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NANOG is required to form the epiblast and maintain pluripotency in the bovine embryo

M. Sofia Ortega^{1,*}, Andrew M. Kelleher^{1,2}, Eleanore O'Neil¹, Joshua Benne¹, Raissa Cecil¹, Thomas E. Spencer¹

¹Division of Animal Sciences, University of Missouri, Columbia, MO, 65211

²Current Address: University of Texas Southwestern Medical Center, Dallas, TX, 75390

Abstract

During preimplantation development, the embryo undergoes two consecutive lineage specifications. The first cell fate decision determines which cells give rise to the trophectoderm and the inner cell mass. Subsequently, the inner cell mass differentiates into hypoblast and epiblast, the latter giving rise to the embryo proper. The transcription factors that govern these cell fate decisions have been extensively studied in the mouse, but are still poorly understood in other mammalian species. In the present study, the role of *NANOG* in the formation of the epiblast and maintenance of pluripotency in the bovine embryo was investigated. Using a CRISPR-Cas9 approach, guide-RNAs were designed to target exon 2, resulting in a functional deletion of bovine *NANOG* at the zygote stage. Disruption of *NANOG* resulted in the embryos that form a blastocoel and an inner cell mass composed of hypoblast cells. Furthermore, *NANOG*-null embryos showed lower expression of epiblast cell markers *SOX2* and *HA2AFZ*, and hypoblast marker *GATA6*, without affecting the expression of trophectoderm markers *CDX2* and *KRT8*. Results indicate that *NANOG* has no apparent role in segregation or maintenance of the trophectoderm, but it is required to derive and maintain the pluripotent epiblast and during the second lineage commitment in the bovine embryo.

Keywords

Preimplantation development; pluripotency; transcription factors

Introduction

During mammalian preimplantation development, the embryo undergoes two consecutive lineage commitments. First, the cells differentiate into the trophectoderm (TE) or the pluripotent inner cell mass (ICM), which give rise to the placental tissues, and the embryo proper, respectively (Betteridge & Fléchon, 1988; Chen, Wang, Wu, Ma, & Daley, 2010). A second lineage specification occurs at the blastocyst stage, the ICM will differentiate into

*Correspondence: Division of Animal Sciences, University of Missouri, 920 East Campus Drive, Columbia, MO, 65211, USA. ortegaobandom@missouri.edu.

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the hypoblast (ruminants) or primitive endoderm (mice), which give rise to the yolk sac (Artus & Chazaud, 2014; Chen et al., 2010; Galdos-Riveros, Favaron, Will, Miglino, & Maria, 2015), and the epiblast, which remains pluripotent and will constitute the embryo proper (Artus & Chazaud, 2014; Betteridge & Fléchon, 1988; Chazaud & Yamanaka, 2016).

Spatio-temporal expression of several transcription factors can be used to differentiate the TE from the ICM. Common markers of the TE markers include caudal type homeobox 2 (*Cdx2*), Gata binding protein 3 (*Gata3*), and TEA domain family member 4 (*Tea4*) (Kuijk et al., 2008; Strumpf et al., 2005), whereas pluripotency markers for the ICM include the POU Domain, Class 5, Transcription Factor 1 (*Pou5f1*), Homeobox Transcription Factor Nanog (*Nanog*), and SRY (sex determining region Y)-box2 (*Sox2*) (Cao, 2013; Chazaud & Yamanaka, 2016). Of note, in the mouse *Pou5f1* and *Cdx2* antagonize each other's expression, resulting in loss of *Cdx2* expression in the ICM, and segregation of the TE lineage as a consequence (Niwa et al., 2005; Strumpf et al., 2005). Interestingly, *Pou5f1*-null mouse embryos establish a CDX2 positive TE and ICM. However, this ICM cannot maintain pluripotency and complete the second lineage differentiation. This data indicates that *Pou5f1* is not essential for the initiation of pluripotency but critical for embryonic development (Frum et al., 2013; Wu & Schöler, 2014).

In contrast, *POU5F1* in the bovine and porcine embryos is expressed in both TE and ICM suggesting a difference in mechanism of the earliest lineage segregation between species (Kuijk et al., 2008; Berg et al., 2011; Sakurai et al., 2016). When *POU5F1* is deleted, *CDX2* is detected only in the TE cells confirming that *POU5F1* does not regulate *CDX2* in the bovine embryo cell fate decisions (Simmet et al., 2018). It is clear that the basic mechanisms regulating the first lineage commitment are different in the mouse and the bovine, but the mechanisms required to maintain pluripotency may be conserved. *NANOG*, a pluripotency marker that appears during the second lineage commitment (Strumpf et al., 2005; Wicklow et al., 2014), is absent in *POU5F1*-null bovine embryos, suggesting that *POU5F1* is required to maintain pluripotency in both murine and bovine embryos (Frum et al., 2013; Simmet et al., 2018; Wu & Schöler, 2014). During the second lineage differentiation, *Nanog* and *Gata6* have been proposed as the defining transcription factors controlling differentiation of the epiblast and primitive endoderm in the mouse. *Gata6* regulates primitive endoderm genes while antagonizing *Nanog* in the blastocyst (Koutsourakis, Langeveld, Patient, Beddington, & Grosveld, 1999; Chazaud, Yamanaka, Pawson, & Rossant, 2006; Keramari et al., 2010). Mouse embryos that lack *Nanog* are able to establish and maintain TE lineage and form a primitive endoderm, but fail to form an epiblast (Mitsui et al., 2003). Here, a genetic approach was utilized to establish the role of *NANOG* in the formation of the epiblast as well as the maintenance of pluripotency in the bovine preimplantation embryo.

Material and methods

Design and Construction of CRISPR/Cas9 Guide RNAs

The coding region of *NANOG* consists of 4 exons with the homeodomain located in Exon 2 and Exon 3. Therefore, guide RNAs (gRNAs) were designed to target the region of Exon 2 before the homeodomain of the gene (Figure 1), using the GPP portal from the Broad Institute (<https://portals.broadinstitute.org/gpp/public/analysis-tools/sgRNA-design>). To select

gRNAs, two criteria were used: (1) those with the highest on-target score after design; and (2) those having the least similarity with other bovine sequences containing a protospacer adjacent motif (PAM). Three gRNAs with the fewest predicted off-targets were selected for further testing. The selected gRNAs ordered within a gBlock® Gene Fragment (IDT technologies, San Jose, CA, USA) containing a promoter region for T7 RNA polymerase (Brooks, Burns, & Spencer, 2015).

Guide RNAs were *in vitro* transcribed using the MEGAshortscript T7 Transcription kit (Thermo Fisher Scientific, Waltham, MA, USA) and isolated using the MEGAclear Transcription Clean-Up kit (ThermoFisher). Prior to zygote injection, the gRNAs were combined to a final concentration of 50 ng/μl gRNAs and 20 ng/μl Cas9 mRNA (IDT technologies). Selected gRNAs were tested by simultaneously injecting 2 guides (expected cut) into bovine zygotes: gRNAs 1+3 (~88 bp cut), gRNAs 1+2 (225 bp cut), and gRNAs 2+3 (~140 bp cut).

Production of embryos in vitro and zygote microinjection

Embryos were produced in vitro with a single sire known to be of high fertility. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Oocyte washing medium consisted of Tissue Culture Medium-199 with Hanks salts and 25 mM HEPES. Oocyte maturation medium consisted of Tissue Culture Medium-199 with Earle salts (Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.2 mM sodium pyruvate, 2 mM L-glutamine, 50 ng/ml recombinant human epidermal growth factor (Thermo Fisher Scientific), and 5.0 μg/ml of follicle-stimulating hormone (Folltropin; Bioniche Animal Health, Athens, GA, USA). Fertilization media consisting of Tyrode's albumin lactate pyruvate (TALP) solutions including HEPES-TALP, and IVF-TALP and culture medium were prepared as described (Ortega et al., 2017).

Cumulus-oocyte complexes (COC) were retrieved by follicular aspiration of ovaries collected at a commercial abattoir (DeSoto Biosciences, Seymour, TN, USA). COC with at least three layers of compact cumulus cells and homogeneous cytoplasm were placed in groups of 50 COC into 2 ml glass sterile vials containing 1 ml of oocyte maturation medium equilibrated with air containing 5% (v/v) CO₂ covered with mineral oil. Tubes with COC were shipped overnight in a portable incubator (Minitube USA Inc., Verona, WI, USA) at 38.5°C to the University of Missouri. After 20-22 h of maturation, up to 200 COC were washed 3 times in HEPES-TALP medium and placed in a 35-mm dish containing 1.7 ml of IVF-TALP. Sperm were purified from frozen-thawed straws using a gradient [50% (v/v) and 90% (v/v)] of Isolate (Irvine Scientific, Santa Ana, CA), washed two times by centrifugation at 100 × g using HEPES-TALP and diluted in IVF-TALP to achieve a final concentration of 1 × 10⁶/ml in the fertilization dish. In addition, 80 μl of penicillamine-hypotaurine-epinephrine solution was added to each fertilization dish to improve sperm motility and promote fertilization. Fertilization proceeded for 17 to 19 h at 38.5°C in a humidified atmosphere of 5% (v/v) CO₂ (Ortega et al., 2018, 2017).

At the end of fertilization, putative zygotes (oocytes exposed to sperm) were denuded from the surrounding cumulus cells at the end of fertilization by vortexing for 5 min in 400 μl of

HEPES-TALP. Zygotes were split in three groups: non-injected, injected only with CAS9 mRNA, and injected with gRNA/CAS9 mRNA against *NANOG*. Putative zygotes to be injected were placed in manipulation medium consisting of Tissue Culture Medium-199 with Hanks salts plus 0.595 mM NaHCO₃, 3.160 mM HEPES, 30.03 mM NaCl, 3 mg/ml bovine serum albumin, 0.05 mg/ml penicillin, 0.06 mg/ml streptomycin, and 7 µg/ml cytochalasin B. After injection, zygotes were cultured in four-well dishes in groups of up to 50 zygotes in 500 µl of SOF-BE2 (Ortega et al., 2017), covered with 300 µl of mineral oil per well at 38.5°C in a humidified atmosphere of 5% (v/v) O₂ and 5% (v/v) CO₂. Percentage of putative zygotes that cleaved was determined at day 3 of development (day 0 = day of insemination) and blastocyst rate at day 8 of development.

Embryo collection and genotyping

To determine if embryos were effectively edited, blastocysts from four different in vitro embryo production procedures (n=255) were collected individually at day 8 of culture, and kept at -20 °C until genotyped. Embryos were processed by adding 6 µL of Embryo Lysis Buffer (ELB) [40 mM Tris at pH 8.9, 0.9% (v/v) Triton X-100, 0.9% (v/v) Non-Idet P-40 (NP40), and 32 Units/ml of Proteinase K]. Embryos in ELB were subjected to 30 m at 60°C, followed by 10 m at 85 °C in an Eppendorf Mastercycler @ Nexus (Eppendorf, Hauppauge, NY, USA). Lysed embryo solution was used as template DNA. For genotyping, the region surrounding the target sequenced of the gRNAs was amplified by endpoint PCR using the primer forward- 5' TCCCAACAGTCTCTCCTCTT 3', and reverse- 5' CGATTCTTGGTCAGGGAAGTAG 3', resulting in a 398 bp product. Each PCR reaction consisted of 1 µL of DNA sample, 2 µL of 10X PCR buffer, 1.6 µL of dNTPs mix (0.4 mM each dNTP), 0.4 µL of each primer (10 µM primer solution), 0.25 µL of Hot-Start Taq-polymerase (Takara Bio, Mountain view, CA, USA), and 14.35 µL of nuclease free water for a total volume reaction of 20 µL. Amplification conditions were: 95°C for 2 m, 40 cycles at 95°C for 10 sec, 55.2°C for 30 sec, 72°C for 1 m, followed by 72°C for 10 m. PCR product (398 bp) was separated and visualized in a 1.5% (w/v) agarose gel.

Amplified PCR product from samples with biallelic edits, and samples with no apparent edits (WT) in the agarose were cloned into a plasmid vector using the TOPO®TA cloning kit (Thermo Fisher Scientific) following manufacturer instructions. The resulting reaction was then transformed into DH5α competent cells (Thermo Fisher Scientific) following manufacturer instructions. Transformed cells were plated in a Lennox-broth agar culture plate and incubated overnight at 37 °C. After incubation, 8 individual colonies were collected, and genotyped as described above. PCR product was then cleaned up using the Qiagen PCR purification kit and sent for Sanger sequencing.

Immunolocalization of GATA6 and NANOG

Embryos were subjected to immunolocalization of GATA6 and NANOG. Solutions used included: blocking buffer [PBS with 5% (w/v) BSA fraction V], antibody dilution buffer [PBS with 0.1% (v/v) Tween20, and 1% (w/v) BSA], wash buffer [PBS with 0.05% (v/v) Tween20], and permeabilization buffer [PBS with 0.5% (v/v) Triton X-100]. Briefly, embryos were collected at day 8 washed three times in cold PBS containing 0.2% (w/v) polyvinylpyrrolidone and fixed in 4% (w/v) paraformaldehyde in PBS for 20 min. Following

fixation, embryos were permeabilized for 30 min and subsequently incubated in blocking buffer for 1 h at room temperature. Embryos were next co-incubated overnight at 4°C with rabbit anti-human monoclonal GATA6 antibody (Cell Signaling Technology, Danvers, MA, USA), and mouse anti-human monoclonal NANOG antibody (Thermo Fisher Scientific, Waltham, MA, USA) at 0.5 µg/ml and 1 µg/ml concentrations in antibody dilution buffer, respectively. Embryos were then washed 6 times in wash buffer and incubated 1 h at room temperature in the dark with 1 µg/ml of goat anti-mouse IgG conjugated to Alexa Fluor 555, and 1 µg/ml goat anti-rabbit IgG conjugated to Alexa Fluor 488 (Thermo Fisher Scientific) in antibody dilution buffer. Embryos were washed 6 times in wash buffer followed by nuclear labeling with Hoechst 33342 (1 µg/ml in ddH₂O) for 10 min in the dark. Finally, embryos were rinsed three times in ddH₂O and placed on a slide containing 1 drop of SlowFade Gold antifade reagent (Thermo Fisher Scientific), covered with a coverslip, and imaged using a Leica DM5500 epifluorescence microscope. Analysis of the images was performed using ImageJ V. 1.6 (National Institutes of Health, Bethesda, MD).

Embryo cell lineage markers qPCR

To assess mRNA of markers for the different cell lineages in the embryo, pre-validated primers were purchased from BioRad (Hercules, CA, USA) for *NANOG* (qBtaCE D0009621), *SOX2* (qBtaCE D0011417), *GATA6* (qBtaC1 D0017221), *H2AFZ* (qBtaCE D0009884), *HNF4A* (qBtaC1 D0005555), *KRT8* (qBtaCE D0014411). In addition, primers previously validated in bovine for *POU5F1* (M. Ozawa et al., 2012), *CDX2* (Manabu Ozawa & Hansen, 2011), *GATA6* (M. Ozawa et al., 2012), *GATA2* and *GATA3* (Bai et al., 2011), the housekeeping genes *GAPDH* and *SDHA* (Kannampuzha-Francis, Tribulo, & Hansen, 2016), were bought from IDT biotechnologies.

Briefly, day 8 individual embryos that formed a blastocoel were washed three times in HEPES-TALP, and placed in micro drops of approximately 50 µL of HEPES-TALP for biopsy. A portion of the trophectoderm was cut using a needle blade (Fine Sciences Tools, Foster City, CA, USA) following procedures explained by Bredbacka et al (1995). The biopsy was collected in a microcentrifuge tube and frozen at -20 °C for genotyping. Through the physical force applied during the biopsy procedure, the embryo was expelled from the zona pellucida; the remaining embryo was only washed three times in cold sterile PBS-PVP, placed in a microcentrifuge tube, snap-frozen in liquid nitrogen, and stored at -80 °C for gene expression analysis. This procedure was also performed for Cas-9 injected embryos as controls.

Biopsies were genotyped as mentioned before. Only embryos biopsies showing biallelic edits in an agarose gel (considered *NANOG*-null) were further used. After genotyping, embryos were pooled in groups of 4, and a total of 6 pools of WT embryos and 6 pools of biallelic edited were used for analysis. Gene expression analysis was performed by Real-time PCR in a CFX384 Touch Real Time System (Bio-Rad) using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Each sample was run in duplicate in 10 µL reactions each one consisting of 0.5 µL of 10 µM primers, 5 µL of supermix, 2.5 µL of nuclease-free water, and 2.0 µL of cDNA. PCR conditions were: activation, 95°C for 2 min; 40 cycles of 95°C for 5 seconds; 60°C for 30 seconds; and 72 for 30 sec. At the end of

amplification, a melt curve was created to assess whether a single product was amplified.

CT was estimated as the difference between the cycle threshold (CT) for the gene of interest and geometric mean of the CT for the reference genes. For visualization fold change was calculated relative to the reference genes (2^{-CT}). Gene expression was analyzed by ANOVA using the GLM procedure of SAS.

Results

Guide selection and editing efficiency

The editing efficiency of three combination of gRNA were evaluated. Of the three combinations of gRNAs tested, gRNAs 1+3 edited embryos consistently, gRNAs 1+2 did not edit, and gRNAs 2+3 edited at a very low efficiency (Figure 1). Thus, the experiments carried out in the remainder of the studies utilized only the combination of gRNAs 1+3. The total editing efficiency from full blastocysts and biopsies genotyped (n=255) is shown in Figure 2. More than 30% of the embryos were either bi-allelic edited or had small deletions that resulted in stop codons based on Sanger Sequencing. As expected, there were no edits in embryos injected only with the *Cas9* mRNA.

Embryonic development

Zygotes injected either with gRNA+ *Cas9* mRNA, or just with *Cas9* mRNA had lower ($P = 0.04$) cleavage rate than non-injected embryos (Table 1). There was no difference ($P = 0.23$) in the percentage of cleaved embryos that reached the blastocyst stage among non-injected embryos, embryos only injected with *Cas9* mRNA, or embryos injected with gRNA against *NANOG* and *Cas9* mRNA. However, embryos injected gRNA against *NANOG* and *Cas9* mRNA ha significantly lower blastocyst rate than their non-injected counterparts ($P < 0.05$).

Immunolocalization of Nanog and Gata6

At day 8 of culture, blastocysts were collected for immunolocalization of NANOG and GATA6. In *Cas9* injected (n=30), the inner cell mass was well defined and contained NANOG and GATA6 positive cells. Embryos that were injected with *CAS9* mRNA and gRNAs against *NANOG* (n=40) did not maintain a defined inner cell mass, and, as expected, *NANOG* was completely absent in the embryo. *NANOG*-null embryos were in general smaller than the control embryos and formed poorly defined ICM composed by all *GATA6* positive cells indicating complete differentiation into hypoblast (Figure 3).

Embryo cell lineage markers

There was little to no expression ($P = 0.003$) of *NANOG* in *NANOG*-null embryos compared to WT embryos. This result indicates that the biallelic edit blocked *NANOG* at the level of transcription. In the same regard, expression of epiblast markers *SOX2* and *H2AFZ* was lower ($P < 0.005$) in *NANOG*-null embryos compared to control (Figure 4). *POU5F1* expression was not different ($P = 0.10$) between groups. For hypoblast markers, *GATA6* showed lower expression ($P = 0.03$), while *HNF4A* tended ($P = 0.053$) to have lower expression in *NANOG*-null embryos compared to WT embryos. Trophectoderm markers *CDX2* and *KRT8* were not different ($P > 0.10$), *GATA3* tended to be lower ($P = 0.06$), and *GATA2* was lower ($P = 0.03$) in *NANOG*-null compared to control embryos.

Discussion

Preimplantation embryonic development involves a series of orchestrated events that control the balance between pluripotency and differentiation (Loh et al., 2006; Wolf, Serup, & Hyttel, 2011; Fogarty et al., 2017). In the mouse, *Nanog* along with *Pou5f1* and *Sox2* are key players in the pluripotency cascade and are necessary for the formation of the ICM in the preimplantation embryo, as well as self-renewal of pluripotent embryonic stem cells in vitro (Mitsui et al., 2003; Loh et al., 2006; Olariu, Lökvist, & Sneppen, 2016; Fogarty et al., 2017).

Here, deletion of *NANOG* in the bovine zygote resulted in defects in the formation of the epiblast and caused downregulation of genes normally enriched in the ICM in the bovine embryo (Negrón-Pérez, Zhang, & Hansen, 2017; Manabu Ozawa et al., 2012). In cattle, *NANOG* is detected in the bovine embryo beginning at 8-cell stage, before establishment of polarization and compaction of the blastomeres (Pfeffer, 2018) and becomes restricted to a subpopulation of the ICM at the blastocyst stage (Goissis & Cibelli, 2014; Graf et al., 2014). Interestingly, deletion of *NANOG* at the zygote stage does not impair initial compaction and formation of the blastocoel (Figure 3, Figure 5), indicating that the establishment of the ICM does not require *NANOG* expression. This is in agreement with previous findings in the mouse in which the ICM of *Nanog*-null embryos only differentiate into extraembryonic endoderm lineage (Artus & Chazaud, 2014; Loh et al., 2006).

Recently, Simmet et al (2018) showed that in the bovine, deletion of *POU5F1* does not affect first lineage differentiation, but is necessary for pluripotency maintenance and *NANOG* expression. In this study, *POU5F1* expression was not significantly reduced, which is expected as *POU5F1* is expressed in all cell lineages in the bovine embryo (Berg et al., 2011; Simmet et al., 2018). The third transcription factor involved in the formation of the ICM in the mouse is *Sox2* (Loh et al., 2006; Keramari et al., 2010; Wicklow et al., 2014). In the present study *SOX2* expression was significantly reduced in *NANOG*-null embryos. That result is in agreement previous reports where knockdown of *SOX2* in the bovine embryo led to the formation of a blastocyst with reduced expression of *NANOG*, suggesting a mutual regulation of *SOX2* and *NANOG* in the bovine embryo (Rodda et al., 2005; Keramari et al., 2010; Goissis & Cibelli, 2014; Mistri et al., 2018).

Furthermore, *H2AFZ* which is enriched in the bovine epiblast (Negrón-Pérez et al., 2017; Manabu Ozawa et al., 2012), was reduced in both *NANOG*-null in this study, and in *POU5F1*-null embryos (Simmet et al., 2018). In the mouse, embryos lacking *H2afz* form abnormal ICM and have defects shortly after implantation (Faast et al., 2001). Likewise, *HNF4A*, a downstream gene for the FGF signaling pathway in human pluripotent stem cells (Twaroski et al., 2015), and enriched in the epiblast and hypoblast in the bovine embryo (Negrón-Pérez et al., 2017) was also reduced in the *NANOG*-null embryos providing further evidence that deletion of *NANOG* impairs pluripotency maintenance in the bovine embryo. *NANOG*-null embryos formed blastocysts with a poorly defined ICM composed only by hypoblast lineage (Kuijk et al., 2012). Interestingly, hypoblast marker *GATA6* was downregulated in *NANOG*-null embryos, further research is required to understand the role of *NANOG* in hypoblast formation. As expected, there was no difference in expression of

TE markers such as *CDX2* and *KRT8*, as differentiation of TE lineage has proven not to be severely affected by deletion of pluripotency markers such as *POU5F1* and knockdown of *SOX2* (Goissis & Cibelli, 2014; Simmet et al., 2018).

Interestingly, transcription factors *Gata2* and *Gata3*, which are expressed in trophoblast cells in both mouse and human (Ng, George, Engel, & Linzer, 1994; Ma & Linzer, 2000) and that are involved in the regulation of trophoblast-specific gene expression in bovine CT-1 cells (Bai et al., 2011), were reduced in *NANOG*-null embryos. In the human, when *NANOG* expression is absent *GATA2* is also downregulated (Fogarty et al., 2017). In the mouse, trophoblast populations are derived from different regions of trophectoderm. The polar trophectoderm, which is adjacent to the ICM gives rise to the proliferating trophoblast and subsequently secondary giant cells. Those produce essential secretory hormones including placental lactogen I and proliferin, and which require *Gata2* and *Gata3* to be expressed (Lee, Talamantes, Wilder, Linzer, & Nathans, 1988; Faria, Ogren, Talamantes, Linzer, & Soares, 1991; Ng et al., 1994; Ma & Linzer, 2000). It is plausible then, that the lower expression of *GATA2* and *GATA3* indicate reduced potential of the TE cells in *NANOG*-null embryos for later proliferation and differentiation.

Taken together, our results indicate that deletion of *NANOG* in the bovine embryo, does not impair compaction and formation of the blastocoel, but impairs formation of a proper epiblast in the bovine embryo. As in the mouse and the human, maintenance of pluripotency in the bovine embryo seems to require *POU5F1*, *NANOG* and *SOX2*. Further research is required to elucidate the molecular mechanisms orchestrating pluripotency during early stages (before compaction) in the bovine embryo, and their possible implications on stem cell biology.

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References

- Artus J, & Chazaud C (2014). A close look at the mammalian blastocyst: Epiblast and primitive endoderm formation. *Cellular and Molecular Life Sciences*, 71(17), 3327–3338. 10.1007/s00018-014-1630-3 [PubMed: 24794628]
- Bai H, Sakurai T, Someya Y, Konno T, Ideta A, Aoyagi Y, & Imakawa K (2011). Regulation of trophoblast-specific factors by *GATA2* and *GATA3* in bovine trophoblast CT-1 Cells. *Journal of Reproduction and Development*, 57(4), 518–525. 10.1262/jrd.10-186K [PubMed: 21606631]
- Berg DK, Smith CS, Pearton DJ, Wells DN, Broadhurst R, Donnison M, & Pfeffer PL (2011). Trophectoderm lineage determination in cattle. *Developmental Cell*, 20(2), 244–255. 10.1016/j.devcel.2011.01.003 [PubMed: 21316591]
- Betteridge KJ, & Fléchon J (1988). The anatomy and physiology of the pre-attachment bovine embryos. *Theriogenology*, (29), 155–187.
- Bredbacka P, Kankaanpää A, & Peippo J (1995). PCR-sexing of bovine embryos: A simplified protocol. *Theriogenology*, 44(2), 167–176. 10.1016/0093-691X(95)00166-6 [PubMed: 16727716]

- Brooks K, Burns G, & Spencer TE (2015). Biological roles of hydroxysteroid (11-Beta) dehydrogenase 1 (*HSD11B1*), *HSD11B2*, and glucocorticoid receptor (NR3C1) in sheep conceptus elongation. *Biology of Reproduction*, 93(2). 10.1095/biolreprod.115.130757
- Cao Y (2013). Regulation of germ layer formation by pluripotency factors during embryogenesis. *Cell & Bioscience*, 3, 15 10.1186/2045-3701-3-15 [PubMed: 23497659]
- Chazaud C, & Yamanaka Y (2016). Lineage specification in the mouse preimplantation embryo. *Development*, 143(7), 1063–1074. 10.1242/dev.128314 [PubMed: 27048685]
- Chazaud C, Yamanaka Y, Pawson T, & Rossant J (2006). Early lineage segregation between epiblast and primitive endoderm in mouse blastocysts through the Grb2-MAPK Pathway. *Developmental Cell*, 10(5), 615–624. 10.1016/j.devcel.2006.02.020 [PubMed: 16678776]
- Chen L, Wang D, Wu Z, Ma L, & Daley GQ (2010). Molecular basis of the first cell fate determination in mouse embryogenesis. *Cell Research*, 20(9), 982–993. 10.1038/cr.2010.106 [PubMed: 20628366]
- Faast R, Thonglairoam V, Schulz TC, Beall J, Wells JRE, Taylor H, Matthaai K, Rathjen PD, Tremethick DJ, Lyons I (2001). Histone variant H2A.Z is required for early mammalian development. *Current Biology*, 11(15), 1183–1187. 10.1016/S0960-9822(01)00329-3 [PubMed: 11516949]
- Faria TN, Ogren L, Talamantes F, Linzer DIH, & Soares MJ (1991). Localization of placental lactogen-I in trophoblast giant cells of the mouse placenta. *Biology of Reproduction*, 44(2), 327–331. 10.1095/biolreprod44.2.327 [PubMed: 2009333]
- Fogarty NME, McCarthy A, Snijders KE, Powell BE, Kubikova N, Blakeley P, ... Niakan KK (2017). Genome editing reveals a role for *OCT4* in human embryogenesis. *Nature*, 550(7674), 67–73. 10.1038/nature24033 [PubMed: 28953884]
- Frum T, Halbisen MA, Wang C, Amiri H, Robson P, & Ralston A (2013). Oct4 cell-autonomously promotes primitive endoderm development in the mouse blastocyst. *Developmental Cell*, 25(6), 610–622. 10.1016/j.devcel.2013.05.004 [PubMed: 23747191]
- Galdos-Riveros AC, Favaron PO, Will S. E. a. L., Miglino MA, & Maria DA (2015). Bovine yolk sac: From morphology to metabolomic and proteomic profiles. *Genetics and Molecular Research: GMR*, 14(2), 6223–6238. 10.4238/2015.June.9.8 [PubMed: 26125823]
- Goissis MD, & Cibelli JB (2014). Functional Characterization of *SOX2* in Bovine Preimplantation Embryos1. *Biology of Reproduction*, 90(2). 10.1095/biolreprod.113.111526
- Graf A, Krebs S, Zakhartchenko V, Schwalb B, Blum H, & Wolf E (2014). Fine mapping of genome activation in bovine embryos by RNA sequencing. *Proceedings of the National Academy of Sciences*, 111(11), 4139–4144. 10.1073/pnas.1321569111
- Kannampuzha-Francis J, Tribulo P, & Hansen PJ (2016). Actions of activin A, connective tissue growth factor, hepatocyte growth factor and teratocarcinoma-derived growth factor 1 on the development of the bovine preimplantation embryo. *Reproduction, Fertility, and Development*. 10.1071/RD16033
- Keramari M, Razavi J, Ingman KA, Patsch C, Edenhofer F, Ward CM, & Kimber SJ (2010). *Sox2* is essential for formation of trophectoderm in the preimplantation embryo. *PLoS ONE*, 5(11), e13952 10.1371/journal.pone.0013952 [PubMed: 21103067]
- Koutsourakis M, Langeveld A, Patient R, Beddington R, & Grosveld F (1999). *GATA6* is essential for early development. *PLoS ONE*, 5(11), e13952.
- Kuijk EW, Puy LD, Tol HTAV, Oei CHY, Haagsman HP, Colenbrander B, & Roelen BAJ (2008). Differences in early lineage segregation between mammals. *Developmental Dynamics*, 237(4), 918–927. 10.1002/dvdy.21480 [PubMed: 18330925]
- Kuijk EW, van Tol LTA, de Velde HV, Wubbolts R, Welling M, Geijsen N, & Roelen BAJ (2012). The roles of FGF and MAP kinase signaling in the segregation of the epiblast and hypoblast cell lineages in bovine and human embryos. *Development*, 139(5), 871–882. 10.1242/dev.071688 [PubMed: 22278923]
- Lee S-J, Talamantes F, Wilder E, Linzer DIH, & Nathans D (1988). Trophoblastic giant cells of the mouse placenta as the site of proliferin synthesis. *Endocrinology*, 122(5), 1761–1768. 10.1210/endo-122-5-1761 [PubMed: 3359962]

- Loh Y-H, Wu Q, Chew J-L, Vega VB, Zhang W, Chen X, ... Ng H-H (2006). The *Oct4* and *Nanog* transcription network regulates pluripotency in mouse embryonic stem cells. *Nature Genetics*, 38(4), 431. 10.1038/ng1760 [PubMed: 16518401]
- Ma GT, & Linzer DIH (2000). GATA-2 restricts prolactin-like protein A expression to secondary trophoblast giant cells in the mouse. *Biology of Reproduction*, 63(2), 570–574. 10.1095/biolreprod63.2.570 [PubMed: 10906066]
- Mistri TK, Arindrarto W, Ng WP, Wang C, Lim LH, Sun L, ... Robson P (2018). Dynamic changes in *Sox2* spatio-temporal expression promote the second cell fate decision through Fgf4/Fgfr2 signaling in preimplantation mouse embryos. *Biochemical Journal*, BCJ20170418. 10.1042/BCJ20170418
- Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, ... Yamanaka S (2003). The homeoprotein *Nanog* is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell*, 113(5), 631–642. 10.1016/S0092-8674(03)00393-3 [PubMed: 12787504]
- Negrón-Pérez VM, Zhang Y, & Hansen PJ (2017). Single-cell gene expression of the bovine blastocyst. *Reproduction*, REP-17-0345. 10.1530/REP-17-0345
- Ng YK, George KM, Engel JD, & Linzer DI (1994). GATA factor activity is required for the trophoblast-specific transcriptional regulation of the mouse placental lactogen I gene. *Development*, 120(11), 3257–3266. [PubMed: 7720565]
- Niwa H, Toyooka Y, Shimosato D, Strumpf D, Takahashi K, Yagi R, & Rossant J (2005). Interaction between *Oct3/4* and *Cdx2* Determines Trophectoderm Differentiation. *Cell*, 123(5), 917–929. 10.1016/j.cell.2005.08.040 [PubMed: 16325584]
- Olariu V, Lövkqvist C, & Sneppen K (2016). *Nanog*, *Oct4* and *Tet1* interplay in establishing pluripotency. *Scientific Reports*, 6 10.1038/srep25438
- Ortega MS, Moraes JGN, Patterson DJ, Smith MF, Behura SK, Poock S, & Spencer TE (2018). Influences of sire conception rate on pregnancy establishment in dairy cattle. *Biology of Reproduction*. 10.1093/biolre/iox141
- Ortega MS, Wohlgemuth S, Tribulo P, Siqueira LGB, Null DJ, Cole JB, ... Hansen PJ (2017). A single nucleotide polymorphism in *COQ9* affects mitochondrial and ovarian function and fertility in Holstein cows. *Biology of Reproduction*, 96(3), 652–663. 10.1093/biolre/iox004 [PubMed: 28339599]
- Ozawa M, Sakatani M, Hankowski KE, Terada N, Dobbs KB, & Hansen PJ (2012). Importance of culture conditions during the morula-to-blastocyst period on capacity of inner cell-mass cells of bovine blastocysts for establishment of self-renewing pluripotent cells. *Theriogenology*, 78(6), 1243–1251.e2. 10.1016/j.theriogenology.2012.05.020 [PubMed: 22898023]
- Ozawa Manabu, & Hansen PJ (2011). A novel method for purification of inner cell mass and trophectoderm cells from blastocysts using magnetic activated cell sorting. *Fertility and Sterility*, 95(2), 799–802. 10.1016/j.fertnstert.2010.10.006 [PubMed: 21055741]
- Ozawa Manabu, Sakatani M, Yao J, Shanker S, Yu F, Yamashita R, ... Hansen PJ (2012). Global gene expression of the inner cell mass and trophectoderm of the bovine blastocyst. *BMC Developmental Biology*, 12(1), 33. 10.1186/1471-213X-12-33 [PubMed: 23126590]
- Pfeffer PL (2018). Building principles for constructing a mammalian blastocyst embryo. *Biology*, 7(3). 10.3390/biology7030041
- Rodda DJ, Chew J-L, Lim L-H, Loh Y-H, Wang B, Ng H-H, & Robson P (2005). Transcriptional regulation of *Nanog* by *Oct4* and *Sox2*. *The Journal of Biological Chemistry*, 280(26), 24731–24737. 10.1074/jbc.M502573200 [PubMed: 15860457]
- Sakurai N, Takahashi K, Emura N, Fujii T, Hirayama H, Kageyama S, ... Sawai K (2016). The Necessity of *OCT4* and *CDX2* for early development and gene expression involved in differentiation of inner cell mass and trophectoderm lineages in bovine embryos. *Cellular Reprogramming*, 18(5), 309–318. 10.1089/cell.2015.0081 [PubMed: 27500421]
- Simmet K, Zakhartchenko V, Philippou-Massier J, Blum H, Klymiuk N, & Wolf E (2018). *OCT4/POU5F1* is required for *NANOG* expression in bovine blastocysts. *Proceedings of the National Academy of Sciences*, 201718833. 10.1073/pnas.1718833115

- Strumpf D, Mao C-A, Yamanaka Y, Ralston A, Chawengsaksophak K, Beck F, & Rossant J (2005). *Cdx2* is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. *Development*, 132(9), 2093–2102. 10.1242/dev.01801 [PubMed: 15788452]
- Twaroski K, Mallanna SK, Jing R, DiFurio F, Urlick A, & Duncan SA (2015). FGF2 mediates hepatic progenitor cell formation during human pluripotent stem cell differentiation by inducing the WNT antagonist NKD1. *Genes & Development*, 29(23), 2463–2474. 10.1101/gad.268961.115 [PubMed: 26637527]
- Wicklow E, Blij S, Frum T, Hirate Y, Lang RA, Sasaki H, & Ralston A (2014). HIPPO pathway members restrict *SOX2* to the inner cell mass where it promotes ICM fates in the mouse blastocyst. *PLoS Genet*, 10(10), e1004618 10.1371/journal.pgen.1004618 [PubMed: 25340657]
- Wolf XA, Serup P, & Hyttel P (2011). Three-dimensional localization of NANOG, OCT4, and E-cadherin in porcine pre- and peri-implantation embryos. *Developmental Dynamics*, 240(1), 204–210. 10.1002/dvdy.22491 [PubMed: 21089074]
- Wu G, & Schöler HR (2014). Role of *Oct4* in the early embryo development. *Cell Regeneration*, 3(1). 10.1186/2045-9769-3-7

Figure 1.

(A) Schematic drawing of bovine *NANOG*, black-filled block represents coding regions, white filled box untranslated regions and lines represent introns. (B) Genomic sequence of exon 2 of *NANOG*, lowercase letters represent introns, uppercase letters represent exon, highlighted in gray are the target sequences of the gRNAs 1 and 3. (C) Editing ability of gRNAs. Each lane is 1 individual embryo. Left lane: molecular weight marker, lanes 2-4: embryos injected with guides 1 and 3, 5-9: embryos injected with guides 1 and 2, 10-14: embryos injected with guides 2 and 3.

Figure 2.

Editing efficiency of microinjection of bovine embryos with guides 1 and 3. (A) Overall editing efficiency of genotyped blastocysts over four replicates. (B) Example of agarose visualization of genotyped embryos for NANOG editing bi-allelic edits are marked with (*). (C) Sanger sequencing results of embryos that appear as biallelic edits or WT in agarose showed in (B), out of 24 embryos showed in B and C, 6 had biallelic edits, 5 monoallelic edits, 7 had small deletions (1-3 nucleotides) determined by sequencing and 7 were not edited.

Figure 3.

Representative images of deletion of NANOG in bovine embryos. First and the second rows are embryos only injected with CAS9 mRNA, third and fourth rows examples of NANOG-null embryos. GATA6 is shown in green, NANOG in red, Nuclei in blue. The sample size was 30 for the CAS9 injected n=30, NANOG-null=40.

Figure 4.

Relative gene expression of cell lineage markers in NANOG-null vs control embryos. Expression is presented as fold change relative to housekeeping genes. There were 6 pools of 4 embryos evaluated for each treatment. Significant differences were considered at $P < 0.05$ (**), and tendency at $P < 0.10$ (*). Values are presented as least-squares means $\pm$ SEM.

Figure 5.

Representative model of NANOG deletion and its effect on lineage specification on the bovine embryo. (A) Illustration of *NANOG*-null embryos development through cell fate decisions. (B) Model of lineage specification in the bovine embryo.

Table 1.

Developmental rates of edited embryos

Group	Oocytes (n) [‡]	Cleaved (%) ^{‡,§}	Blastocyst (%)	Blastocyst/cleaved (%)
<i>NANOG</i> gRNA	1119	68.51 ± 2.45 ^a	22.78 ± 1.99 ^a	33.74 ± 2.52 ^a
<i>Cas9</i> mRNA	511	70.23 ± 3.06 ^a	26.88 ± 2.49 ^a	37.78 ± 3.15 ^{ab}
Non-injected	362	81.10 ± 3.47 ^b	37.01 ± 2.82 ^b	45.55 ± 3.57 ^b

[‡]Total number the oocytes across four replicates[‡]Values are presented as least-squares means ± SEM[§]Values with different superscripts differ ($P < 0.05$)