonadal niche to support donor-derived gametogenesis. Donor cells that can be used for germline complementation include blastomeres, stem cells (such as embryonic stem cells (ESC), induced pluripotent stem cells (iPSC), and SSC), and PGC-like cells (PGCLCs) (Bishop and Van Eenennaam, 2020, Ledesma et al., 2023, Mueller and Van Eenennaam, 2022, Oback and Cossey, 2023). Blastomeres are totipotent but not self-renewing, whereas stem cells have self-renewal capabilities. SSCs are unipotent cells isolated from testes capable of spermatogenesis. ESCs are derived from the inner cell mass of blastocysts, while iPSCs are reprogrammed somatic cells (Bishop and Van Eenennaam, 2020, Ledesma et al., 2023, Oback and Cossey, 2023). Both ESCs and iPSCs can be induced to become PGCLCs, which in mice have been developed into *in vitro* gametes capable of producing live offspring (Hayashi et al., 2012). Although induction of livestock PGCLCs to produce gametes has not yet been achieved, the availability of livestock ESCs opens up the possibility for this approach (Ledesma et al., 2023).

Germline complementation can be achieved through two strategies: testis complementation and embryo complementation, both resulting in the formation of chimeras whose germ cells are exclusively derived from a single donor genotype (Bishop and Van Eenennaam, 2020, Ledesma et al., 2023, Oback and Cossey, 2023). The timing of germ cell loss in the host and donor cell source determines which complementation strategy can be used. Testis complementation involves injecting donor SSCs or PGCLCs into the gonads of a juvenile or adult host, leading to the

generation of "secondary" chimeras after organogenesis. On the other hand, embryo complementation involves combining donor blastomeres or PSCs with a germline-ablated host during embryonic development, resulting in "primary" chimeras where genetically different cell populations coexist during embryogenesis (Bishop and Van Eenennaam, 2020, Ledesma et al., 2023, Oback and Cossey, 2023). The term "blastocyst complementation" is often used to refer to embryo complementation, although donor cells can be injected at different stages of embryo development depending on the species and goals of complementation (Dechiara et al., 2009). To date, germline complementation to produce live absolute transmitters resulting in the production of live donor-derived offspring has been accomplished in rodents and non-mammalian food animals, including fish and chickens. In chickens, PGCs isolated from embryonic blood or gonadal tissues can be injected into the blood stream of developing embryos, generating germline chimeras (Hu et al., 2022, van de Lavoir et al., 2006). Similarly, transplantation of fish PGCs and gonial cells into sterile hosts at different developmental stages, including blastula, larvae, and adults, has successfully achieved donor gametogenesis in various fish species (Goto and Saito, 2019).

Both testis and embryo complementation, the two strategies for germline complementation in mammalian livestock, present distinct challenges related to donor cell isolation, culture, genetic modification, and integration with the host. Transplantation of SSC into the sterile testes of males has been achieved in *Dazl* KO mice and rats (Richardson et al., 2009, Speed et al., 2003), *Etv5* KO mice (Zhang et al., 2021), *NANOS2* KO mice, boars, bucks, and bulls (Ciccarelli et al., 2020, Latham et al., 2023), *NANOS3* KO boars (Wang et al., 2023), and *Tscd22d3* KO mice (Zhou et al., 2019). While SSC testis complementation was able to restore natural fertility levels in *NANOS2* KO mice, much lower rates of spermatogenesis were observed in boars and bucks (Ciccarelli et al., 2020). Although a key finding was that success was higher when complementation was

performed in juvenile hosts compared to adults. Additionally, Ciccarelli et al. (2020) proposed that simple technical refinements to the injection procedure, such as optimizing the volume of cell suspension transferred, number of donor cells, and route of injection, are likely to generate surrogate males that could be used in a natural breeding scheme (Ciccarelli et al., 2020). Most recently, it was reported that an SSC-transplanted *NANOS2* KO bull produced semen with normal sperm concentration and motility (Latham et al., 2023). Moreover, the donor-derived bull sperm successfully *in vitro* fertilized bovine oocytes resulting in donor-derived embryos, and pregnancies were achieved via natural mating, but to date no live donor-derived offspring have been reported. However, there is currently a lag between SSC transfer and successful sperm production, of ~3 months in mice and ~4 months in boars and bucks, although the timing from SSC transfer to donor-derived sperm production has not yet been reported for bulls (Ciccarelli et al., 2020, Latham et al., 2023, Oback and Cossey, 2023).

Germline complementation via embryo complementation has been achieved in *NANOS3* KO male mice (Miura et al., 2021), a *NANOS3* KO heifer of fetal-age (Ideta et al., 2016), *Prdm14* KO rodents (Kobayashi et al., 2021), and *Tscd22d3* KO mice (Koentgen et al., 2016). When donor mouse ESCs were injected into *nanos3* KO mouse host embryos, and the resulting mice had a 100% donor-derived germline, were fertile, and were able to produce donor-derived offspring via natural mating (Miura et al., 2021). Embryo complementation via microinjection of bovine donor blastomeres donor blastomeres into *NANOS3*-null bovine host morulas resulted in donor-derived primary oocytes in the ovaries of a bovine female fetus (Ideta et al., 2016). A comparison of germline complementation studies indicates that embryo complementation produced a higher proportion of fertile animals transmitting the donor-derived genotype compared to testis complementation (Oback and Cossey, 2023). Additionally, chimeric mice sires do not seem to

differ from regular sires in terms of onset of sexual maturity and fertility. Embryo complementation presents a unique challenge known as sex chimerism, where female donors may unintentionally be combined with male hosts or vice versa, potentially resulting in a hermaphrodite phenotype. However, this concern can be effectively addressed by exclusively combining host and donors of the same sex, either through using sexed sperm or by employing PCR-based sex identification techniques for precise selection of complementation partners.

There are other important considerations for applications of each germline complementation method in livestock. Testicular biopsies required to obtain livestock SSCs and the testis complementation process itself are invasive and require handling of juvenile animals. In contrast, embryo complementation is performed in vitro before ET, thus reducing animal handling. Embryo complementation results in primary chimeras with potentially chimeric somatic components, while testis complementation would result in 100% host-derived somatic components. Testis complementation typically represents cloning of the current generation via SSCs, while embryo complementation usually represents cloning of the next generation, which is important for accelerating rates of genetic gain. Additionally, ESC-based embryo complementation could allow for trait stacking through sequential GnEd and allows for genomic selection of superior lines. Moreover, embryo complementation offers the potential for a diverse founder population as it is more feasible to produce a large number of donor embryos compared to live animals. While germline transmission through ESC-based embryo complementation has yet to be achieved in livestock, the increasing availability and variety of embryonic-derived pluripotent stem cells in livestock species brings this approach closer to realization.

The genes encoding key RNA-binding proteins, *DAZL, NANOS2*, and *NANOS3* have emerged as ideal targets for germline ablation in livestock due to their success in eliminating the

germline while preserving the gonads, simplicity of gene KO strategy, and demonstrated conserved role in livestock (Oback and Cossey, 2023). In contrast, other genes targeted only in rodents, such as *Etv5, Prdm14*, and *Tscd22d3*, have limitations that make them less suitable (Kobayashi et al., 2021, Koentgen et al., 2016, Soto and Ross, 2021, Zhang et al., 2021). *Etv5* impairs Sertoli cell function, *Prdm14* has an unclear conserved function in germline development outside of mice, and *Tscd22d3* requires a complicated conditional KO strategy to maintain the desired genotype.

Specifically, we propose that *NANOS3* is an ideal target for generating germline-ablated hosts in cattle for germline complementation, for two primary reasons. Firstly, *NANOS3* is one of the earliest genes expressed specifically in PGCs. Therefore, its disruption would eliminate PGCs at an early stage compared to other targets like *NANOS2* or *DAZL*, which enables the use of embryo complementation. Secondly, we have demonstrated that *NANOS3* plays an essential role in both male and female germ cell development, making *NANOS3* KO cattle viable hosts for producing donor-derived germ cells in both sexes. This presents an opportunity to expand the availability of gametes from both genetically desirable sires and dams, thus reducing the genetic lag that exists between the seedstock and commercial sectors of the beef industry. Importantly, if the donor line is unedited, the offspring of GnEd surrogate hosts would not carry the edit and would be classified as null-segregants from a regulatory perspective. However, editing of the donor line might be advantageous in some situations, especially in the generation of homozygous GnEd offspring of both sexes which would be of particular importance for the introduction of recessive GnEd traits into a breeding program.

5. LIMITATIONS OF THIS STUDY & FUTURE DIRECTIONS

Although we identified large on-target deletions (>1 kb), that may contribute to the observed phenotypes, it is important to consider the possibility of even larger deletions in our samples. Off-target effects were not investigated in this study as we carefully selected the guides to minimize this risk based on established criteria in previous bovine studies aimed to minimize this risk (Hennig et al., 2020). Nevertheless, with additional expenditures of both time and money, whole genome sequencing could be used to comprehensively assess for the presence or absence of other deletions and/or off-target edits, if warranted by the identification of a reasonable path to harm resulting from such genetic variations.

Similarly, while we successfully demonstrated the elimination of *NANOS3* at the DNA and RNA levels, the lack of a commercially available and validated NANOS3 antibody specific to bovine samples hindered the direct confirmation of *NANOS3* protein elimination in the KO samples and this study did not provide evidence for the presence of *NANOS3*-positive germ cells in the fertile edited bull. However, given that *NANOS3* was only found to be expressed in a small proportion (≤ 2%) of 90d PGCs and 283d gonocytes, we would likely not see positive staining for *NANOS3* at these timepoints. In contrast, we would expect positive NANOS3 staining in the control 41d gonadal samples and potentially in the undifferentiated spermatogonia of the control and edited (#3964) 15mo testes. . Efforts to develop or validate a specific NANOS3 antibody for bovine samples would significantly contribute to our understanding of bovine germline development and the effects of *NANOS3* disruption. Furthermore, to strengthen the evidence of seminiferous cord structures and Sertoli cell presence in the *NANOS3* KO males immunohistochemistry using established markers for Sertoli cells, Leydig cells, and PTM cells could be used. This analysis would provide a more comprehensive and definitive validation of the

presence and functionality of these specific cell types and structures in the absence of *NANOS3*. However, it is worth noting that the hormone assays conducted in this study do provide indirect evidence of endocrinologically functional cells in the *NANOS3* KO and edited bulls.

Additionally, this study did not analyze hormone levels during fetal development, which limits our understanding of potential impacts on germline development. Investigating hormone profiles during fetal development would provide a more comprehensive picture of the effects of *NANOS3* disruption, particularly in female animals where germline development progresses further during this stage compared to males. Additionally, the absence of a female control for hormone assays in this study hinders a direct comparison of hormone profiles with the *NANOS3* KO heifer. Moreover, hormone sampling in this study was conducted on a monthly basis, which may have missed the peaks of pulsatile hormones, such as testosterone and female cyclical hormones. Although the monthly hormone sampling in this study may have limitations, it is important to highlight that these samples were crucial in demonstrating the endocrine function in *NANOS3* KO bulls, which had not been previously investigated in mice or livestock.

Furthermore, since the evaluation in this study began at 41 days, which falls after the expected completion of PGC migration, future studies should delve into the mechanism and timing of fetal germ cell loss. Employing techniques such as tunnel assays for apoptosis would provide valuable insights into the fate of PGCs, shedding light on the number of PGCs that reach the genital ridge before undergoing cell death. To further elucidate the role of *NANOS3* in cattle, future studies could focus on targeting specific mutations in the N-terminal and zinc finger domains of the protein. This approach would help determine if either or both of these domains have essential roles, similar to what has been observed in mice, and provide insights into the functional importance of specific regions of the NANOS3 protein.

Ultimately, to fully understand the potential applications and suitability of *NANOS3* KO cattle, germline complementation experiments should be conducted. Comparisons can be made between testis complementation in juvenile bulls and embryo complementation to assess the success of restoring gametogenesis in the sterile host animals. Such studies would provide valuable information on the functionality and fertility potential of *NANOS3* KO animals and their suitability as hosts for germline complementation and transmission.

6. CONCLUSIONS

In conclusion, our study demonstrates that the absence of *NANOS3* in cattle leads to the specific deficiency of both male and female germ cells. The elimination of germ cells in *NANOS3* KO testes as early as 41 days of fetal age suggests a conserved role of *NANOS3* in promoting bovine PGC survival, similar to its function in mice. Notably, we also provide evidence supporting the haplosufficiency of bovine *NANOS3*. Importantly, we demonstrate that despite the lack of germ cells, seminiferous tubule development was not impaired in *NANOS3* KO bovine testes during fetal, perinatal, and adult stages. Furthermore, the live *NANOS3* KO bull exhibited normal reproductive development and pre-pubertal hormone levels despite the absence of germ cells. These findings highlight the potential of *NANOS3* KO bulls as hosts in germline complementation strategies. In addition, our successful production of a live, germline ablated, *NANOS3* KO, heifer combined with the successful generation of a blastocyst complemented *NANOS3* KO bovine female fetus (Ideta et al., 2016), support the potential for *NANOS3* KO heifers to also serve as hosts in germline complementation strategies. Therefore, *NANOS3* KO cattle could be hosts for donor-derived exogenous germ cell production in both sexes, which could provide an opportunity to expand the availability of gametes from both genetically desirable sires and dams, potentially enabling the efficient generation of absolute transmitters of homozygous GnEd gametes of both

sexes. Overall, our findings contribute to the understanding of *NANOS3* function in cattle and have valuable implications for the development of novel breeding technologies using germline complementation.

7. ADDITIONAL INFORMATION

7.1. Acknowledgements

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