The role of NANOG during bovine preimplantation development

von Claudia Andrea Springer

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von Claudia Andrea Springer

aus Starnberg

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Aus dem Veterinärwissenschaftlichen Department der Tierärztlichen
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Für meine Familie

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INDEX OF ABBREVIATIONS

3D	Three-dimensional
AETE	Association of Embryo Technology in Europe
AI	Artificial insemination
AMH	Anti-Müllerian hormone
ART	Assisted reproductive technology
BME	Basal Medium Eagle’s amino acids solution
BSA	Bovine serum albumin
CLSM	Confocal laser scanning microscope
COC	Cumulus-oocyte complex
Ctrl	Control
d0	Day 0
DSB	Double strand break
E	Embryonic day
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EPI	Epiblast
ESC	Embryonic stem cells
ET	Embryo transfer
FGFR	FGF-receptor
FSH	Follicle-stimulating hormone
GE	Genetically engineered
HDR	Homology-directed repair
HYPO, HB	Hypoblast
ICM	Inner cell mass

ICSI	Intracytoplasmic sperm injection
IFOT	Intrafollicular oocyte transfer
IVF	In vitro fertilization
IVM	In vitro maturation
IVP	In vitro production
JAK/STAT	Janus kinase/signal transducer and activator of transcription
KO	Knockout
MAPK	Mitogen-activated protein kinase
MEM	Minimum Essential Medium
NHEJ	Non-homologous end joining
OPU	Ovum pick-up
OCS	Estrous cow serum
PD032	PD0325901
PE	Primitive endoderm
PHD	Post-hatching development
RNA-seq	RNA-sequencing
RTK	Receptor tyrosine kinase
SCNT	Somatic cell nuclear transfer
scRNA-seq	Single-cell RNA-sequencing
SD	Standard deviation
TE	Trophectoderm
ZGA	Zygotic genome activation
ZI	Zygote injection

I. INTRODUCTION

Beginning with just a few cells, the start of embryogenesis sets in motion a long journey until a fully developed offspring is born. The emergence of three distinct cell lineages marks the first specification events, where the epiblast (EPI), trophectoderm (TE) and hypoblast (HB) develop. During formation of the blastocyst, the first lineage differentiation is defined by the segregation of outer cells from the inner cell mass (ICM), building the surrounding TE and defined by CDX2 expressing cells. The specification of a subset of cells within the ICM characterizes the second lineage differentiation. Some cells will express NANOG and the others GATA6 and SOX17, markers for EPI and HB precursor cells, respectively. The pluripotent EPI will become the embryo itself and will give rise to all cell lineages needed for a grown organism. The TE builds the embryonic part of the placenta and is vital for the embryo's implantation in the uterus. The yolk sac development depends on the formation of the HB (called primitive endoderm, PrE in the mouse) (CHAMBERS et al., 2003; MITSUI et al., 2003; CHAMBERS et al., 2007; CAI et al., 2008; PLUSA et al., 2008; MESSERSCHMIDT & KEMLER, 2010; MORRIS et al., 2010; FRANKENBERG et al., 2011).

Little is known about the regulation of the first and second lineage segregation, especially with regard to models other than mouse. While many aspects of early embryo development are conserved between mammals, species-specific differences exist between mouse and human with regard to gene expression patterns and lineage specification. Domestic animals share many similarities with human embryos and since assisted reproductive technologies (ARTs) in cattle is highly developed, we designate bovine as an optimal model organism to study early embryo development (reviewed in PILISZEK & MADEJA, 2018; SPRINGER et al., 2021b).

The aim of this study was to investigate the role of NANOG during bovine embryo development in the time before implantation. For this, I created *NANOG*-KO embryos by using the CRISPR/Cas9 system in bovine cells to induce a disruption of exon 2 in *NANOG*. These modified cells were used in somatic cell nuclear transfer (SCNT) (ZAKHARTCHENKO et al., 1995), resulting in

NANOG-deficient bovine embryos.

Parts of this dissertation have been published:

- First Publication:

  Springer, C.; Wolf, E.; Simmet, K. A New Toolbox in Experimental Embryology—Alternative Model Organisms for Studying Preimplantation Development. J. Dev. Biol. **2021**, 9, 15. https://doi.org/10.3390/jdb9020015

- Second Publication:

  Springer, C.; Zakhartchenko, V.; Wolf, E.; Simmet, K. Hypoblast Formation in Bovine Embryos Does Not Depend on NANOG. *Cells* **2021**, *10*, 2232. https://doi.org/10.3390/cells10092232

In the first publication, I review the need to study preimplantation development in alternative model organisms with focus on pig and bovine. I discuss all the relevant techniques we used in this thesis and the pros and cons of various ART procedures. Additionally, I highlight differences in regulating the second lineage differentiation between species, which is relevant for our results and therefore serves as part of the review of literature in this thesis.

The second publication is the research I performed in bovine embryos and shows the effects on the resulting phenotype of *NANOG*-KO day 8 embryos. By applying reverse genetic studies and combining this approach with modulation of the FGF/MEK pathway, I was able to highlight differences in hypoblast formation between bovine and mouse. The second publication serves as the results section in this dissertation.

During preparation of this thesis another group published a paper, where a *NANOG*-KO was induced using zygote injection (ZI) in bovine embryos (ORTEGA et al., 2020). As I used a different method and applied experiments regarding modulation of the second lineage differentiation, I could enhance the knowledge about the effect of *NANOG*-KO during the first and second lineage differentiation in bovine embryos.

II. REVIEW OF THE LITERATURE

1. The preimplantation development of the mammalian embryo

1.1. Fertilization

When sperm and egg (haploid gametes) unite, the process is called fertilization. As a result, genetically unique individuals develop (reviewed in FLORMAN & DUCIBELLA, 2006; HYTTEL et al., 2009). For this, various steps need to be processed: spermatozoa's and oocyte's integrity and the capability of fertilization, their transportation, the appropriate time frame, sperm capacitation and penetration into the oocyte, the development of male and female pronuclei, their union, activation and the segmentation of the fertilized oocyte, which is now called the zygote, need to occur (CHANG & PINCUS, 1951). After ovulation, the cumulus-oocyte complex (COC), which consists of the oocyte, the zona pellucida and cumulus cells enters the infundibulum and will be fertilized in the ampullary region of the oviduct (COY et al., 2012). After ejaculation, the sperm first needs to fulfill capacitation in order to be able to fertilize the oocyte. For this, spermatozoa reside in the female genital tract and capacitation involves adaption of mostly its plasma membrane in a number of complex processes. Subsequently, the capacitated spermatozoa will adhere and bind to the zona pellucida (HARTMANN et al., 1972; BEDFORD, 1983; HARRISON, 1996). Then, the induction of the acrosome reaction begins where the acrosome of the sperm head releases hydrolytic enzymes and thus penetrates the zona pellucida. Afterwards, sperm fuse with the plasma membrane of the oocyte and this triggers oocyte activation, which prevents polyspermic fertilization, establishes resumption of meiosis, and the initiation of embryo development. Mixing maternal and paternal chromosomes by dissolving the two pronuclei results in a completed meiosis. A unique embryonic genome has been created and returns to mitotic cell divisions (reviewed in KUPKER et al., 1998).

1.2. Early cell divisions

The first mitosis of the zygote results in a 2-cell embryo where both cells, also called blastomeres, occupy their full copy of the embryonic genome. The zona

pellucida is still surrounding the embryo for some days, but the period differs between species (reviewed in HYTTEL et al., 2009). The following mitotic divisions arise almost without cellular growth, thus each blastomere becomes smaller in every division and this process is called cleavages (reviewed in ZERNICKA-GOETZ, 2005). During the embryo's transport through the oviduct, the first cleavages happen at species-specific timepoints. The same counts for the embryos' passage into the uterus, where timepoints are species-specific as well, e.g. 8–16 cell stage in cattle (reviewed in HYTTEL et al., 2009). For the first cleavage, maternal proteins and transcripts are necessary until later activity of the embryonic genome is of the essence and governs exclusive control of its development (reviewed in LI et al., 2013). Therefore, two phases of embryonic or zygotic genome activation (ZGA) need to process: the minor and the major ZGA (reviewed in SVOBODA, 2018). After a few divisions the morula, a mulberry shaped form, has developed with blastomeres of the same size (reviewed in ARTUS & CHAZAUD, 2014).

1.3. First and second lineage segregation

Compaction induces a smoother surface of the morula as outer totipotent blastomere cells differentiate to an epithelium, called the trophectoderm (TE) or trophoblast (VAN SOOM et al., 1997). After morula stage, a subsequent process of blastulation takes place where a fluid-filled cavity forms and builds the blastocyst. Eventually, inner blastomeres of this early embryo polarize and form the inner cell mass (ICM), which is now pluripotent and completes the first lineage segregation (JEDRUSIK et al., 2008). This process is conducted by the Hippo signaling pathway, which directs outer cells to form the TE via positional signals that eventually surround the ICM (NISHIOKA et al., 2009). The trophectoderm will give rise to the embryonic part of the placenta (MENCHERO et al., 2018). The ratio of ICM cells to TE cells is around 1:3. Due to osmotic pressure inside the blastocyst's cavity the blastocyst gradually expands until the zona pellucida ruptures and the embryo can escape. In bovine, this process is called hatching. During this late blastocyst stage, the second lineage differentiation occurs. Within the ICM, epiblast cells (EPI) divide from the primitive endoderm (called PE in mouse) or hypoblast precursor cells (called HB in bovine, human). The EPI will become the embryo proper and is characterized by expression of the transcription factor NANOG. The PE or HB will give rise to

the yolk sac and GATA6 was found to be a key regulator (reviewed in GERRI et al., 2020).

1.4. Elongation and implantation

The polar TE covering the EPI (Rauber's layer) is eventually lost and the embryo exposed to the environment in the uterus. Together with the underlying HB, it forms first a transparent circle, then an oval structure, called embryonic disc (PFEFFER et al., 2017; RAMOS-IBEAS et al., 2020). Special for bovine and pig is the elongation of the blastocyst, where the embryo is expanding. Thereby, the embryo becomes ovoid, then tubular and later filamentous (CHANG, 1952; GREENSTEIN et al., 1958; GEISERT et al., 1982; BETTERIDGE & FLECHON, 1988; HUE et al., 2001; MADDOX-HYTTEL et al., 2003). Especially in pig the growth rate is massive, as from day 10 until day 13, the embryo expands its size from one centimeter to about one meter in a filamentous structure (GEISERT et al., 1982; ALBERTINI et al., 1987; MATTSON et al., 1990). The implantation is species-specific and occurs around day 19–21 in bovine (GUILLOMOT, 1995; MAMO et al., 2012), day 13 in pig (OESTRUP et al., 2009), day 7 in human (WILCOX et al., 1999) and on embryonic day (E) E4.5 in mouse (WANG & DEY, 2006).

2. The pluripotency regulating transcription factor NANOG

2.1. NANOG in the preimplantation mouse embryo and mouse embryonic stem cells

MITSUI et al. (2003) first described the homeobox transcription factor NANOG in mice. The name is based on the mythological term "Tir Na Nog" which means "land of the ever young". *NANOG* exists of four exons with three introns, which is conserved in different species (CLARK et al., 2004). The expression of NANOG begins at morula stage and becomes restricted to the ICM during blastocyst formation (MITSUI et al., 2003; HART et al., 2004). NANOG was found to be a vital part of the pluripotency network next to OCT4 and SOX2, as it promotes ESC pluripotency and self-renewal while repressing genes that are needed for differentiation (BOYER et al., 2005). ICM cells express NANOG, OCT4 and SOX2 that are required for formation of the epiblast (NICHOLS et al., 1998; AVILION et al., 2003; CHAMBERS et al., 2003; MITSUI et al., 2003). In murine ESC it was shown that NANOG binds to the GATA6 promotor and thus directly

represses GATA6 (SINGH et al., 2007). To repress NANOG the Grb/MEK pathway is needed, which also regulates the GATA6 expression (HAMAZAKI et al., 2006). A mutually exclusive expression in a so-called salt-and-pepper distribution of NANOG and GATA6 occurs in the ICM of early blastocysts (CHAZAUD et al., 2006). In mouse ESC, NANOG expression fluctuates as low NANOG levels directed cells towards differentiation, but did not result in commitment (CHAMBERS et al., 2007). A heterogenous expression of NANOG in mouse ESC was reported, where cells were flow-sorted in terms of their NANOG expression level. After cultivation of those sorted cells, they changed again to their original heterogeneous state (SINGH et al., 2007). Mouse embryos without functional NANOG still differentiate towards TE cells and express GATA6 ubiquitously in the ICM but show no epiblast formation (MITSUI et al., 2003; FRANKENBERG et al., 2011). Morphologically, *Nanog*-mutant mouse embryos appear normal and decidualization is induced but peri-implantation lethality occurs. The isolation of ESC from those embryos fails. Blastocyst outgrowths from *Nanog*-deficient ICM cells show a parietal endoderm-like morphology (MITSUI et al., 2003). In contradiction to the originally described PE outgrowth, an alternative phenotype of the *Nanog*-KO was described, as ICM cells were supposed to be blocked in a transitional stage and were neither able to remain in the pluripotent ground state nor to differentiate. As a result, it was proposed that *Nanog*-KO ICM cells could only differentiate towards TE fate or else experience apoptosis (SILVA et al., 2009). Interestingly, in contrast to the ubiquitous expression of GATA6, later markers of the PE such as SOX17 and GATA4 (PLUSA et al., 2008; MORRIS et al., 2010; NIAKAN et al., 2010) are lost in mouse *Nanog*-KO embryos (SILVA et al., 2009; MESSERSCHMIDT & KEMLER, 2010; FRANKENBERG et al., 2011). In the mouse, supplementing exogenous FGF4, which is released by NANOG-expressing cells, was able to rescue SOX17 in the ICM of *Nanog*-KO embryos (FRANKENBERG et al., 2011). When the MEK pathway was blocked in presence of *Nanog*-KO mouse embryos and mouse ESC, their viability was compromised and cell death occurred (FRANKENBERG et al., 2011; HASTREITER et al., 2018).

2.2. NANOG in the preimplantation bovine embryo

In cattle, NANOG is first expressed at the 8-cell stage and becomes restricted to the subpopulation of the ICM during blastocyst development (KUIJK et al., 2008; KHAN et al., 2012; MADEJA et al., 2013; GRAF et al., 2014). Recently, a *NANOG*-KO in bovine embryos was performed using zygote injection (ZI) to induce a frameshift mutation in exon 2 of *NANOG*. *NANOG*-deficient embryos were found to have a similar blastocyst rate compared to the Cas9-injected control group. The size appeared to be smaller although the authors did not quantify this. The embryos were able to develop a blastocoel but the ICM was poorly defined displaying no epiblast. The ICM ubiquitously expressed GATA6 but later markers of the hypoblast were not investigated. Quantitative PCR revealed a significantly reduction of mRNA transcripts of GATA6 and the pluripotency markers SOX2 and H2AFZ in *NANOG*-deficient embryos, whereas OCT4 was similar in both *NANOG*-KO and WT groups. It was hypothesized that NANOG is necessary for maintaining pluripotency in the bovine preimplantation embryo, similar to mouse. Expression of TE markers such as CDX2 was not affected by disruption of NANOG (ORTEGA et al., 2020).

3. Review Paper: A new toolbox in experimental embryology – alternative model organisms for studying preimplantation development

The following part of the thesis has been published in the Journal of Developmental Biology on 2nd of April in 2021.

Springer, C.;Wolf, E.; Simmet, K. A New Toolbox in Experimental Embryology—Alternative Model Organisms for Studying Preimplantation Development. J. Dev. Biol. **2021**, 9, 15. https://doi.org/10.3390/jdb9020015

Abstract: Preimplantation development is well conserved across mammalian species, but major differences in developmental kinetics, regulation of early lineage differentiation and implantation require studies in different model organisms, especially to better understand human development. Large domestic species, such as cattle and pig, resemble human development in many different aspects, i.e., the timing of zygotic genome activation, mechanisms of early lineage differentiations and the period until blastocyst formation. In this article, we give an overview of different assisted reproductive technologies, which are well established in cattle and pig and make them easily accessible to study early embryonic development. We outline the available technologies to create genetically modified models and to modulate lineage differentiation as well as recent methodological developments in genome sequencing and imaging, which form an immense toolbox for research. Finally, we compare the most recent findings in regulation of the first lineage differentiations across species and show how alternative models enhance our understanding of preimplantation development.

Keywords: embryo; cattle; pig; ART; SCNT; genome editing

3.1. Introduction

To study the events during preimplantation development, a look beyond the most commonly used mouse model can be vital to discover the often still unknown molecular pathways that regulate the first steps of embryo development. Where the mouse shows unique regulatory mechanisms, other animals share great

similarities in their developmental plan. The moment of zygotic genome activation, the first lineage differentiations and maintenance of pluripotency are some aspects that are not always conserved between species, but are very similar in cows and pigs compared to humans (reviewed in [1–3]). Furthermore, the scarcity of human embryos and the ethical and logistical challenges increase the need to work with other models. At first glance, working with large domestic species to study preimplantation development may appear laborious and impractical. However, looking closer, there are numerous benefits that come from highly developed assisted reproductive technologies (ARTs) in these species. To produce embryos regularly, it is sufficient to have a nearby abattoir, where ovaries can be obtained. Using in vitro techniques, an unlimited amount of research material can be produced without the need of housing experimental animals. If embryos at developmental stages beyond our current in vitro culture capabilities are required, protocols for embryo transfer (ET) and recovery are available.

With recent breakthroughs in genome editing, it is now possible to perform a wide range of reverse genetics studies in large domestic species in a time and resource efficient manner. In combination with the different ARTs that are available, a plethora of possible studies may be conducted to increase our knowledge about mechanisms and dynamics during preimplantation development in alternative model organisms. Here, we describe in detail the different ARTs and their benefits or disadvantages for studying preimplantation development and we show, when and how manipulations of the embryo can be performed to shed light on the regulation of the first lineage differentiations.

3.2. State of the ART - assisted reproductive technologies in cattle and pigs

Artificial insemination (AI) and other ARTs have revolutionized the cattle and pig industry. The use of AI enabled tremendous genetic improvement of dairy cows by dissemination of superior bulls, increasing the milk yield per year 3.8-fold from 2400 kg to 9200 kg in only 57 years (1950–2007) in the USA [4]. The pig industry increased the number of piglets weaned per sow per year from 20 to 30 over the last three decades [5]. ARTs, i.e., ET, ovum pick-up (OPU) and in vitro fertilization (IVF), have also been developed to increase the number of offspring of uniparous animals such as cows ([6,7], reviewed in [8]). These techniques enable the use of cattle and pigs as model organisms for developmental biology

and biomedical research. In this chapter, we concentrate on ARTs in cows and pigs and provide an overview about the different techniques, the advantages and limitations of these procedures.

3.2.1. Superstimulation and embryo transfer

The principle of superstimulation regarding its commercial use is to increase the number of offspring of cows with superior breeding values. Multiple oocytes ovulate and after AI, the uterus of the superstimulated cow is flushed and the obtained embryos are transferred to recipients. Hence, more calves with superior genetics will be born in a shorter time range. For this purpose, follicle-stimulating hormone (FSH) or similar gonadotropins are administered. After ovulation, superstimulated females release large numbers of oocytes into the oviduct which are then fertilized via AI, develop in vivo and can be flushed non-surgically directly from the uterus (reviewed in [9]). Additionally, it is possible to collect in vivo matured oocytes by flushing the oviduct, but a surgical [10–12] or transvaginal endoscopic approach [13] is necessary. Although the ovarian response varies a lot among individual donors and treatment protocol, this technique enormously increases the numbers of retrieved embryos or oocytes. Two extensive studies showed an average of 6.9 embryos collected from beef cows [14] and 5.1 to 5.4 viable embryos from lactating dairy cows [15]. According to the Association of Embryo Technology in Europe (AETE), an average of 6.9 embryos per collection from dairy and beef cows was achieved in 2019 [16]. An indicator for the population of antral follicles in both human and cow is anti-Müllerian hormone (AMH). Concentrations of AMH in the plasma may predict a cow's response to superovulatory treatment [17,18].

The fertilization rate after AI in heifers is decreased after superstimulation compared to spontaneous ovulation (72% vs. >80%), suggesting an impact on oviductal function [19]. Effects on embryos derived from superovulation procedures were investigated by Gad et al. [20], illustrating a reduced competence for preimplantation development in vivo and altered gene expression patterns.

In pigs, superovulation is only rarely performed, as it is a multiparous species. In breeds with physiologically low ovulation rates, such as Duroc, it can help to increase the embryo yield, with normal embryonic and fetal development [21,22].

With ET, it is possible to remove one or more embryos from the reproductive tract of a donor female and subsequently transfer them to surrogates. The nonsurgical transfer into the bovine uterus is the standard technique when using either fresh or cryopreserved day 7 blastocysts. Of importance is the synchronous reproductive cycle of the recipients (reviewed in [8]). If earlier stages need to be transferred, Besenfelder and Brem [23] developed a transvaginal endoscopic technique to insert early tubal stage embryos (day 1–2) into the oviduct. This technique allows embryos to passage through the oviduct during the period when major epigenetic reprogramming and major embryonic genome activation take place ([24,25], reviewed in [26]). These processes are easily disturbed by changes in the environmental conditions [27]. Interestingly, transfer of early cleavage stage embryos into the uterus of domestic animals leads to impaired development and results in low pregnancy outcomes [28], whereas in humans, uterus transfer with zygotes or early cleavage stage embryos is commonly performed [29].

In pigs, ET is often used in combination with somatic cell nuclear transfer (SCNT) to produce genetically modified livestock, using a minimal invasive laparoscopic method ([30], reviewed in [31]). Additionally, a nonsurgical method for deep uterine embryo transfer was established, which could pave the way for a commercial use [32,33].

3.2.2. Ovum Pick-Up (OPU)

In 1988, a Dutch team first collected cumulus-oocyte complexes (COCs) from cattle by using transvaginal ultrasound-guided follicle aspiration, giving rise to a new procedure called OPU [7]. OPU is combined with in vitro production (IVP) of bovine embryos and is an alternative to superstimulation. There are many advantages: in contrast to superovulation, the reproductive status of the donor is irrelevant, it can even be pregnant, acyclic, or having genital tract infections. Furthermore, heifers that are not responding to the superstimulation treatment can be used as well. As OPU can be performed twice a week, it can increase the yield of transferable embryos immensely [34]. It is performed regularly over a long period and donors with a high number of COCs seem to perform steadily on a high level [35]. Still, between breeds and different animals, the number of retrieved COCs per OPU session is variable [36]. The collected COCs are then used for IVP of embryos (see Section 3.2.5, adapted by the author).

3.2.3. Intrafollicular Oocyte Transfer (IFOT)

Recently, a technique for intrafollicular oocyte transfer (IFOT) in cows has been established [37]. Here, immature COCs derived from abattoir ovaries or by OPU are transferred directly into a pre-ovulatory follicle of synchronized heifers to enable maturation in vivo prior to AI. This procedure circumvents the disadvantages of in vitro maturation (IVM) of oocytes and results in higher blastocyst rates (40.1 vs. 29.3% after IFOT and IVM, respectively) [38]. IFOT allows the production of a high number of embryos in a complete in vivo system without any hormonal superstimulation or extensive laboratory facilities [37,39]. However, pregnancy rates were rather low when using cryopreserved embryos derived from IFOT (15.4%) [38].

3.2.4 Intracytoplasmic sperm injection (ICSI)

Intracytoplasmic sperm injection (ICSI) describes a microfertilization technique of the direct injection of a single spermatozoon or sperm head (nucleus) into the ooplasm. It is possible to use immobilized or dead sperm, making it especially interesting as an alternative to in vitro fertilization to overcome male infertility in humans (reviewed in [40]). The first offspring from ICSI-derived embryos was described by Martin [41] in pigs and by Goto, et al. [42] in cattle. In bovine, ICSI has not been established for commercial use, as IVF protocols are very efficient; therefore, it is used for research interest only [36]. The same is true for ICSI in pigs, where costs and effort cannot be compensated by the low success rates, which makes it impractical for pig production [41,43–46]. Nevertheless, as polyspermy is a common phenomenon in IVF in pigs (see Section 3.2.5, adapted by the author), ICSI is a considerable alternative (reviewed in [40]). Furthermore, ICSI-mediated gene transfer can be used for genetic modification of porcine embryos (see Section 3.3, adapted by the author). Besides humans and pigs, ICSI is merely interesting for horses, because methods for capacitating sperm in vitro have not been developed so far [47].

3.2.5. In vitro production (IVP) of embryos

Since many decades, IVP protocols exist for bovine embryos and they have been improved constantly, while in pig the procedure still requires improvement. The aim is to generate embryos in the laboratory via fertilization of oocytes, which have been matured either in vitro (well established for cows and pigs) or in vivo

(mostly for mouse and human). In domestic species, COCs can be derived from abattoir ovaries, making it possible to procure great amounts of oocytes without much effort. If ex vivo derived oocytes are desired, superstimulation or OPU can be performed.

After collecting the COCs, the first step is their IVM. Gonadotropins, such as FSH and luteinizing hormone (LH) are supplemented to simulate the preovulatory surge of those hormones to achieve an expansion of cumulus cells and resumption of meiosis. The hormones are combined with serum, bovine serum albumin (BSA) or epidermal growth factor (EGF), which help to stimulate maturation and cumulus expansion. As serum may vary dependent on its batch, serum-free media are preferred [48]. Additionally, serum–containing media may induce a shift towards a higher proportion of male bovine embryos [49]. Subsequently, after 22–24 h (bovine) or 44–48 h (pig), the matured oocytes can be fertilized. A defined sperm concentration without undesirable semen components enables continuity in IVF procedures. In preparation of sperm for IVF, centrifugation through a Percoll density gradient is the most conventional method in cattle, but other procedures such as swim-up, centrifugation on BSA, or Sephadex column separation are available [50,51]. Heparin, which is found in the genital tract of females, supports fertilization of matured oocytes by inducing sperm capacitation [52]. Subsequently, presumptive zygotes are placed into embryo culture medium after removing excess sperm cells and cumulus cells to avoid the presence of degenerating cells that may decrease the efficiency of the culture system [50]. Seven days after insemination, bovine blastocysts can be cryopreserved or used for ET (reviewed in [8]).

For basic research, it is possible to maintain bovine embryos until day 8 or 9 in culture, when they have developed to blastocysts that hatch from the zona pellucida. Routinely achieved day 8 blastocyst rates in bovine are approximately 30–40% [12,53]. Approaches to prolong development in vitro have been elaborated recently. In the post-hatching development (PHD) system, embryos are cultured in an agarose-coated dish in serum- and glucose-enriched medium (PHD medium) until day 15 or 16, when they show epiblast (EPI)-derived cells, a Rauber’s layer and some degree of proliferation of hypoblast (HYPO) cells. Although trophectoderm (TE) cells can grow in the PHD medium, HYPO migration along the entire inner embryo surface was not achieved, apoptosis and

necrosis were visible and EPI formation was compromised in this system. Therefore, PHD medium supports proliferation of the TE but is incapable to maintain embryo development beyond the blastocyst stage [54–57]. In a different approach, embryos were cultured in N2B27 medium (used in mouse and human primed and naïve stem cell culture) and reduced oxygen (5%) until day 15. Embryos were routinely obtained and showed HYPO formation and varying amounts of EPI, with several embryos displaying a SOX2 positive EPI disc [58]. Recently, a three-dimensional (3D)-printed oviduct-on-a-chip platform was created, which mimicked the oviductal environment in vitro. In this culture chamber, oviductal epithelial cells were incubated, thus fertilization and early embryo development resembled the physiological situation more closely, leading to bovine zygotes with a similar transcriptome profile compared to in vivo produced zygotes [27].

Of importance is the difference between both human and mouse compared to domestic animals regarding the peri-implantation development. In cattle, the embryo will elongate up to 20 cm via rapid trophoblast development that dramatically alters the blastocyst morphology prior to implantation and similar growth is seen in pigs, whereas in human and mouse, there is no elongation ([59–61], reviewed in [62]). Therefore, to study peri-implantation development in humans, large domestic animals may not serve as optimal model organisms.

In small ruminants (goats and sheep), IVP protocols are also accessible, where embryos can be cultured until day 8 with similar outcomes as in cattle ([63], reviewed in [64]).

It is important to compare IVP embryos with their in vivo equivalents. Whereas IVP shows a fertilization rate of up to 80% and a blastocyst rate of 30–40% [12,53], a fertilization rate by AI of over 90% in ovulated oocytes is described, with most of the resulting zygotes developing to blastocysts [65]. More differences comparing in vitro versus in vivo embryos are seen regarding the ultrastructure [66], microvilli [67], lipid content [68], cryoresistance [67], and most importantly the gene expression profile. Altered transcript levels in IVP embryos are connected to metabolism and growth as well as altered fetal development after transfer [69–71]. The large variety of media used in IVP is a problem when comparing results of different research groups. Using serum in medium can modulate the gene expression pattern and decrease cryoresistance of

bovine IVP blastocysts [68,72]. Regarding bovine blastocyst yield and quality, there was no difference between media containing estrous cow serum or BSA [73].

In pig, IVP is not as developed as in cattle, leading to highly variable success rates that are below those achieved in bovine IVP [74–76]. Blastocysts derived by IVP procedures show an inferior number of cells and lower ability to produce pregnancies compared to their ex vivo counterparts [77]. Nevertheless, it is feasible to culture porcine embryos until day 6–8 and progress has been made in implementing 3D culture systems to investigate elongated stages [78–80]. A yet unresolved problem in pig IVP protocols is the high proportion of polyspermy. Imbalanced nuclear and cytoplasmatic maturation as well as a low quality of oocytes and increased sperm concentrations are discussed as factors causing polyspermic penetration of porcine oocytes. Polyspermic embryos are aneuploid, show abnormal cleavage patterns, reduced growth of the inner cell mass (ICM), and cannot develop to term, thereby decreasing the IVP efficiency [81–84].

3.2.6. Somatic cell nuclear transfer (SCNT)

During SCNT, the nucleus of a somatic donor cell is introduced into an oocyte whose own nuclear DNA has been removed (enucleation). This reconstructed embryo is activated to progress embryonic development and emerging embryos can be transferred to a recipient, enabling development to term. The nuclear genome of the resulting offspring is identical to the respective donor cell, whereas the mitochondrial DNA is mostly or completely derived from the recipient oocyte [85]. In agriculture, cloning can help to preserve genetic resources and to expand the distribution of breeding livestock, reviewed in [86,87]. As genome editing efficiency has improved immensely in recent years, it is now feasible to use SCNT for producing genetically engineered (GE) livestock to enhance demanded traits such as improved product quality, rapid growth or resistance of diseases [36,88,89]. Tsunoda, et al. [90] reported a general blastocyst rate of 10–40% in bovine SCNT experiments, of which 10–30% developed into calves upon transfer to recipients. SCNT may serve as an important key tool for studying preimplantation development, when combined with gene editing procedures (see Section 3.3, adapted by the author).

In pig, blastocyst rates of SCNT embryos vary between 20 and 40% [91–94], but

the overall cloning efficiency—defined as the number of cloned piglets born per transferred SCNT embryos—is low at 1–5%, as shown in an extensive study over three years [95].

Despite numerous promising advantages, SCNT is not only impeded by its low efficiency, but cloned animals may also suffer from various developmental defects. Problems occurring when conducting SCNT are micromanipulation trauma, oocyte incompetence, in vitro culture-induced anomalies and failed epigenetic reprogramming of the transferred nucleus (reviewed in [96]). As a result, physiological development is considered to be impaired as abnormal epigenetic profiles and gene expression may occur (reviewed in [97]). After transfer of bovine SCNT embryos to recipients, placental failure has frequently been observed, likely due to abnormal embryo-maternal communication during peri-implantation [98,99], giving a possible explanation for the high rate of pregnancy failures. The so-called “large offspring syndrome” is connected to cloned cattle and sheep neonates with unusually large bodies and sometimes associated organ defects, but the syndrome is also described in IVP embryos [100]. In pigs, aberrant cleft lips or teat numbers were found in surviving SCNT animals [101]. Cao, et al. [102] described a delayed zygotic genome activation (ZGA) and altered gene expression patterns in pig embryos produced by SCNT. Despite its limitations, SCNT has tremendous advantages, particularly for the generation of genetically engineered/genome edited large animal models, and further progress in modulating the epigenome could improve nuclear reprogramming (reviewed in [97]).

3.3. Genetic manipulations

A great variety of possible experiments emerges when researchers combine different ART procedures with new tools (Figure 1) which precisely edit the genome, such as CRISPR/Cas9 (reviewed in [103–106]). This RNA-guided nuclease induces double strand breaks (DSBs) at a defined target region and thus causes small insertions or deletions during non-homologous end joining (NHEJ) repair, which can induce a knockout of a gene of interest. Precise edits or knock-ins can be achieved through homology-directed repair (HDR) of a DSB if a suitable repair template is offered. Due to its high efficiency and ease of use, CRISPR/Cas9 is currently the method of choice for creating genome alterations in animal models. Together with highly developed ARTs, an unlimited set of

possible applications arises, making large animals a valuable and very accessible model for gaining a deeper understanding of mammalian preimplantation development ([107], reviewed in [108]).

Genetically modified embryos may be produced by SCNT, where the modifications have been induced in the primary cells that serve as donors of nuclei, or directly in zygotes using zygote injection (ZI) or electroporation. When using SCNT, all embryos have a uniform genotype, show no mosaicism, and donor cells can be screened thoroughly for possible off-target effects, making this the preferred technique for producing genome edited animals (reviewed in [109]). Nevertheless, cloning artefacts (see Section 3.2.6, adapted by the author) that possibly alter developmental mechanisms must be considered and closely monitored by implementing appropriate controls [110]. A high passage number may impair donor cell viability and SCNT success [111,112], which is often the case as cells must be passaged several times in order to produce clonal cells with the desired modifications for SCNT.

A different approach is ZI, where a desired mutation can be induced by injecting the CRISPR/Cas components into a pronucleus or the cytoplasm of a zygote. More recently, successful use of electroporation to manipulate porcine and bovine zygotes has been reported [113–115]. Zygote injection or electroporation require less technical effort compared to SCNT and may induce mutations at a high rate. Nevertheless, a tremendous problem is the common effect of mosaicism. When DNA replication precedes CRISPR-mediated genome edition, mosaicism occurs and therefore greatly reduces the odds for generating embryos with a uniform genetic modification. Additionally, the type of mutation is unknown during development and the only narrow genomic material per sample hampers in-depth investigations, especially when further analysis via imaging techniques or transcriptome analyses are needed. Therefore, repeatability and analysis can be a problem when performing ZI [116–119], but despite the possible drawbacks, it has been recently shown that a knock-in calf can be produced in one step using ZI [120]. Injection of CRISPR/Cas into M-phase oocytes concurrent with ICSI can increase editing efficiency and reduce mosaicism in mouse and human embryos [121].

Genetically modified bovine embryos can be cultured in vitro up to day 8. If later developmental stages are of interest, manipulated embryos may be transferred to

the uterus of a recipient cow and flushed non-surgically until shortly before implantation. Van Leeuwen, et al. [122] successfully transferred IVP derived embryos to cows at day 7 and flushed them again at day 11–15, showing the opportunity to examine gene edited embryos at later developmental stages, which at the moment cannot be produced bona fide in vitro.

Other techniques for genetic modification include ICSI- and sperm-mediated or lentiviral gene transfer. Sperm as a vector can be employed during ICSI-mediated gene transfer, where semen is co-incubated with an exogenous transgene before conducting ICSI. This is especially of interest in pig [123–125], but the vector may also be used in bovine for IVF [126,127] and even AI for both pig and bovine, as well [128,129]. Unfortunately, these techniques come with high variability in success and unprecise modifications (reviewed in [130]). With lentiviral gene transfer, complex retroviruses are disabled to serve as a vector and can infect both dividing and non-dividing cells. The vector naturally fuses with the cell (oocyte or zygote) and is internalized, making it less damaging compared to microinjection techniques. Lentiviral constructs can be injected into the perivitelline space of a zygote or by co-culture with a zona-free zygote. Transgenesis rates are extremely high with up to 100% in various animal species [131,132]. However, the “cargo size” is limited (6–8 kb at most), multiple integrations at different loci may occur and transgenerational silencing has been reported ([133,134], reviewed in [135]).

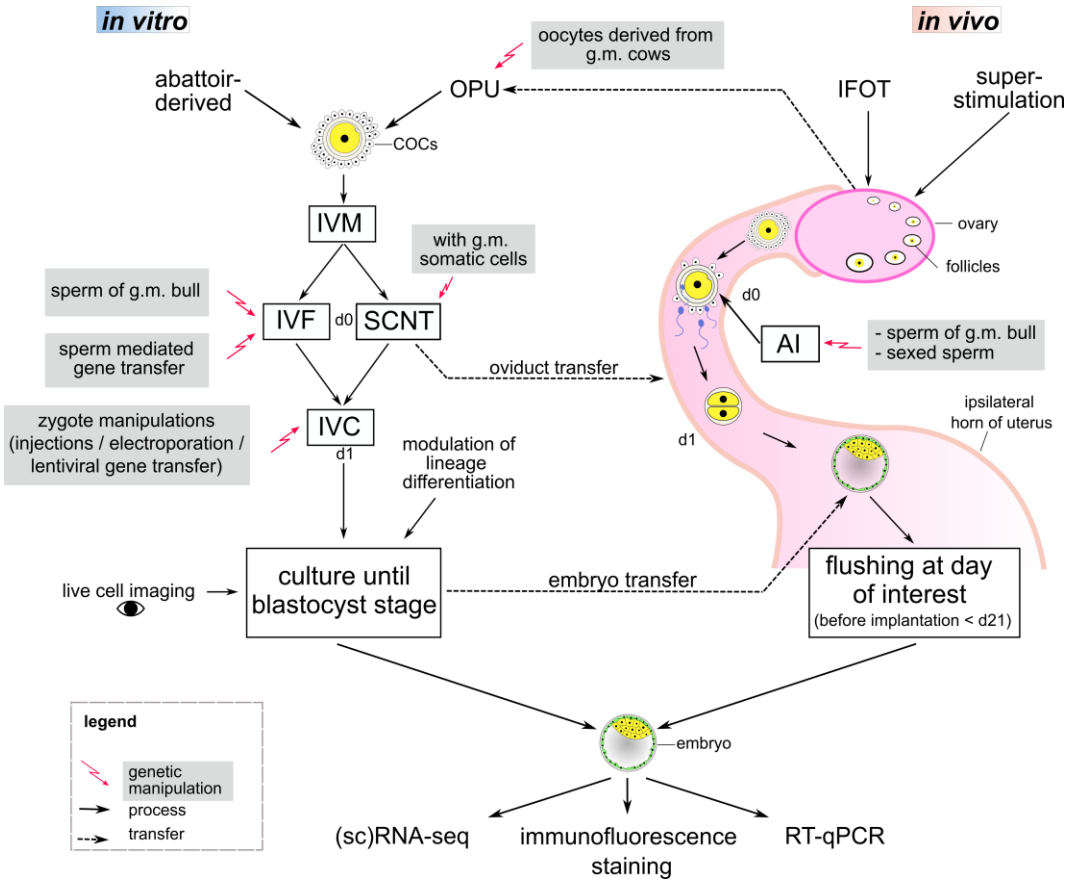

Figure 1: Studying preimplantation development with combined artificial reproduction technologies (ARTs) and genetic manipulation tools in cows. For in vitro production, cumulus-oocyte complexes (COCs) can either be derived from the abattoir or by ovum pick-up (OPU), where oocytes from genetically modified (g.m.) cows may be used. After in vitro maturation (IVM) of COCs, oocytes are in vitro fertilized (IVF) or reconstructed embryos from somatic cell nuclear transfer (SCNT) are activated, marking day 0 (d0) of embryo development. To study preimplantation development, genetic modifications can be introduced during IVF via sperm, by using g.m. donor cells in SCNT, or by direct manipulation of zygotes. These modifications enable, e.g., reverse genetics studies of specific gene functions or tagging of lineage-specific proteins with fluorescent markers. Embryos can then be transferred into the oviduct of a cow or cultured in vitro from day 1 (d1) until day 8. During culture, modulation of lineage differentiation and live cell imaging is feasible. If later stages are of interest, embryo transfer into the uterus can be carried out on day 7. For in vivo production, intrafollicular oocyte transfer (IFOT) or superstimulation increase the oocyte yield and artificial insemination (AI) with g.m. or sexed sperm can be performed. After in vivo development, embryos may be flushed non-surgically from the uterus at the day of interest until shortly before implantation on day 21 (d21). Embryos derived from in vitro or in vivo can be further examined by (single-cell) RNA-sequencing ((sc)RNA-seq), immunofluorescence staining or reverse transcriptase-quantitative PCR (RT-qPCR).

3.4. New insights into preimplantation development from alternative model organisms

It is of outmost importance to compare preimplantation development between species to get a comprehensive understanding about different regulatory systems, especially when deciphering the role of various transcription factors during early mammalian development. Two lineage differentiations pave the way during mammalian preimplantation development. First, outer and inner cells of the morula diverge, giving rise to the surrounding CDX2-expressing TE and the ICM. Second, within the ICM the pluripotent NANOG-expressing cells form the EPI and segregate from the differentiated primitive endoderm (PE) or HYPO expressing GATA6 or SOX17 ([136–142], reviewed in [143]).

In the mouse, the HIPPO/YAP signaling pathway is crucial for the specification of ICM and TE, as outer cells at the 16-cell stage with less cell-to-cell contact polarize and down-regulate the HIPPO signaling pathway. Subsequently, YAP localizes to the nuclei in outside cells and activates TEAD4, leading to the expression of TE-specific genes, such as Gata3 or Cdx2 ([144,145], reviewed in [146]). Despite recent gene expression analysis, which indicated differences in early lineage specification in the mouse and other mammals, such as human [147–150] and cow [151], Gerri et al. [152] found an evolutionary conserved molecular cascade that initiates TE segregation in human, cow and mouse embryos. HIPPO signaling pathway effectors and TE-associated factors are conserved in cells that initiate the TE program in morula stage embryos of these species, which was confirmed by single-cell RNA-sequencing (scRNA-seq) datasets, immunofluorescence staining and inhibition of modulators of the first lineage segregation. Nevertheless, the group confirmed a different expression pattern of SOX2, a specific marker of the ICM. In the mouse morula, the transcription factor SOX2 is restricted to the inner cells via the HIPPO pathway and considered to be the first marker of pluripotency [153]. In bovine embryos, SOX2 was detected in some blastomeres from the 8-cell stage on, whereas in human embryos SOX2 was expressed in all nuclei. Expression of SOX2 in nuclei of human and cow morulae continues until formation of the expanded blastocyst, where it is finally restricted to cells of the ICM. This is in contrast to mouse, where the restriction starts earlier [152].

In the mouse, HIPPO/YAP signaling also plays a crucial role during EPI

formation, where the TEAD-YAP dependent variable expression of pluripotency factors, such as SOX2, induces formation of EPI in the ICM. Variations in TEAD activity resulted in a higher proportion of unspecified cells, which are eliminated by cell competition, resulting in a high-quality EPI [154].

The modulation of signaling during lineage differentiations with exogenous factors or inhibitory small compounds is a widely used strategy in developmental studies. During the second lineage differentiation, FGF4/MAPK signaling is vital for PE formation and blocks NANOG expression, resulting in a salt-and-pepper distribution of EPI and PE precursor cells in the ICM (Figure 2). In mouse embryos, inhibition of this pathway leads to a complete ablation of GATA6 and all cells express NANOG [155,156]. However, in bovine embryos inhibition of FGF4/MAPK signaling increases the number of NANOG expressing cells, but only partially blocks GATA6 expression [157]. A more precise marker of the HYPO in bovine embryos is SOX17, as it is mutually exclusive with NANOG already by day 8. Inhibition of MAPK in N2B27 medium showed a dose-dependent response, where increasing the concentration of the inhibitor eventually completely ablated SOX17 expression [158]. When bovine embryos are cultured in the 2i system, which activates the WNT pathway and inhibits MAPK, NANOG expression is increased and GATA6 still present [159]. Therefore, FGF4 signaling is not crucial for GATA6 expression in cattle and a different, so far unknown factor needs to be considered. Interestingly, in human embryos no effect of MAPK inhibition is seen, thus representing an FGF4-independent formation of the HYPO in contrast to other species [157,160]. Similar to cattle, pig embryos treated with MAPK inhibitors showed a severely decreased number of HYPO cells, whereas the number of EPI cells remained unchanged [161,162]. In rabbit, MAPK inhibition has no effect on the expression of EPI markers, but PE markers, such as SOX17, are lost, increasing the proportion of cells that show neither EPI nor PE identity. GATA6 expression on the other hand remained unchanged, indicating that maturation of this cell lineage requires FGF signaling in rabbit ([163], reviewed in [164]).

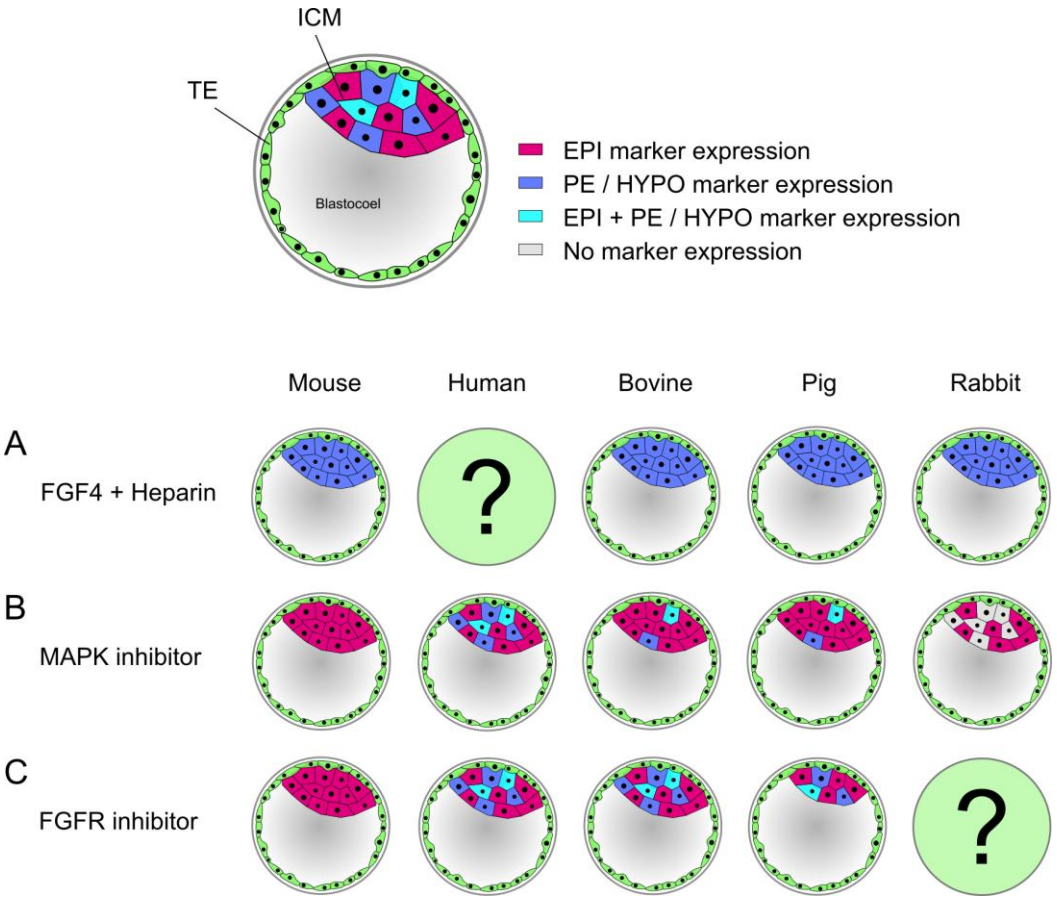

Figure 2: Effects of modulators during second lineage specification vary between mammals. (**A**) Supplementation of FGF4 and heparin leads to a ubiquitous expression of primitive endoderm (PE) and hypoblast (HYPO) markers (dark blue) in mouse and bovine, pig, and rabbit blastocysts, respectively. In human, no experiment has been reported. (**B**) Inhibition of MAPK induces a pan-ICM epiblast (EPI) marker expression (magenta), in sharp contrast to human, where it has no effect. In bovine and pig blastocysts, inhibition of FGF/MAPK pathway does not prevent formation of HYPO precursor cells, though the number of HYPO marker expressing cells is significantly reduced. In bovine, a significant shift towards EPI identity is seen (in pig not significant). Rabbit embryos treated with MAPK inhibitors show no effect on EPI marker expressing cells, but HYPO marker expression was abolished, hence leaving cells with no marker expression (gray), where the identity is unknown. (**C**) Treatment of embryos upstream of MAPK pathway with FGF-receptor inhibitors display a homogenous PE marker expression in mouse blastocysts, similar to MAPK inhibition. In human and bovine embryos, FGFR inhibitors have no effect, whereas in pig embryos, a decreased ICM was reported, but showing a normal distribution of EPI and HYPO markers. In rabbit embryos, the effect is still unknown.

When treating embryos with exogenous FGF4 and heparin, mouse, bovine, pig and rabbit embryos show the same effect: the ICM completely consists of GATA6 or SOX17 expressing cells, suggesting that FGF4 signaling directs GATA6 expression in these embryos ([156,157,162,163], reviewed in [164]). To block the

pathway upstream of MAPK, FGF-receptor (FGFR) inhibitors can be used. While in the mouse the ICM again consists only of NANOG expressing cells [165], in human and bovine there is no effect on the lineage precursor cells [157,166]. In the pig, the ICM decreases in cell number while showing an unchanged expression pattern of EPI and HYPO markers [162]. These findings illustrate, that only in the mouse differentiation of the PE is entirely dependent on FGF4/MAPK, while all other examined species seem to regulate this process in an alternative manner.

Recently, MAPK/ERK signaling dynamics were investigated more closely via single-cell resolution in the mouse model, which was for the first time able to show a transient inactivation of ERK. First, active ERK was present in both ICM and TE as a consequence of FGF signaling. Subsequently, a subset of mitotic events resulted in short pulses of ERK inactivity in both daughter cells, which later showed elevated NANOG and decreased GATA6 levels. By contrast, non-sister cells exhibited a different signaling pattern, similar to expression patterns reported in embryonic stem cells (ESC) [167,168]. A high ERK activity is found throughout all stages of murine preimplantation development, and only during blastocyst formation a transient ERK inhibition in a subset of cells was found, supporting reports that suggested a low ERK activity resulting in EPI specification, while high ERK activity induces PE formation. This transient ERK inactivation indicates a coordination of cell cycle, signaling and differentiation during embryo formation [168,169].

Another pathway, which plays a vital role in maintaining pluripotency is the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway. In the mouse, FGF activates JAK/STAT and increases transcription of ground state pluripotency targets. In bovine, the JAK/STAT pathway was found to be crucial for ICM formation and expression of pluripotency factors, similar to mouse [170].

With regard to OCT4/POU5F1—a key pluripotency transcription factor important for lineage differentiation and maintenance of pluripotency—differences during differentiation between rodent and both human and bovine became apparent. In the mouse, *Oct4* is actively silenced by CDX2 in the TE [151], which is unique, because all other examined species co-express both factors in the TE, reviewed in [108]. These unique regulatory networks might have evolved from different implantation and placentation strategies ([151], reviewed in [171]). OCT4

deficiency in mouse blastocysts causes lack of GATA6 and NANOG persistence [172,173], whereas bovine and human *OCT4*-KO blastocysts lack NANOG, while GATA6 is still expressed [110,150]. In human and mouse, the HYPO or PE specific marker SOX17 is not expressed in the absence of OCT4, indicating a cell-autonomous requirement of OCT4 during the second lineage differentiation [174].

A *SOX2*-KO model in pigs underlined the importance of SOX2 for ICM formation and cell proliferation in porcine early stage embryogenesis in consistence with the mouse model, where targeted embryos formed a blastocoel but failed to form an ICM. Conversely, Sox2 overexpression in murine 1- and 2-cell embryos led to developmental arrest before the morula stage, whereas in porcine 2-cell embryos, SOX2 overexpression did not hamper blastocyst formation [175–177]. It was speculated that the expression of exogenous SOX2 via a DNA-lipofectamine system is delayed by ZGA, which starts at 4-cell stage in pigs and thus did not affect early embryonic gene expression [175]. Alternatively, high levels of SOX2 could lead to differentiation as seen in human ESCs, where SOX2 overexpression led to differentiation towards TE cells [178].

With scRNA-seq, another tool is now available to analyze developmental processes in an unprecedented manner. The transcriptome of each single cell within the embryo may now be examined, be it the modulation of various signaling pathways as shown above, or the existence of a naïve pluripotency signature in the morula (day 4–5) and ICM of early blastocyst (day 5–6) in pig [161]. In bovine embryos, scRNA-seq showed an asynchronous blastomere development during the phase of major genome activation [179]. ScRNA-seq opens the way for new approaches to delineate cell fate progression in embryos of large animals. In human and mouse embryos, a characterization of embryogenesis on a genome wide molecular level has already been reported [180,181]. By comparison of rodent, human, and marmoset embryos, a considerable portion of mouse pluripotency associated factors was not found in the ICM of human and non-human primate blastocysts [180]. As mentioned above, scRNA-seq data was used to declare a conserved TE initiation program in mouse, human, and bovine embryos [152].

Live cell imaging is another new instrument which expands the available toolbox. As bovine and porcine embryos show a lipid-rich dark cytoplasm, time-lapse cinematography is limited, making confocal microscopy the method of choice

when nuclear or chromosomal dynamics are of interest. Yao et al. [182] performed zygote injections in bovine IVF embryos using mRNA for α-tubulin tagged with enhanced green fluorescent protein (EGFP) as a microtubule marker and histone H2B fused with mCherry as a chromatin marker. This enabled the analysis of nuclear or chromosomal integrity from 1-cell up to blastocyst stage even in spite of the dark cytoplasm. Thus, it was possible to detect a relationship between nuclear abnormalities with embryonic development and morphological quality. A combination of live cell imaging, scDNA-seq and genetic manipulations was used to investigate mitotic divisions and chromosome segregation in bovine embryos, shedding light on the molecular pathways that regulate chromosome fidelity during the error-prone cleavage stage of mammalian embryogenesis [183].

In mouse embryos, live cell imaging revealed new insights in kinetics of transcription factors during cell segregation in vivo. A fluorescence decay after photoactivation assay monitored the location and movement as well as the decay of OCT4, revealing two subpopulations in the early embryo. Cells with slower OCT4 kinetics were more likely to give rise to a pluripotent cell lineage in the ICM, whereas cells with faster OCT4 kinetics segregated to outer cells, indicating that cells of the embryo differ in accessibility of target genes before the physical segregation in inner and outer cells [184]. In the 4-cell embryo, SOX2 engaged in more long-lived interactions with the DNA than OCT4 and varied between cells. Blastomeres displaying more SOX2 binding to DNA were found to contribute more progeny to the pluripotent inner cells of a murine 16-cell stage embryo, thus SOX2-DNA binding predicts cell fate as early as the four-cell stage. This highlights the benefit of this noninvasive imaging method to relate heterogeneities in transcription factor binding with the first cell fate determination ([185,186], reviewed in [187]).

3.5. Conclusions and outlook

The embryo as research specimen to study preimplantation development in domestic species can be produced in many ways. In choosing the optimal protocol, the focus lies on the production efficiency, reproducibility and the generation of bona fide samples. The embryo that most closely resembles the biology of preimplantation development is produced in vivo without implementation of any ART. While this would generate bona fide samples, the

low efficiency especially in uniparous species and no access for experimental procedures make this approach impractical. To increase the yield during in vivo production in cattle, superstimulation offers a long established and easy to perform method. Drawbacks are the animals' variable response to hormonal treatment and alteration of gene expression patterns in the embryos. IFOT in cows may also increase the yield and provides access to immature COCs before transfer, but variation in blastocyst rates and a high technical effort are disadvantages of this procedure.

IVP of embryos has a great efficiency regarding blastocyst rates and the availability of ovaries from a nearby abattoir is the only requirement for conducting IVP on a regular basis without any animal husbandry. Every step in the development of an embryo can be observed and manipulated during in vitro culture, raising the opportunity to conduct countless different experiments with great sample sizes and thus a high reproducibility. Embryos from IVP show a different transcriptome signature when compared to their ex vivo counterparts and the culture environment has a great impact on development, which must be considered when designing experiments.

In several mammals, including human [188], mouse [189], cow [190,191], pig [192] and sheep [193], it was shown that IVP derived male embryos develop faster to the blastocyst stage compared to female embryos. Variable growth, metabolism, and (epi)genetic programming before implantation may be due to different responses of males and females to changing conditions in environment, including female X-chromosome dosage compensation [194,195]. X-inactivation in mammals is still a topic with open questions and species-specific differences were reported [196,197]. In cloned embryos, abnormal development in both sexes was shown and a connection to variations in X-inactivation was established (reviewed in [97]). These differences between sexes and the effect of in vitro culture on kinetics and epigenetic reprogramming must be considered. Variable mechanisms regarding X-inactivation should be kept in mind when comparing X-linked gene expression of different species.

Components in the culture medium may also bias experiments, e.g., BSA was reported to alter the effect of exogenous FGF4 on mouse embryos [198].

It is possible to perform genetic manipulations directly in the embryo during IVP

using zygote injection or electroporation, where mutations can be induced at a high frequency and also more complex alterations can be achieved. Besides using genetically modified animals combined with in vivo development of the embryo, this method offers the specimen closest to the biology of preimplantation development, while enabling genetic studies and the advantages of in vitro culture. Nevertheless, potential drawbacks are the frequent occurrence of mosaicism and only little available material to thoroughly investigate the genotype while simultaneously conducting experiments. SCNT provides the possibility to genetically modify somatic donor cells, which can be clonally expanded and genotyped including possible off-target effects. As every embryo generated then has the exact same genotype, great reproducibility is achieved, and albeit the SCNT procedure being the most artificial technique in producing embryos with its known effects on the embryo, this procedure opens the door especially to more complex experiments. When proper controls are implemented in the experimental setup, the observed effects on embryo development can be traced back to either being due to the actual experiment or the SCNT procedure. A combination of IVP or SCNT with transfer to the oviduct or uterus of a surrogate provides a natural environment and the possibility of studying developmental stages that at the moment cannot be sustained in vitro.

Modern gene editing tools in combination with highly developed ART in domestic large animals offer a platform to challenge the open questions in mammalian preimplantation development. As an example, the role of maternal Oct4 transcripts stored in the oocyte has been investigated in the mouse using a conditional knockout of *Oct4* in oocytes [172,173]. To achieve this in bovine, female primary transgenic cells expressing Cre-recombinase under the control of the ZP3 promoter, which is active in growing oocytes, and a floxed OCT4 gene are required. These cells are used for SCNT to produce a cow, from which using OPU a great number of oocytes can be retrieved for IVP of embryos. Together with sperm from a heterozygous *OCT4* knockout bull, it would be then possible to produce embryos where neither maternal nor zygotic OCT4 is present.

Cutting-edge research in the mouse helped us to better understand how the first events of differentiation are induced and regulated and how pluripotency is maintained during preimplantation development. However, species-specific differences during early preimplantation development strengthened the

importance of models other than mouse.

Very recently, the first model of a human embryo was introduced, which was developed by reprogramming fibroblasts into in vitro 3D models of the human blastocyst, called iBlastoids, which could help to overcome the scarcity of human material in the future [199].

Nevertheless, bovine and pig models are excellent alternative model organisms to be studied, not only for their similarities to human development, but also for their availability and the established ARTs in combination with the phenomenal tools of gene editing. Together with newly developed analysis techniques, such as single-cell RNA-sequencing for live cell imaging, a comprehensive toolbox is now available which supports the potential of large domestic animals in the field of developmental biology. Bovine and pig embryos are more than an alternative—they are crucial for a complementary understanding of mammalian preimplantation development.

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Abbreviations

3D	Three-dimensional
AETE	Association of Embryo Technology in Europe
AI	Artificial insemination
AMH	Anti-Müllerian hormone
ART	Assisted reproductive technology
BSA	Bovine serum albumin
COC	Cumulus-oocyte complex
d0	Day 0
DSB	Double strand break
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EPI	Epiblast
ESC	Embryonic stem cells
ET	Embryo transfer
FGFR	FGF-receptor
FSH	Follicle-stimulating hormone
GE	Genetically engineered
HDR	Homology-directed repair
HYPO	Hypoblast
ICM	Inner cell mass
ICSI	Intracytoplasmic sperm injection
IFOT	Intrafollicular oocyte transfer
IVF	In vitro fertilization
IVM	In vitro maturation

IVP	In vitro production
JAK/STAT	Janus kinase/signal transducer and activator of transcription
NHEJ	Non-homologous end joining
OPU	Ovum pick-up
PE	Primitive endoderm
PHD	Post-hatching development
RNA-seq	RNA-sequencing
SCNT	Somatic cell nuclear transfer
scRNA-seq	Single-cell RNA-sequencing
ZGA	Zygotic genome activation
ZI	Zygote injection

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III. PUBLICATION

The following part of the thesis has been published in Cells (MDPI) on 28 August 2021.

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Hypoblast Formation in Bovine Embryos Does Not Depend on NANOG

Abstract: The role of the pluripotency factor NANOG during the second embryonic lineage differentiation has been studied extensively in mouse, although species-specific differences exist. To elucidate the role of NANOG in an alternative model organism, we knocked out *NANOG* in fibroblast cells and produced bovine *NANOG*-knockout (KO) embryos via somatic cell nuclear transfer (SCNT). At day 8, *NANOG*-KO blastocysts showed a decreased total cell number when compared to controls from SCNT (NT Ctrl). The pluripotency factors OCT4 and SOX2 as well as the hypoblast (HB) marker GATA6 were co-expressed in all cells of the inner cell mass (ICM) and, in contrast to mouse *Nanog*-KO, expression of the late HB marker SOX17 was still present. We blocked the MEK-pathway with a MEK 1/2 inhibitor, and control embryos showed an increase in NANOG positive cells, but SOX17 expressing HB precursor cells were still present. *NANOG*-KO together with MEK-inhibition was lethal before blastocyst stage, similarly to findings in mouse. Supplementation of exogenous FGF4 to *NANOG*-KO embryos did not change SOX17 expression in the ICM, unlike mouse *Nanog*-KO embryos, where missing SOX17 expression was completely rescued by FGF4. We conclude that NANOG mediated FGF/MEK signaling is not required for HB formation in the bovine embryo and that another—so far unknown—pathway regulates HB differentiation.

Keywords: NANOG; SOX17; bovine preimplantation development; MEK; second lineage differentiation

1. INTRODUCTION

Before implantation, mammalian embryos undergo two consecutive lineage specifications. First, outer and inner cells in the morula form the surrounding CDX2-expressing trophectoderm (TE) and the inner cell mass (ICM), respectively, during blastocyst development. Second, within the ICM, NANOG-expressing cells form the pluripotent epiblast (EPI) and exhibit a mutually exclusive expression pattern with differentiated cells expressing GATA6 and SOX17 from the primitive endoderm (PE) or hypoblast (HB) in bovine and human embryos. Consequently, three distinct cell lineages arise: the EPI, which will give rise to the embryo proper, the PE/HB, which will form the yolk sac, and the TE, responsible for extraembryonic tissues and implantation [1–8], reviewed in [9]. While these landmarks of preimplantation embryonic development are conserved between mammalian species, fundamental differences exist regarding the regulation of the second lineage segregation. Human and bovine *OCT4*-knockout (KO) embryos lose NANOG and maintain GATA6 expression, whereas mouse *Oct4*-KO embryos still express NANOG and fail to develop a PE [10–13]. Additionally, FGF4 signaling via the mitogen-activated protein kinase (MAPK) pathway, also called MEK pathway, has different roles in the regulation of the second lineage differentiation between species. It is known that in mouse, EPI precursor cells express FGF4, which via the FGF receptors 1/2 (FGFR) and the MEK-pathway induces and regulates the formation of the PE (reviewed in [14]). Inhibition of the MEK pathway or the FGFR in mouse embryos results in an ICM only expressing NANOG, while GATA6 expression is completely lost [15–17]. In both human and bovine embryos, inhibition upstream of MEK via FGFR inhibitors has no effect on EPI or HB formation [18]. In bovine embryos, MEK inhibition increases NANOG expression and reduces HB markers, but HB marker expression is still present. In human embryos, MEK inhibition has no effect, suggesting that bovine and human HB formation is partly or completely independent of this pathway, and that these species regulate the second lineage differentiation differently [18–21].

Recently, a dosage-dependent effect of the MEK-inhibitory compound PD0325901 (PD032) was found in bovine embryos. A concentration of 2.5 µM eliminated expression of the later HB-marker SOX17 completely [22], while previous studies observed maintenance of the early marker GATA6 at

concentrations of 0.5 and 1 µM [18,20], challenging the hypothesis of a partly MEK-independent HB formation in bovine.

Supplementing exogenous FGF4 from morula to blastocyst stage leads to ubiquitous GATA6 expression in mouse, bovine, pig and rabbit embryos (reviewed in [23]), revealing a non-cell-autonomous role for FGF4.

NANOG is a member of the homeobox family of DNA binding transcription factors that is known to maintain the pluripotency of embryonic stem cells (ESCs) together with OCT4 and SOX2 [6,24]. In mouse, *Nanog*-KO does not affect the formation of the blastocyst, but during the second lineage differentiation, the EPI lineage fails. Thereby, ubiquitous expression of the early PE marker GATA6 within the ICM was reported, whereas the late PE markers SOX17 and GATA4 were lost [1,2,25]. SOX17 expression in mouse *Nanog*-KO embryos is rescued by supplementing exogenous FGF4, confirming the crucial role of FGF4 expressed by EPI cells for PE differentiation [2]. Mouse *Nanog*-KO embryos and ESCs lose viability in the presence of MEK inhibitors, resulting in early cell death [2,26]. So far, the phenotype of *NANOG*-KO in human embryos or ESCs has not been investigated, but a *NANOG*-knockdown experiment in human ESCs showed that, while NANOG represses embryonic ectoderm differentiation, it does not influence the expression of OCT4 or SOX2 [27]. In bovine embryos, NANOG is first expressed at the morula stage and becomes specific to EPI precursor cells in the ICM at day 7 [18,28,29]. Only recently, a *NANOG*-KO via zygote injection was first described in bovine, displaying pan-ICM GATA6 expression and reduced transcript levels for the pluripotency factors SOX2 and H2AFZ [30].

In the present study, we addressed the role of NANOG in bovine preimplantation embryos using a reverse genetics approach. After induction of a NANOG frameshift mutation in fibroblasts and production of embryos via somatic cell nuclear transfer (SCNT), we characterized the *NANOG*-KO phenotype by immunofluorescence staining of day 8 blastocysts for markers of EPI and HB precursor cells. We further addressed the roles of FGF4 and the MEK pathway by treating *NANOG*-KO embryos with exogenous FGF4 and with an inhibitor of the MEK pathway, respectively, revealing new insights into the second lineage differentiation in bovine embryos.

2. MATERIAL & METHODS

2.1. CRISPR/Cas9-mediated knockout of *NANOG* in adult fibroblasts

We induced a frameshift mutation in a non-homologous end joining approach with an sgRNA (5'-CTCTCCTCTTCCCTCCTCCA-3') designed by Synthego software (V2.0) using ENSBTAT00000027863 (NANOG) as reference gene (design.synthego.com, accessed on 15 July 2021). The sgRNA targeting exon 2 of NANOG was cloned into pSpCas9(BB)-2A-Puro (PX459) V2.0, a gift from Feng Zhang [31]. All experiments are based on a cell line with origin in bovine adult ear fibroblast cells that were isolated in the laboratory in the Chair for Molecular Animal Breeding and Biotechnology, Ludwig-Maximilians-Universität München, 85764 Oberschleissheim, Germany. Bovine fibroblasts were transfected with the Nucleofector device (Lonza; Basel, Switzerland) according to the manufacturer's instructions. After selection with 2 µg/mL puromycin (Sigma-Aldrich; St. Louis, MO, USA) for 48 h, we produced single-cell clones as described previously [32]. After PCR amplification with primers 5'-GGAAGGGATTCCTGAAATGAG-3' (forward) and 5'-GTGGGATCTTAGTTGCGACAT-3' (reverse), gene editing-induced modifications in the NANOG alleles and naturally occurring SNPs were examined by Sanger sequencing using the primers 5'-AAGGTCTGGGTTGCAATAGG-3' (forward) and 5'-CCACCAGGGAAATCCCTTATTT-3' (reverse). All primers were synthesized by Biomers.net (Ulm, Germany; accessed on 15 July 2021).

2.2. Production and analysis of SCNT and IVP embryos

SCNT and in vitro production (IVP) procedures were performed as described previously [33]. Briefly, bovine ovaries were collected at a slaughterhouse, and retrieved cumulus-oocyte-complexes were matured in vitro. After SCNT or fertilization of the oocytes (day 0), fused complexes and presumptive zygotes were cultivated in synthetic oviductal fluid including Basal Medium Eagle's amino acids solution (BME, Sigma-Aldrich), Minimum Essential Medium (MEM, Sigma-Aldrich) and 5% estrous cow serum (OCS) from day 0 or day 1 up to day 8 for SCNT or IVP zygotes, respectively. After 8 days of culture, the zona pellucida was removed enzymatically using Pronase (Merck Millipore; Burlington, MA, USA), and embryos were fixed in a solution containing 2% paraformaldehyde [34].

2.3. Modulation of signaling pathways

Growth factors or inhibitors were supplemented from day 5 morula stage until day 8 blastocyst stage. The MEK1 and MEK2 inhibitor PD032 (Tocris; Bristol, UK) was used at 0.5 or 2.5 µM, and controls were cultured in equal amounts of DMSO (Sigma-Aldrich). 1 µg/mL human recombinant FGF4 (R&D Systems; Minneapolis, MN, USA) was added to synthetic oviductal fluid with 1 µg/mL heparin (Sigma-Aldrich).

2.4. Immunofluorescence staining and confocal laser scanning microscopy

Before staining, embryos were incubated for 1 h at room temperature in a blocking solution containing 0.5% Triton X-100 (Sigma-Aldrich) and 5% donkey serum (Jackson ImmunoResearch; West Grove, PA, USA) or fetal calf serum (Thermo Fisher Scientific; Waltham, MA, USA) or both sera sequentially, depending on the species origin of the secondary antibodies. Double staining for either NANOG/GATA6, NANOG/SOX17, SOX17/SOX2, OCT4/SOX2 or GATA6/CDX2 was achieved by incubation overnight at 4 °C in primary antibody solution and transfer to secondary antibody solution at 37 °C for 1 h after washing 3 times. The antibodies used and the applied dilutions are presented in Supplementary Table S1. Labeled embryos were mounted in Vectashield mounting medium containing 40,6-diamidino-2-phenylindole (DAPI, Vector Laboratories; Burlingame, CA, USA) in a manner that conserved the 3D structure of the specimen [35]. Z-stacks of optical sections with an interval of 1.2 µm were recorded using an LSM710 Axio Observer confocal laser scanning microscope (CLSM; Zeiss, Jena, Germany) with a 25 × water immersion objective (LD LCI Plan-Apochromat 25×/0.8 Imm Korr DIC M27) or a Leica SP8 CLSM (Leica;Wetzlar, Germany) with a 40 × water immersion objective (Leica; 1.1NA), respectively. DAPI, Alexa Fluor 488, 555, and 647 were excited with laser lines of 405 nm, 499 nm, 553 nm, and 653 nm (LSM710), respectively, or with a white light laser (SP8).

2.5. Statistical analysis

Statistical analysis was performed using Graphpad Prism 5.04. After checking normal distribution of data with a Kolmogorov–Smirnov test, we performed nonparametric tests. For pairwise comparisons, a two-tailed Mann–Whitney U test

was performed, whereas for three experimental groups, a Kruskal–Wallis test and subsequent Dunn's multiple comparisons test as post hoc test was applied. The level of significance was set to $p < 0.05$. Data are presented as mean ± standard deviation (SD). For quantitative image analysis, the ImageJ (V 1.53c) cell counter plugin was used [36]; control embryos with less than 8 cells in the ICM were excluded from the analysis.

3. RESULTS

3.1. *NANOG*-KO has no effect on blastocyst rate but results in reduced total cell number

After selection with puromycin, we generated 57 single-cell clones and achieved a mutation rate of 54.4% including 8 homozygous mutations (14.0%). Two different cell clones with an identical homozygous insertion of a single nucleotide, which induces a frameshift mutation, were used for SCNT to produce embryos without NANOG (*NANOG*-KO). SCNT embryos from two different cell clones with no mutation from the same transfection experiment (NT Ctrl) and embryos produced by in vitro fertilization (IVP Ctrl) served as controls. There were no differences regarding blastocyst rates between embryos from all four cell clones. SCNT embryos from both *NANOG*-KO cell clones showed consistent alterations as described below. The group of NT Ctrl embryos derived from the two *NANOG*-intact cell clones was phenotypically homogeneous. To verify the absence of NANOG protein, *NANOG*-KO blastocysts (n = 9) were stained for NANOG using two different antibodies, and no positive cells were observed (Supplementary Figure S1A). There was no significant difference between *NANOG*-KO and Ctrl embryos regarding blastocyst rates. *NANOG*-KO embryos were able to expand but appeared to be smaller than NT Ctrl blastocysts (Supplementary Figure S1B), with significantly decreased diameters of day 8 *NANOG*-KO compared to NT Ctrl blastocysts (Supplementary Figure S1C). We analyzed the total cell number and the number of ICM and TE cells using SOX2 and CDX2 as markers, respectively. We found a significant reduction in both lineages in *NANOG*-KO embryos, while no significant difference in the ratio of ICM to total cell number was seen, showing a proportionally normal distribution of cells to ICM and TE during the first lineage differentiation in the absence of NANOG (Table 1).

Table 1. Developmental rates and cell numbers of day 8 *NANOG*-knockout (*NANOG*-KO), nuclear transfer control (NT Ctrl), and in vitro-produced control (IVP Ctrl) embryos. Data are presented as mean ± standard deviation. Different superscript letters (a, b) within a row indicate significant differences ($p < 0.05$). Data were analyzed by Kruskal–Wallis test with Dunn's multiple comparisons test as post hoc test.

Experimental Group	*NANOG*-KO	NT Ctrl	IVP Ctrl
No. of experiments	8	5	17
Blastocysts/zygotes [%]	29.0 ± 12.9	36.1 ± 9.9	28.3 ± 4.2
No. of analyzed embryos	25	32	51
Total cell number	66.4 ± 27.3 [a]	148.6 ± 65.6 [b]	177.4 ± 52.2 [b]
ICM/total cell number [%]	23.8 ± 11.3	29.2 ± 11.0	29.0 ± 7.3
ICM number	15.8 ± 10.5 [a]	43.5 ± 24.5 [b]	51.3 ± 20.0 [b]
TE cell number	50.6 ± 22.6 [a]	105.1 ± 47.2 [b]	126.1 ± 40.5 [b]

3.2. NANOG is dispensable for expression of pluripotency factors and hypoblast markers

We stained *NANOG*-KO, NT Ctrl, and IVP Ctrl day 8 blastocysts for GATA6/CDX2 (Figure 1A) and SOX17/SOX2 (Figure 1B). In both control groups, embryos showed consistent co-expression of CDX2 with GATA6, and a subset of the CDX2 negative ICM cells was also GATA6 negative.

Staining of NT Ctrl (n = 9) and IVP Ctrl blastocysts (n = 18) for NANOG and GATA6 (Supplementary Figure S2A) confirmed that GATA6 negative cells express NANOG, resulting in the previously reported mutually exclusive expression of these lineage markers [16,18]. SOX2 was expressed in the entire ICM, and a subset of cells already expressed the late HB marker SOX17. In *NANOG*-KO day 8 blastocysts, an ICM was clearly discernible by CDX2 negative cells, while GATA6 was expressed ubiquitously with no negative cells in the ICM or TE. The ratio of SOX17 positive cells within the ICM increased significantly compared to NT Ctrl (61.6% ± 25.7% vs. 38.6% ± 19.6%, respectively) but not IVP Ctrl (56.1% ± 13.5%), while cells with exclusive SOX2 expression were still present, albeit at reduced numbers (Figure 1D).

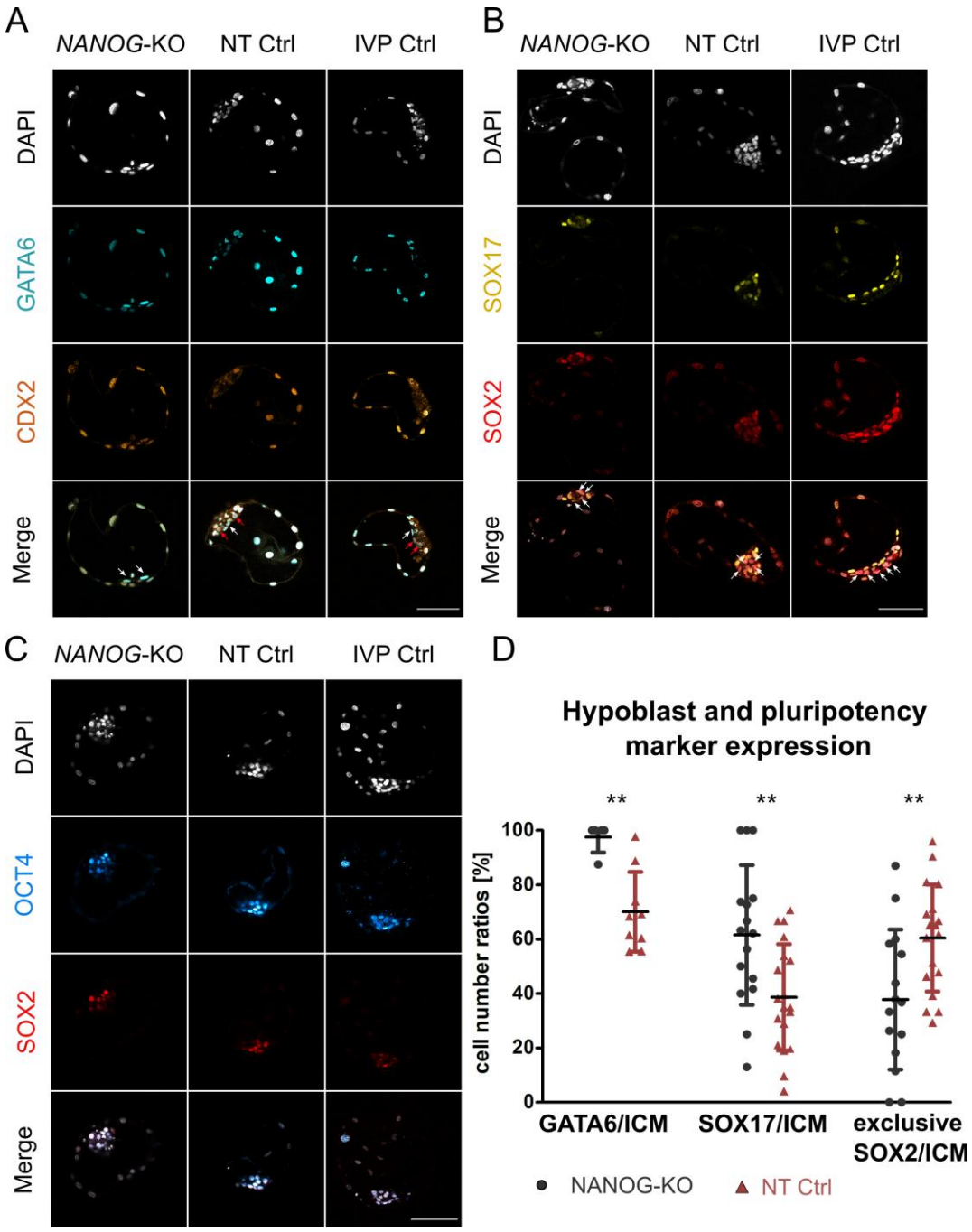

Figure 1 (legend on next page).

Figure 1: Expression of hypoblast and pluripotency markers in *NANOG*-KO and control groups. Representative confocal planes of day 8 blastocysts stained for GATA6/CDX2 (**A**) and SOX17/SOX2 (**B**). Sample sizes of GATA6/CDX2 were n = 5, 9, 14 and of SOX17/SOX2 n = 16, 18, 10 for *NANOG*-KO, NT Ctrl, and IVP Ctrl, respectively. White arrows indicate ICM cells with GATA6 expression (**A**) or exclusive SOX2 expression (**B**), red arrows indicate GATA6/CDX2 double negative cells (**A**) in the ICM. (**C**) Expression of pluripotency factors OCT4 and SOX2. Sample sizes were n = 4, 9, 4 for *NANOG*-KO, NT Ctrl, and IVP Ctrl, respectively. Color codes were: Grey (DAPI), cyan (GATA6), orange hot (CDX2), yellow (SOX17), red (SOX2), and cyan hot (OCT4). (**D**) The ratio of GATA6, SOX17, and SOX2 positive cells within the ICM of *NANOG*-KO (black) and NT Ctrl (red) embryos. SOX2 served as ICM marker in the quantification of SOX17. SOX2 exclusive expression represents cells positive for SOX2 while negative for SOX17. Data were analyzed using a two-tailed Mann–Whitney U test and are presented as mean (%) ± standard deviation. Asterisks (**) indicate significant differences between groups ($p < 0.01$). Sample sizes of GATA6 were n = 5, 9; of SOX17 n = 16, 19; and of SOX2 exclusive n = 15, 18 for *NANOG*-KO and NT Ctrl embryos, respectively. Scale bars indicate 100 µm.

We conclude that NANOG is required for the repression of GATA6 in the ICM. In contrast to mouse *Nanog*-KO embryos that show complete loss of SOX17 [2], we still found SOX17 positive cells in the ICM. However, absence of NANOG and a ubiquitous GATA6 expression is not sufficient to induce a pan-ICM expression of SOX17 in bovine blastocysts. Staining for OCT4 and SOX2 showed that in NT Ctrl and IVP Ctrl embryos, both factors are co-expressed throughout the entire ICM and that in the absence of NANOG, this pattern is maintained (Figure 1C). None of the embryos showed OCT4 expression in the TE at day 8.

3.3. Inhibition of MEK induces cell death in *NANOG*-KO embryos

In the next step, we aimed to investigate the effect of *NANOG*-KO while inhibiting the MEK signaling pathway. Because previous reports on the effect of the MEK 1/2 inhibitor PD032 in bovine embryos are in conflict [18,22], we first set out to test the effect of different dosages on the expression of NANOG and GATA6 in IVP Ctrl embryos. There was no difference between the DMSO control (n = 11) and the dosages 0.5 (n = 4) and 2.5 µM (n = 10) PD032 regarding the blastocyst per morula rate (45.6% ± 12.5%, 46.8% ± 6.1%, 50.0% ± 16.3%, respectively) and the ratio of ICM to total cell number (30.4% ± 5.8%, 29.2% ± 7.8%, 32.5% ± 8.2%, respectively). The number of ICM cells was determined without a specific staining on the basis of the embryos' morphology. In agreement with Kuijk et al. [18], the proportion of NANOG positive cells was markedly

increased, while the expression of GATA6 was reduced but not completely switched off at both concentrations (Figure 2A). Similarly to GATA6, SOX17 was significantly reduced but still present at a concentration of 2.5 µM (Figure 2B). At 2.5 µM, both HB markers were always co-expressed with NANOG and thus failed to establish a mutually exclusive expression pattern.

As a higher dosage (2.5 µM) of PD032 did not affect blastocyst development or cell numbers, we performed inhibition of the MEK pathway in SCNT embryos using this concentration. We found similar blastocyst per morula rates ($p > 0.05$) of NT Ctrl in DMSO ($n = 3$; 52.4% ± 16.7%) and PD032 ($n = 4$; 42.5% ± 14.8%). The expression pattern of NT Ctrl embryos incubated with the MEK inhibitor was comparable to that of IVP Ctrl embryos that underwent the same treatment, as HB markers were still present (Figure 2B). Although treatment of *NANOG*-KO embryos with DMSO did not affect the blastocyst per morula rate ($n = 3$, 57.8% ± 16.2%) when compared to NT Ctrl treated with DMSO, incubating *NANOG*-KO embryos in the presence of PD032 ($n = 5$) resulted in severely compromised viability, and all embryos died. This agrees with findings in mouse embryos and mouse ESCs, where loss of NANOG and inhibition of MEK also result in cell death [2,26].

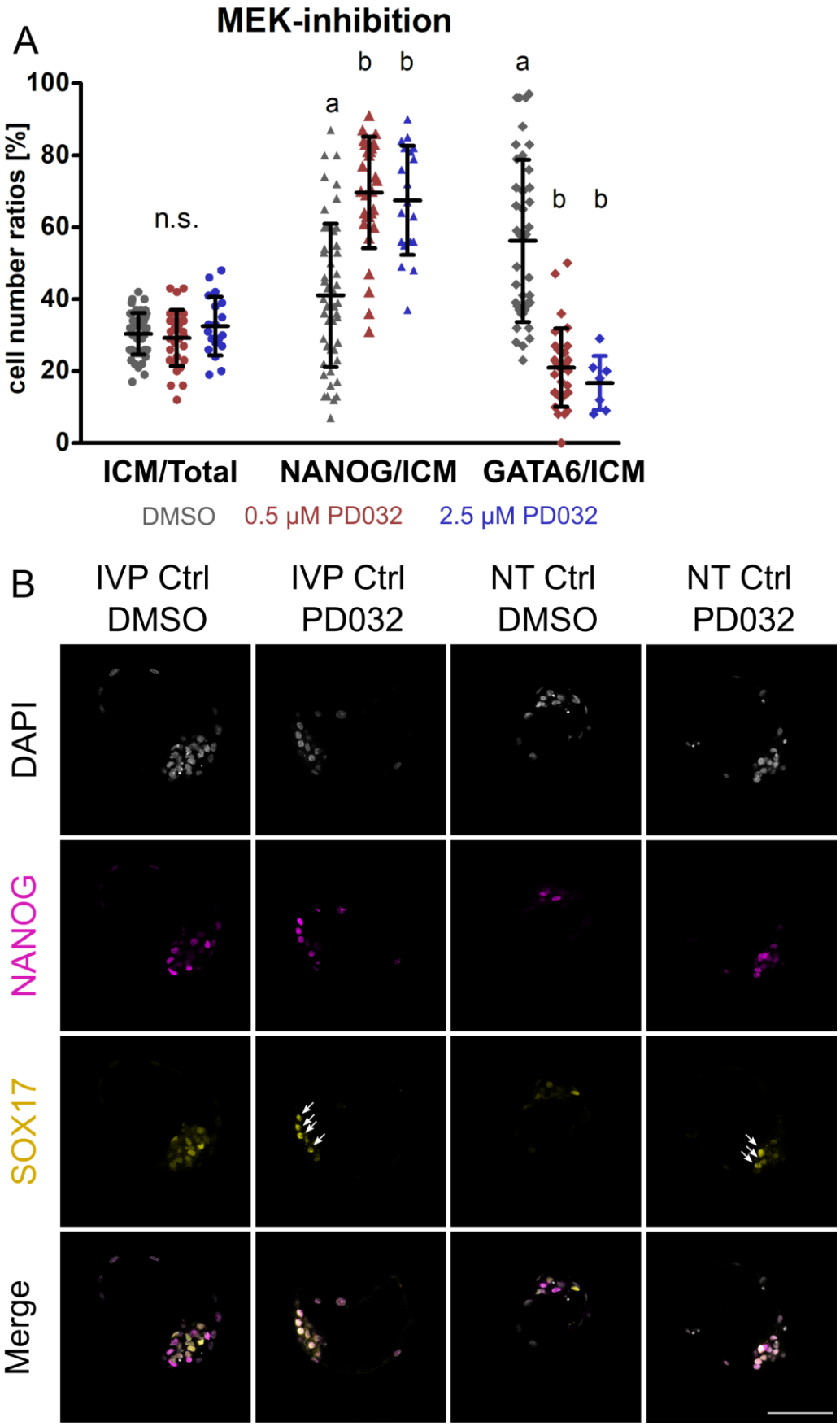

Figure 2: (legend on next page).

Figure 2: The effect of different dosages of MEK-inhibitor PD0325901 (PD032) on the expression of NANOG, GATA6, and SOX17. (**A**) The ratio of ICM/Total, NANOG/ICM, and GATA6/ICM in the presence of 0.5 and 2.5 µM PD032 in IVP Ctrl embryos. The proportion of ICM to total cell number (Total) is shown, and the number of NANOG and GATA6 expressing cells was set in relation to the number of ICM cells. Embryos were cultured from day 5 morula to day 8 blastocyst in the presence of 0.5 and 2.5 µM PD032. Data were analyzed by Kruskal–Wallis with Dunn's multiple comparisons test as post hoc test and are presented as mean ± standard deviation. Different superscripts (a, b) indicate significant differences between groups ($p < 0.0001$), n.s. = not significant. Sample sizes of ICM/Total and NANOG/ICM were n = 49, 30, 19 and of GATA6/ICM n = 40, 30, 7 for DMSO (grey), 0.5 µM (red) and 2.5 µM (blue) PD032, respectively. (**B**) Representative confocal planes of NANOG and SOX17 expression in IVP Ctrl and NT Ctrl blastocysts cultured with DMSO or 2.5 µM PD032. Sample sizes of embryos stained for NANOG (magenta) and SOX17 (yellow) were n = 49 for IVP Ctrl DMSO, n = 19 for IVP Ctrl PD032, n = 8 for NT Ctrl DMSO, and n = 9 for NT Ctrl PD032. DAPI = grey; arrows indicate SOX17 expression in the presence of 2.5 µM PD032; scale bar indicates 100 µm.

3.4. FGF4 in *NANOG*-KO embryos does not convert the entire ICM to hypoblast precursor cells

Subsequently, we investigated whether exogenous FGF4 can induce full SOX17 expression in *NANOG*-KO bovine embryos. In IVP Ctrl and NT Ctrl embryos, treatment with FGF4 completely switched off the expression of NANOG (Figure 3D), and most ICM cells expressed SOX17 (Figure 3E, Supplementary Figure S2B). FGF4 had no effect regarding blastocyst per morula rate and total cell number, while the ratio of SOX2 positive cells, i.e., the ICM, to total cell number was significantly reduced in both groups (Figure 3A–C). Treatment of *NANOG*-KO embryos with FGF4 did not affect the blastocyst per morula rate or the ICM to total cell number ratio, but the total cell number increased significantly with *NANOG*-KO embryos (117.6 ± 48.7 vs. 66.4 ± 27.3 without FGF4 treatment) reaching a total cell number similar to that of untreated NT Ctrl (148.6 ± 65.6, Table 1). In all embryos treated with FGF4, the ubiquitous expression of SOX2 in the ICM was maintained (Figure 4). As the SOX17 expression increased in FGF4 treated Ctrl groups, the exclusive expression of SOX2 was significantly reduced, whereas in mutant embryos, SOX2 exclusive expression remained unchanged (Figure 3F, Figure 4). The percentage of SOX17 positive cells in the ICM did not increase in *NANOG*-KO embryos (Figure 3E), which is in contrast to mouse *Nanog*-KO embryos, where exogenous FGF4 induces SOX17 expression in most of the ICM cells [2]. We conclude that in bovine, NANOG is required for FGF4

mediated expression of SOX17, as FGF4 alone was not sufficient to convert all ICM cells to SOX17 expressing HB precursor cells.

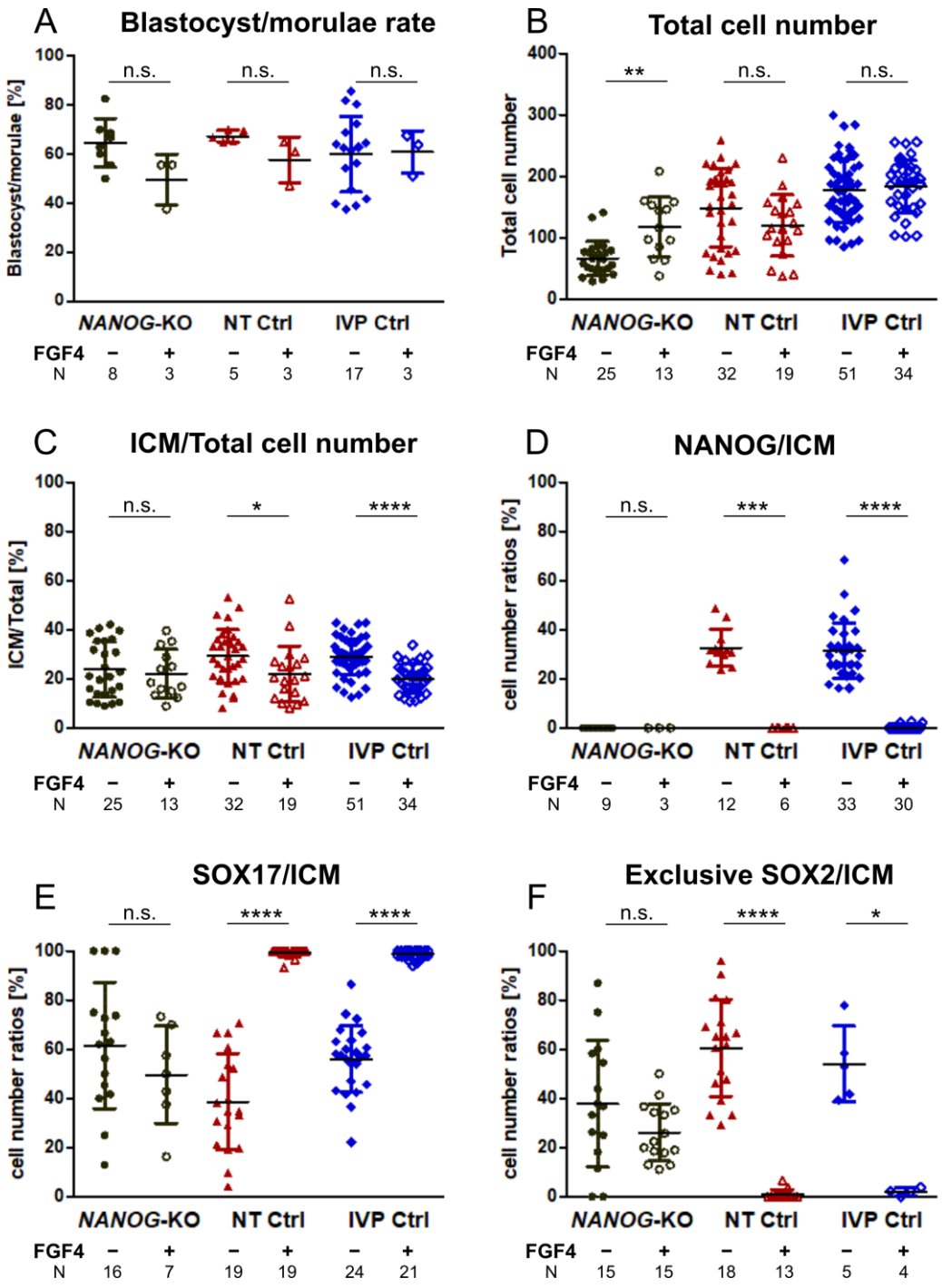

Figure 3: **(legend on next page)**

Figure 3: Developmental rates and cell number ratios of NANOG, SOX17, and exclusive SOX2 in *NANOG*-KO, NT Ctrl, and IVP Ctrl day 8 embryos treated with exogenous FGF4 and heparin. (**A**) Blastocyst per morula rate, (**B**) total cell number, (**C**) proportion of ICM to total cell number, (**D**) ratio of NANOG-positive cells in the ICM, (**E**) ratio of SOX17-positive cells in the ICM, and (**F**) ratio of cells exclusively expressing SOX2 in the ICM of *NANOG*-KO (black), NT Ctrl (red), and IVP Ctrl (blue) embryos without (–) and with (+) FGF4 and heparin are presented. ICM cells were determined by staining of SOX2. Data were analyzed by two-tailed Mann–Whitney U test and are presented as mean (%) ± standard deviation. Asterisks indicate significant effects of FGF4 treatment within embryo group. N = number of analyzed embryos, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; n.s. = not significant.

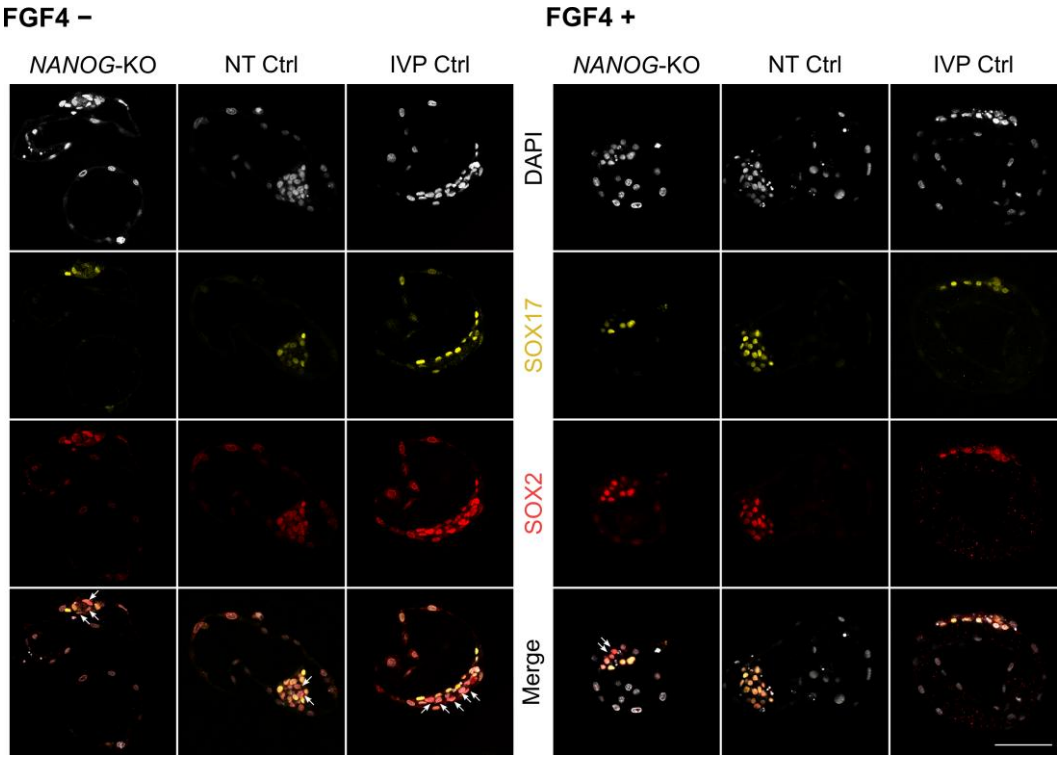

Figure 4: Expression of pluripotency and late hypoblast markers in *NANOG*-KO, NT Ctrl, and IVP Ctrl day 8 embryos treated with exogenous FGF4 and heparin. Representative confocal planes of day 8 blastocysts stained for SOX17/SOX2. Embryos without (–) and with (+) FGF4 and heparin treatment are presented. Arrows indicate ICM cells with exclusive SOX2 expression (SOX17 negative). Color codes are: Grey (DAPI), yellow (SOX17) and red (SOX2). Scale bar indicates 100 µm.

4. DISCUSSION

To investigate the regulation of differentiation and maintenance of pluripotency during mammalian preimplantation development, it is vital to examine models other than mouse, as species-specific differences exist. The bovine embryo is a very suitable alternative, as IVP procedures are highly developed and similarities to human embryo development have been reported (reviewed in [23]).

In this study, we focused on NANOG, because it is not clear whether the role of this pluripotency factor during the second lineage segregation is conserved between mammals. In order to achieve uniform modification of all cells of the embryo, we used SCNT to produce *NANOG*-KO embryos instead of zygote injection, where mosaicism may hamper analyses. To exclude the effects of the SCNT procedure on the phenotype, we implemented two control groups: SCNT embryos generated from transfected cells that maintained the wildtype genotype (NT Ctrl) and embryos from in vitro fertilization (IVP Ctrl). NT Ctrl did not vary from IVP Ctrl embryos in any of the examined parameters, except for the proportion of SOX17 cells in the ICM, which was decreased in NT Ctrl embryos. We set the number of cells expressing lineage marker proteins in relation to the number of ICM cells in order to account for variations due to the different sizes of the embryos, especially the reduced size of *NANOG*-KO embryos. Staining embryos with markers for TE and ICM, i.e., CDX2 and SOX2, respectively, enabled us to quantify reliably the cell numbers in each lineage after the first differentiation. We found that the ratio of ICM to total cell number did not change in *NANOG*-KO embryos, showing that NANOG is not required for proper segregation of TE and ICM, as reported in mouse. On the other hand, we found a significant reduction in total cell numbers, which is in contrast to mouse *Nanog*-KO embryos, where the loss of NANOG does not impede cell proliferation until the E3.5 blastocyst stage [1]. Interestingly, the reduced total cell number in *NANOG*-KO embryos reached normal levels when the embryos were cultivated with exogenous FGF4, where the proliferative impact of FGF4 [17, 37–39] evidently alleviated the reduction of total cell numbers in the absence of NANOG. We hypothesize that in *NANOG*-KO embryos, the absence of FGF4 expressing EPI precursor cells causes the reduced cell number. This suggests that EPI cells express FGF4, which to our knowledge has not been shown yet in bovine but is known in mouse [1].

Although Ortega, et al. [30] found a reduction of SOX2 transcripts in bovine *NANOG*-KO embryos, we detected SOX2 and OCT4 expression in the absence of NANOG on the protein level. To our knowledge, this is the first report on SOX2 expression in the absence of NANOG in a mammalian embryo. We were not able to detect OCT4 in the TE of day 8 blastocysts using a monoclonal antibody, which is in contrast to Berg et al. [40] and Simmet et al. [11], who detected OCT4 in the TE of ex vivo day 11 or in vitro day 7 blastocysts using a different polyclonal antibody, respectively.

In bovine *NANOG*-KO embryos, the ICM ubiquitously expresses the early HB marker GATA6, which agrees with previous reports on bovine and mouse *NANOG*-deficient embryos [2,30]. Interestingly, in bovine *NANOG*-KO embryos, expression of the later HB marker SOX17 was still present, but the absence of NANOG and a ubiquitous GATA6 expression was not sufficient to induce a pan-ICM expression of SOX17, as some cells in the ICM still showed exclusive SOX2 expression, making the regulation of SOX17 in the bovine embryo partly independent of NANOG and GATA6.

This is in sharp contrast to the mouse *Nanog*-KO, where expression of the late PE markers GATA4 and SOX17 completely fails but can be rescued in a chimeric complementation assay or fully induced by exogenous FGF4 [1,2,25].

We further investigated the second lineage segregation in bovine blastocysts by inhibiting the MEK pathway with PD032. In line with previous reports, MEK-inhibition did not completely ablate GATA6 positive cells [18,20], and also SOX17 was still expressed in the ICM. Canizo et al. [22] found a dosage-dependent effect of PD032 with the concentration also applied in this study abolishing all SOX17. The reasons for these contrasting results remain unclear, and we can only speculate that the different embryo culture media have an effect on SOX17 expression in the presence of PD032. Bovine embryos were cultured in PD032 concentrations of up to 100 µM, and reduction of SOX17 transcripts was already achieved at 10 µM, while higher dosages did not further decrease transcript abundance [19]. Treating bovine embryos with a broad-spectrum inhibitor of receptor tyrosine kinases (RTKs) including MEK (BI-BF1120) increased the abundance of SOX17 transcripts, suggesting that SOX17 does not depend on direct activation via the MEK pathway [41]. We further found that HB markers were generally co-expressed with NANOG when the MEK pathway was

blocked. Therefore, we suggest that NANOG mediated repression of HB markers is dependent on MEK signaling. Our data and previous reports indicate that, in bovine embryos, GATA6 and SOX17 are partly independent of the MEK pathway and that a so far unknown factor plays an important role in the regulation of HB differentiation.

When combining inhibition of MEK with loss of NANOG, we found that the viability of those embryos was severely compromised, resulting in cell death. Similar reports exist in *NANOG*-deficient mouse embryos and ESCs, where cell death is observed after adding inhibitors of the MEK-pathway [2,26]. We conclude that HB formation, i.e., expression of GATA6 and SOX17, in the absence of both NANOG and a functioning MEK pathway is associated with cell death. We speculate that apoptosis is induced during the cell sorting process to eliminate cells that do not commit to either EPI or HB, as selective apoptosis was described for appropriate segregation of PE and EPI in mouse blastocysts [5,42]. Nevertheless, our hypothesis cannot explain why the TE is also affected by cell death.

In the mouse, the loss of FGF4 expressing EPI precursor cells leads to complete ablation of late PE marker expressing cells that can be rescued with exogenous FGF4 [2,43]—evidence that FGF4 alone is sufficient to induce PE differentiation. The regulation of SOX17 expression in bovine embryos appears to be different, as FGF4 alone without functional NANOG was not sufficient to convert all ICM cells to SOX17 expressing HB precursor cells. Thus, we conclude that NANOG is required for FGF4 mediated expression of SOX17.

Our results show that in the bovine embryo, the establishment of HB precursor cells is independent of EPI-cell mediated FGF/MEK signaling. This is in sharp contrast to mouse but similar to human, where the FGF/MEK pathway does not regulate the second lineage differentiation [18,21]. An unknown factor induces HB differentiation, and it is of utmost interest to further investigate this pathway and whether it also exists in human embryos as well.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cells10092232/s1, Figure S1: Producing bovine *NANOG*-KO embryos via somatic cell nuclear transfer (SCNT); Figure S2: Expression of epiblast and hypoblast markers in NT Ctrl and IVP Ctrl day 8 embryos; Table S1: Targets, antibodies, suppliers, and applied dilutions for immunofluorescence staining.

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Abbreviations

BME	Basal Medium Eagle’s amino acids solution
CLSM	Confocal laser scanning microscope
EPI	Epiblast
ESCs	Embryonic stem cells
FGFR	FGF-receptor
HB	Hypoblast
ICM	Inner cell mass
IVP	In vitro production
KO	Knockout
MAPK	Mitogen-activated protein kinase
MEM	Minimum Essential Medium
OCS	Estrous cow serum
PD032	PD0325901
PE	Primitive endoderm
RTK	Receptor tyrosine kinase
SCNT	Somatic cell nuclear transfer
SD	Standard deviation
TE	Trophectoderm

5. SUPPLEMENTS

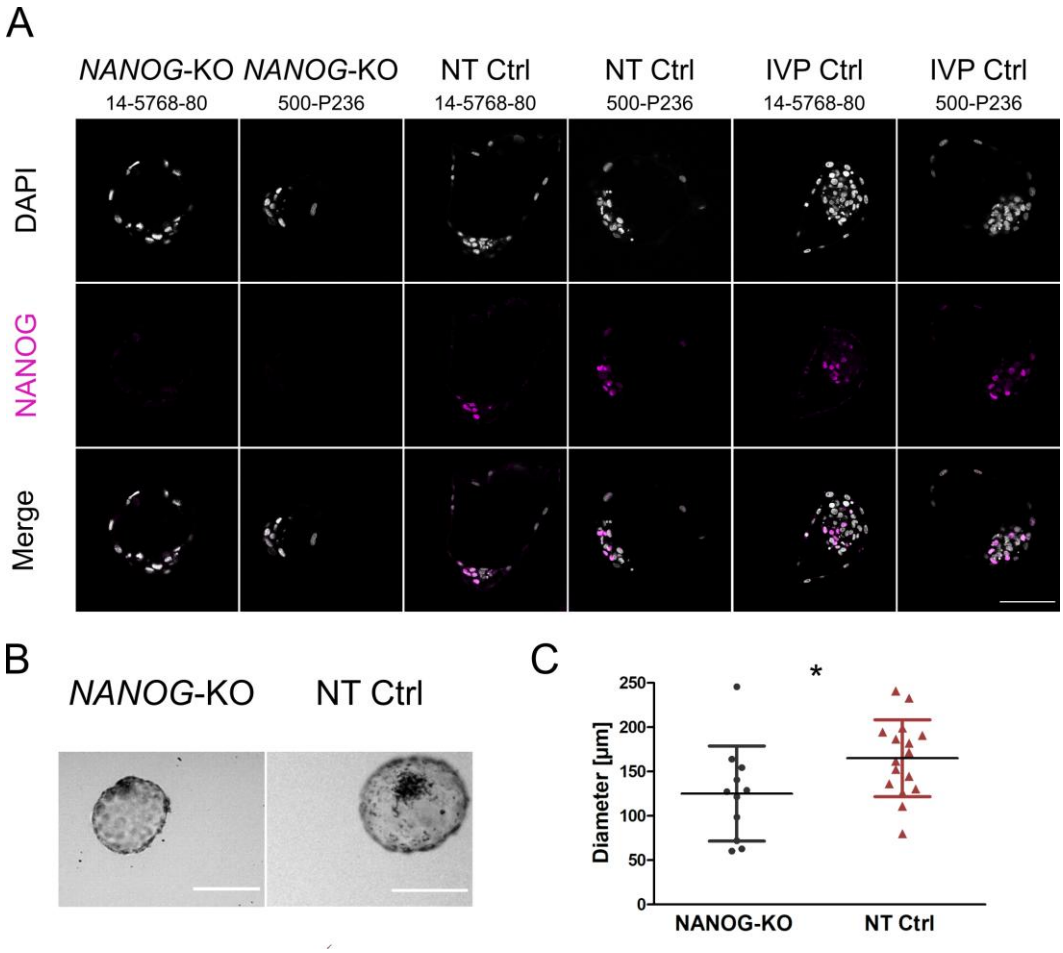

Figure S1: Producing bovine *NANOG*-KO embryos via somatic cell nuclear transfer (SCNT). (A) Representative confocal planes of day 8 *NANOG*-KO and control blastocysts from somatic cell nuclear transfer (NT Ctrl) and in vitro fertilization (IVP Ctrl) stained for NANOG with two different primary antibodies (Table S1). (B) Brightfield images of *NANOG*-KO and NT Ctrl day 8 blastocysts. (C) Mean diameter ± standard deviation of *NANOG*-KO (n=11) and NT Ctrl (n=16) day 8 blastocysts. Data were analyzed by two-tailed Mann-Whitney U test (* $p < 0.05$). All scale bars indicate 100 µm.

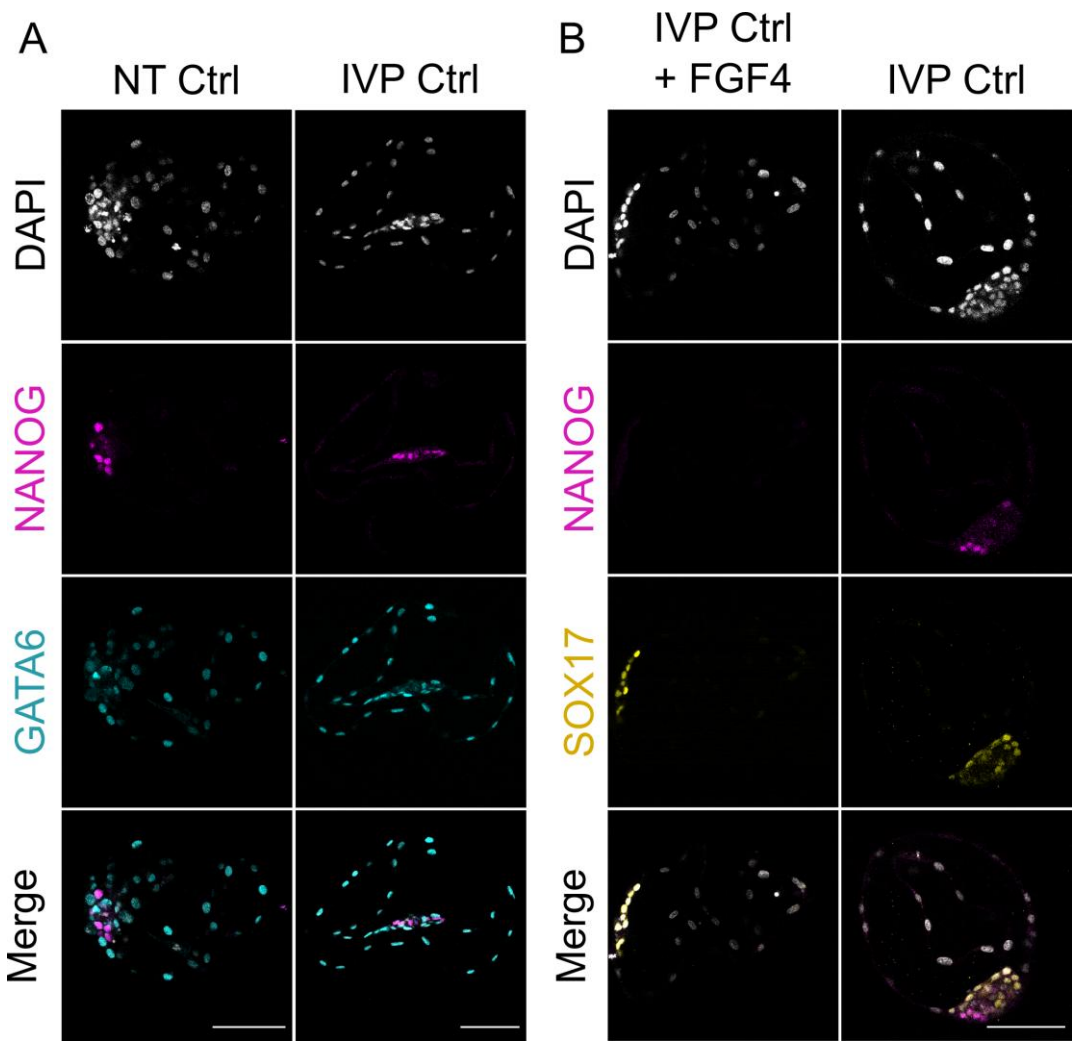

Figure S2: Expression of epiblast and hypoblast markers in NT Ctrl and IVP Ctrl day 8 embryos. (A) Representative confocal planes of day 8 NT Ctrl and IVP Ctrl blastocysts stained for NANOG and GATA6. (B) IVP Ctrl embryos were cultured from morula to day 8 blastocyst in the presence of FGF4 and heparin (each 1 µg/ml) and stained for NANOG and SOX17. Scale bars indicate 100µm.

Table S1: Targets, antibodies, suppliers and applied dilutions for immunofluorescence staining. Ig = immunoglobulin.

Target	Antibody	Supplier	Dilution
NANOG_01	Rabbit anti-human NANOG (500-P236)	Peprotech	1:600
NANOG_02	Mouse anti-human NANOG (14-5768-80)	Thermo Fisher	1:250
GATA6	Goat anti-human GATA6 (AF1700)	R&D Systems	1:500
SOX17	Goat anti-human SOX17 (AF1924)	R&D Systems	1:100
SOX2_01	Goat anti-human SOX2 AF2018	R&D Systems	1:500
SOX2_02	Rabbit anti-human SOX2 (AB5603)	Millipore	1:1000
OCT4	Rabbit anti-human OCT4 monoclonal ab181557	Abcam	1:250
CDX2	Rabbit anti-human CDX22 ab88129	Abcam	1:250
Rabbit Ig	Donkey anti-rabbit Alexa Fluor 555 (ab150074)	Abcam	For NANOG_01: 1:800 For OCT4: 1:800 For SOX2_02: 1:1000
Rabbit Ig	Donkey anti-rabbit 711-605-152 Alexa 647	Jackson Immuno Research	For CDX2: 1:400
Mouse Ig	Donkey anti-mouse Alexa Fluor 647 (715-605-150)	Jackson Immuno Research	For NANOG_02: 1:400
Goat Ig	Donkey anti-goat Alexa Fluor 633 (A212082)	Thermo Fisher	For GATA6: 1:400
Goat Ig	Bovine anti-goat Alexa Fluor 488 (805-545-180	Jackson Immuno Research	For GATA6: 1:1000 For SOX17: 1:200 For SOX2_01: 1:500

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IV. DISCUSSION AND OUTLOOK

Aim of this work was to study the role of the transcription factor NANOG during bovine preimplantation development, as most publications concentrate on mouse. I induced a *NANOG*-KO in bovine fibroblast cells by electroporation with a sgRNA using the CRISPR/Cas9 technology (Figure 1). The insertion of a single nucleotide in both alleles led to a frameshift mutation, resulting in *NANOG*-deficient cells. These cells were used for SCNT to produce *NANOG*-KO bovine embryos. *NANOG*-KO embryos and controls from SCNT (NT Ctrl) and IVP were cultured until day 8 of development and fixed for further analysis via immunofluorescence staining. By investigating the phenotype of *NANOG*-KO and modulation of the second lineage differentiation by supplementing exogenous FGF4 and heparin or inhibition of the FGF/MEK pathway, I was able to show how bovine preimplantation embryos regulate the formation of hypoblast and I found sharp contrasts to the usually used mouse model. To our knowledge, this is the first extensive study on the role of NANOG in bovine embryos using SCNT and further mechanistic studies.

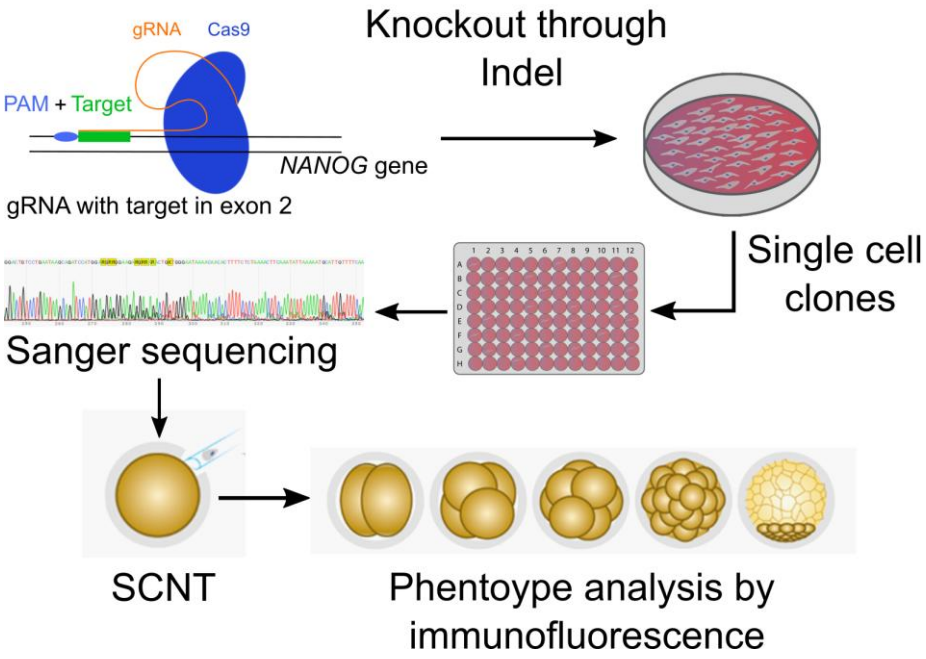

Figure 1: Producing bovine *NANOG*-KO embryos via somatic cell nuclear transfer (SCNT). Experimental design: knockout of *NANOG* was induced in fibroblasts that served as donors for SCNT, phenotype analysis was performed using immunofluorescence staining.

Bovine as a model for studying preimplantation development

So far, most publications used the mouse model to investigate preimplantation embryo development. In recent years it became clear that species-specific differences exist and not all results retrieved from the mouse can equally be applied to human development. The scarcity of human embryos and its ethical problem increase the need to find an appropriate substitute to study regulatory systems in embryo development. Although the mouse is an optimal model in many cases as the gestation period is very short and already implemented mutant mouse lines and breeding can be used to rapidly create interesting studies, the mouse cannot fulfill the demands regarding a model organism to study early preimplantation development. Studies have shown that to some extent, bovine embryos serve as a more appropriate model as they resemble the human preimplantation development more closely (reviewed in SIMMET et al., 2018b; SPRINGER et al., 2021b). Especially concerning the interaction between OCT4 and CDX2 (BERG et al., 2011) and the regulation of the FGF/MEK pathway (KUIJK et al., 2012; ROODE et al., 2012), bovine embryos show a more similar mode of regulation compared to human. This implies that experiments in early bovine embryo development might be a necessary alternative to the mouse model. The ART in domestic animals, and especially in bovine, is highly sophisticated and by using IVP and collecting ovaries in a slaughterhouse nearby, it is a simple and very effective procedure where a variety of experiments, including SCNT, can be performed. Thus, insights can be gathered with almost no additional overhead and by using IVP and SCNT, researchers can contribute to animal welfare as no experiments on animals need to be conducted, compared to mouse (reviewed in SPRINGER et al., 2021b).

Producing bovine *NANOG*-KO embryos

Creating *NANOG*-KO embryos in times of innovative technologies like CRISPR/Cas can be achieved efficiently, when sgRNAs show a high mutation rate (SANDER & JOUNG, 2014). A specific sgRNA directs Cas9 to the *NANOG* locus, where it generates double-strand breaks (DSB) (CONG et al., 2013; JINEK et al., 2013; MALI et al., 2013). DSB are repaired by an error-prone mechanism called non-homologous end joining (NHEJ), which subsequently leads to possible insertions or deletions (indels). As a consequence, the gene function is destroyed due to a shift in the reading frame (JASIN & HABER, 2016). Thereby, the

efficiency in creating mutations varies a lot due to the sgRNA design (WHITWORTH et al., 2014), different cell lines (JEONG et al., 2016), or the targeting of different genes (NI et al., 2014). Thus, to increase efficiency in the rate of those mutations, it is advisable to test suitable sgRNAs before producing single cell clones that serve as donors for SCNT, as this could be more time intensive when experiments need to be repeated due to low knockout rates.

To enhance efficiency, a puromycin resistance cassette was integrated in a plasmid to select successfully transfected cells. The sgRNAs were created with binding sites in either exon 1 or 2, as those exons are included in all known splicing variants of the human *NANOG* transcripts. I used electroporation in a cell suspension including half a million cells for each experiment and tested three different guides. After this, cells were cultivated and selected with puromycin. Most cells surviving this treatment would express the puromycin resistance cassette and thus, had the sgRNA successfully expressed. I froze half the cells to later produce single cell clones and the other half of the mixed cell population served as DNA for analysis of the mutation rate. Results of Sanger sequencing could be inserted in a software called TIDE (Tracking of Indels by Decomposition), which uses an algorithm that identifies the spectrum and frequency of small indels generated in a pool of cells by genome editing tools such as CRISPR/Cas9 (BRINKMAN et al., 2014). Subsequently, I used the most effective sgRNA to produce single cell clones, where a mutation rate of over 54 % was achieved. I checked for known single-nucleotide polymorphisms (SNPs) to ensure that both alleles were amplified during PCR. A cell clone with a homozygous insertion of thymine, which lead to a frameshift mutation of NANOG, was used for SCNT (Figure 2).

Figure 2: Inducing knockout in exon 2 of *NANOG* in bovine fibroblast cells via CRISPR/Cas9. Sanger sequencing revealed an insertion of thymine (T, red box) in bovine single cell clones, which lead to a frameshift mutation and *NANOG*-knockout.

In the review paper (see section II.3.), I described the disadvantages of SCNT procedures regarding altered gene expression patterns (reviewed in SPRINGER et al., 2021b). Despite these limitations, I chose SCNT as this approach guarantees consistent modification of all cells of the embryo and a homogeneous experimental setup with reproducibility of the results. As cloning artefacts have been reported that could alter developmental mechanisms (reviewed in SIMMET et al., 2020), proper controls need to be implemented (SIMMET et al., 2018a).

I used two different control groups, to exclude discrepancy due to the SCNT procedure. For the first control group, I worked with transfected cells with origins in the same experiment that the *NANOG*-KO cells derive from. In these cells, the sgRNA did not lead to frameshift mutations, leaving the cells with a wildtype phenotype expressing NANOG. We created NT Ctrl embryos by using these unmodified cells in SCNT. The second control group consisted of IVP embryos that did not undergo SCNT procedure. By comparing all results and especially developmental rates and protein expression in immunofluorescence staining, I was able to exclude side effects due to the SCNT procedure. Importantly, I did not find any difference in both control groups, except for the expression of SOX17 in the ICM, which was reduced in NT Ctrl embryos. I set the expression of each

investigated protein in relation to the corresponding ICM cell number of the embryo to minimize deviations due to the embryo's size. Less differentially abundant transcripts (DATs) between NT Ctrl and *in vitro* produced bovine preimplantation embryos were found, compared to KO embryos. Thus, by using SCNT, there are negligible effects on expression of EPI, HB and TE lineage in bovine embryos (SIMMET et al., 2018a). This result substantiates the use of SCNT to study early lineage differentiation.

As I performed many experiments, I needed to use two cell clones to produce *NANOG*-KO and NT Ctrl embryos to reduce passaging of single cell clones. It was reported that a high passage number has a negative effect on donor cell viability and SCNT success (reviewed in SPRINGER et al., 2021b).

Additionally, I was able to verify the phenotypes with the second cell clone. I compared blastocyst rates and the phenotype of the different cell clones prior to including them into analysis and found no differences; therefore, I presented the data without differentiating between cell clones.

An alternative to SCNT procedure is ZI or zygote electroporation. The desired mutation can be achieved by directly inserting the CRISPR/Cas components into the zygote. Compared to SCNT, performing ZI comes along with less technical effort and high mutation rates are possible. A drawback is the frequently occurring effect of mosaicism, hampering the repeatability of producing homozygous bi-allelic KO in embryos. Additionally, during development, the genotype of the embryo is unknown and the scarce genomic material impairs in-depth investigation and analysis of the potential KO (YEN et al., 2014; CRISPO et al., 2015; LIANG et al., 2015; BEVACQUA et al., 2016). Overall, the production of bovine *NANOG*-KO embryos is possible with relatively small effort, but it is vital to implement proper control mechanisms.

Phenotype of *NANOG*-KO embryos

I did not see NANOG expression in *NANOG*-KO day 8 embryos produced with both KO cells and by staining with two different NANOG antibodies. Thus, I was able to validate the success of implementing *NANOG*-KO via SCNT. As no maternal NANOG mRNA stores exist in the oocyte (GRAF et al., 2014), NANOG was indeed missing from the time of construction and activation of the early embryo onwards. Little is known in bovine preimplantation embryos about the

impact of NANOG on other pluripotency factors, such as OCT4 or SOX2. Although the only other report about *NANOG*-KO in bovine embryos reported a reduction of transcripts of *SOX2* (ORTEGA et al., 2020), I detected OCT4 and SOX2 expression in the ICM on a protein level. So far, no reports exist about SOX2 expression in the absence of NANOG in another mammal.

In images of immunofluorescence staining of day 8 embryos I did not see OCT4 expression in the TE in neither *NANOG*-KO nor control groups. I was able to test the specificity of this antibody against OCT4, as our group has access to *OCT4*-KO embryos, where OCT4 was not detected using this antibody (Preprint, SIMMET et al., 2021). I used this antibody to stain OCT4 in day 7 and day 9 *ex vivo* derived embryos, where expression in the TE was present at day 7 but no longer detected by day 9 (unpublished data). BERG et al. (2011) and SIMMET et al. (2018a) used a different polyclonal OCT4 antibody (Santa Cruz, sc-9081) in ex vivo day 11 or in vitro day 7 blastocysts, respectively. This antibody is not available anymore. I can only speculate that the polyclonal antibody results in a different expression pattern.

In bovine *OCT4*-deficient blastocysts, NANOG expression is lost (SIMMET et al., 2018a), but vice versa, OCT4 seems to be independent from NANOG expression (SPRINGER et al., 2021a). I could confirm that SOX2 is a reliable marker for the ICM in day 8 bovine blastocysts (GOISSIS & CIBELLI, 2014). Thus, I analyzed the ICM by staining of SOX2 (ICM marker) or CDX2 (TE marker), and could extrapolate ICM cells by comparing with DAPI staining, as this highlights all nuclei in the embryo. If, due to the experimental setup, a staining with GATA6 or CDX2 was not possible, I determined the ICM based on the embryo's morphology.

The total cell number was reduced in *NANOG*-KO compared to NT Ctrl bovine embryos (66.4 ± 27.3 vs. 148.6 ± 65.6, Table 1), which is in contrast to mouse, where the total cell number remains constant (MESSERSCHMIDT & KEMLER, 2010) and was not investigated in the second bovine *NANOG*-null publication (ORTEGA et al., 2020). Furthermore, no significant difference in the ratio of ICM to total cell number in bovine *NANOG*-KO embryos suggests that there was a normal distribution of ICM and TE cells during the first lineage differentiation in the absence of NANOG (SPRINGER et al., 2021a). In consistence to ORTEGA et al. (2020) and publications in mouse (MITSUI et al., 2003; FRANKENBERG et

al., 2011), I found that GATA6 expression was independent of NANOG. In sharp contrast to findings in mouse, the later hypoblast marker SOX17 was still present in bovine *NANOG*-null embryos (SPRINGER et al., 2021a), whereas it was lost in mouse (FRANKENBERG et al., 2011).

Modulation of the second lineage differentiation

KUIJK et al. (2012) found no difference in the expression pattern of EPI or HB lineage in bovine embryos when supplementing MEK-inhibitor PD032 from day 1–8 or day 5–8. Thus, I chose to adapt the experimental setup and pooled all morulae on day 5 and regrouped them with IVC media supplemented with PD032 or control media containing DMSO. For experiments involving DMSO, I could not use the mouse-anti NANOG antibody by Thermo Fisher (14-5768-80) that I used in the other experiments, as there seems to be a cross reaction between DMSO and this antibody, which contains stabilizing factors for long term storage at 4° C. Thus, I chose the alternative antibody by Peprotech (500-P236) that was also used by KUIJK et al. (2012). With this antibody, I could identify NANOG expressing cells and also validated *NANOG*-KO with a second antibody. I found in agreement with aforementioned publication, that the number NANOG-expressing cells increased, whereas GATA6-expressing cell number decreased but was still present in IVP embryos that were treated with PD032 from day 5 to day 8. Thus, I concluded that in bovine preimplantation development, hypoblast formation is independent of the FGF/MEK pathway and that is why there must be another, so far unknown pathway that regulates hypoblast formation in bovine embryos during second lineage differentiation. Mouse embryos seem to be completely dependent, whereas human embryos were found to be independent of the FGF/MEK pathway (reviewed in SPRINGER et al., 2021b). When CANIZO et al. (2019) stated that expression of the later HB marker SOX17 was dosage dependent in bovine embryos and questioned the aforementioned unknown pathway that seemed to regulate hypoblast formation in bovine embryos, I chose to add experiments to examine this matter. Thus, I increased dosage of the MEK inhibitor PD032 to a similar concentration used in CANIZO et al. (2019), and stained for SOX17 in day 8 embryos. I still found the later hypoblast marker SOX17 at a concentration of 2.5 µM PD032. The cause for the discrepancies is unclear, but could be due to the use of a different media for in vitro production. Similar to reports in mouse, I found that *NANOG*-KO embryos undergo cell death,

whereas NT Ctrl embryos showed the same effects as IVP controls. As experiments in mouse demonstrated that FGF4 was the missing factor that helped to rescue the before lost SOX17 expression in *Nanog*-KO blastocysts, I chose to test this hypothesis in *NANOG*-KO embryos. In control blastocysts, that still expressed NANOG, supplementing FGF4 and heparin from day 5 to day 8 resulted in embryos completely lacking NANOG and ubiquitously expressing SOX17 in the ICM. Despite findings in mouse (FRANKENBERG et al., 2011), FGF4 treated *NANOG*-KO embryos did not significantly change SOX17 expression compared to untreated *NANOG*-KO embryos. Therefore, I concluded that NANOG is the missing factor to induce full SOX17 expression via the FGF/MEK pathway.

Outlook

Further research is needed to better understand the preimplantation development in mammals, including bovine and human. In addition to our genetic study, which focused only on day 8 blastocysts, I suggest a time lapse study of the developing hypoblast precursor cells. The onset of this lineage and the temporal and spatial patterns could be monitored closely. In addition, the maturation of the hypoblast precursor cells could be observed, as expression of hypoblast markers SOX17 and GATA4 starts later then GATA6 (FRANKENBERG et al., 2011).

Unfortunately, the IVC of bovine embryos is time limited, thus other approaches are needed to investigate the later role of NANOG in preimplantation development. As I showed in Figure 1 of the review paper, a variety of possible experiments can be combined to prolong studies. Thereby, an oviduct transfer of two to four cell-stage *NANOG*-KO and Control embryos produced by SCNT could be performed (BESENFELDER & BREM, 1998). The oviduct plays a vital role in gene expression patterning as the period of epigentetic reprogramming occurs when the embryo passages the oviduct (reviewed in FEIL, 2009; VENTURA-JUNCA et al., 2015; FRASER & LIN, 2016). So far, IVP procedures cannot offer an appropriate environment for a physiological embryo development (reviewed in MÉNÉZO et al., 2015). Progress has been made by FERRAZ et al. (2018), where a 3D-printed oviduct-on-a-chip was created for bovine embryo cultivation. Retrieved zygotes showed a similar transcriptome profile compared to in vivo produced counterparts. A uterus-on-a-chip is still missing to be able to investigate embryo stages later than the zygote.

Until now, the most physiological way to investigate embryos is cultivation in its usual environment – in vivo in the cow. A less invasive method is the embryo transfer, where embryos from morula stage onwards can be transferred non-surgically into the uterus of a cow. Flushing the embryos at any time before implantation is possible. This circumvents the prolonged in vitro cultivation of embryos and enables researchers to investigate later stage embryos (reviewed in SPRINGER et al., 2021b).

Recently, an interesting approach has been reported about the establishment of an "iBlastoid", a model of a human embryo. By reprogramming human fibroblasts into in vitro 3D models, it was possible to create a human blastocyst (LIU et al., 2021). Future research is needed to implement this technology for other species and genetic alterations need to be monitored closely.

The research in preimplantation embryo development could also give insight into mechanisms that could help to overcome the so far unsuccessful implementation of stem cells in other mammals than mouse (EVANS & KAUFMAN, 1981; MARTIN, 1981), human (THOMSON et al., 1998), primate (THOMSON et al., 1995; THOMSON et al., 1996), rat (IANNACCONE et al., 1994) and rabbit (GRAVES & MOREADITH, 1993; SCHOONJANS et al., 1996). A long time has gone by since the first introduction of mouse ESC in 1981 and no fundamental breakthrough was found to introduce stem cell lines in large domestic animals, e.g. in bovine or pig. This suggests that differences in early embryo development between large domestic animals and other species exist and are the cause for the failure in establishing bovine or porcine ESC. The reverse genetics approach I conducted in this thesis could be adapted in other experiments to decipher the role of various proteins that are vital during early embryo development. Thus, studying preimplantation development in bovine embryos could not only enhance our understanding in regulatory mechanisms in this delicate period during gestation but also give hints for future research concerning embryonic stem cell implementation.

V. SUMMARY

The role of NANOG during bovine preimplantation development

In recent years, species-specific differences regarding regulation of early preimplantation development became apparent. So far, little is known about bovine preimplantation development, but similarities to human embryos and highly developed assisted reproductive technologies (ART) designate bovine as an excellent model to study the first and second lineage differentiation in embryos. I chose to study the role of the transcription factor NANOG in bovine blastocysts, as most publications concentrate on mouse. By using the CRISPR/Cas9 technology, it was possible to induce a homozygous biallelic mutation in exon 2 of NANOG in fibroblast cells, which lead to a frameshift mutation resulting in *NANOG*-deficient embryos. Single cell clones with a knockout (KO) of *NANOG* and control cells with functioning NANOG (NT Ctrl) served as donors for somatic cell nuclear transfer (SCNT). In vitro produced embryos (IVP Ctrl) served as second control group. All embryos were cultured until the blastocyst stage on day 8. Embryos were fixed for further analysis via immunofluorescence staining.

At day 8, *NANOG*-KO embryos displayed a reduction in total cell number compared to NT Ctrl. The inner cell mass (ICM) to total cell number ratio was not changed, suggesting that a normal distribution of cells into ICM and TE happened during the first lineage segregation. I concluded that the first lineage differentiation does not depend on NANOG. The pluripotency factors OCT4 and SOX2 were coexpressed in all cells of the ICM. This is in contrast to another report on *NANOG*-KO in bovine embryos using zygote injection (ZI), as a reduction of SOX2 transcripts was found.

Consistent with the aforementioned report and publications in mouse, I found a pan-ICM GATA6 expression in *NANOG*-KO bovine embryos. Interestingly, I still found SOX17 expression in the ICM of bovine *NANOG*-null embryos. This is in contrast to mouse *Nanog*-KO embryos, where the late hypoblast (HB) marker SOX17 is lost.

I conducted further mechanistic studies by modulation of the second lineage differentiation. For this, I supplemented either the MEK 1/2 inhibitor PD0325901

(PD032) or FGF4 and heparin from day 5 to day 8 in the culture media. I could show that in control embryos with PD032 supplementation, NANOG expressing cells increased and both GATA6 and SOX17 decreased, but were still present. This is in contrast to mouse, where full dependency of the FGF/MEK pathway was reported. Even with a higher dosage of the PD032, I could still see SOX17 expression, indicating no dosage dependent expression of the HB marker. When conducting this experiment with bovine *NANOG*-KO embryos, viability was compromised and all embryos died, similar to mouse.

The lost SOX17 expression in mouse *Nanog*-KO embryos could be rescued by supplementing exogenous FGF4 and heparin. In sharp contrast, I showed that bovine *NANOG*-KO embryos displayed an unchanged expression of SOX17, whereas control embryos supplemented with FGF4 consisted of only SOX17-expressing cells and NANOG was lost.

I conclude that NANOG mediated FGF/MEK signaling is not required for HB formation in bovine embryos. Another, so far unknown pathway must be responsible for HB initiation in bovine embryos during second lineage differentiation.

VI. ZUSAMMENFASSUNG

Die Rolle von NANOG im präimplantativen Rinderembryo

In den letzten Jahren wurden spezies-spezifische Unterschiede in der Regulierung der Entwicklung des Embryos vor der Implantation in den Uterus offensichtlich. Jedoch weiß man bis heute nur wenig über die frühembryonale Entwicklung beim Rind, obwohl Ähnlichkeiten zum menschlichen Embryo und die weit entwickelten Techniken in der assistierten Reproduktion das Rind dazu prädestinieren, es als Modell für die Untersuchung der ersten und zweiten Zelldifferenzierung in Embryonen zu nutzen. Ich entschloss mich die Rolle des Transkriptionsfaktors NANOG in bovinen Blastozysten zu erforschen, da sich die meisten Publikationen auf das Mausmodell stützen. Durch die CRISPR/Cas9 Technologie war ich in der Lage eine homozygote biallelische Mutation in Exon 2 von NANOG in Fibroblasten zu erzeugen, was zu einer Leserahmenverschiebung und letztendlich zu einem Knockout (KO) von *NANOG* führte. *NANOG*-KO Einzelzellklone und Kontrollzellen mit funktionierender NANOG-Expression (NT Ctrl) dienten als Spenderzellen für den somatischen Zellkerntransfer (engl. somatic cell nuclear transfer, SCNT). In vitro produzierte Embryonen (IVP Ctrl) bildeten die zweite Kontrollgruppe. Alle Embryonen wurden bis zum Blastozystenstadium an Tag 8 kultiviert. Die Embryonen wurden zur weiteren Untersuchung mittels Immunfluoreszensfärbung fixiert.

An Tag 8 wiesen *NANOG*-KO Embryonen verglichen zu NT Ctrl eine reduzierte Gesamtzellzahl auf. Das Verhältnis von innerer Zellmasse (engl. inner cell mass, ICM) zur Gesamtzellzahl blieb unverändert, was vermuten lässt, dass eine normale Verteilung der Zellen in ICM und Trophectoderm (TE) während der ersten Zelldifferenzierung stattgefunden hat. Ich schlussfolgerte, dass NANOG für die erste Zelldifferenzierung nicht benötigt wird. Die Pluripotenzfaktoren OCT4 und SOX2 wurden in allen Zellen des ICM ko-exprimiert. Das steht im Widerspruch zu der anderen Publikation bezüglich eines durch Zygoteninjektion (ZI) induzierten *NANOG*-KO in bovinen Embryonen, in der von einer Reduktion von SOX2 Transkripten berichtet wurde.

Wie in der zuvor erwähnten Publikation und anderen Berichten im Mausmodell beschrieben, habe ich eine GATA6 Expression im kompletten ICM von bovinen

NANOG-KO Embryonen beobachtet. Interessanterweise wurde noch immer eine SOX17 Expression im ICM von bovinen *NANOG*-KO Embryonen gefunden. Das steht im Gegensatz zu *Nanog*-KO Embryonen in der Maus, bei der ein kompletter Verlust des späten Hypoblast (HB) Markers SOX17 eintritt.

Ich führte weitergehende mechanistische Experimente durch, indem ich die zweite Zelldifferenzierung modellierte. Dafür habe ich entweder den MEK 1/2 Inhibitor PD0325901 (PD032) oder FGF4 und Heparin von Tag 5 bis Tag 8 in das Kulturmedium hinzugegeben. Ich konnte zeigen, dass in Kontrollembryonen mit hinzugefügtem PD032 die Zahl der NANOG-exprimierenden Zellen stieg, während zwar GATA6 und SOX17 abnahmen, jedoch immer noch vorhanden waren. Das steht im Widerspruch zur Maus, bei der von einer völligen Abhängigkeit vom FGF/MEK Signalweg berichtet wurde. Sogar bei einer erhöhten Konzentration von PD032 konnte ich immer noch eine SOX17 Expression beobachten, was darauf hindeutet, dass es keine konzentrationsabhängige Expression des HB Markers gibt. Als ich das Experiment mit bovinen *NANOG*-KO Embryonen durchführte, war die Überlebensfähigkeit beeinträchtigt und alle Embryonen starben, ähnlich wie bei der Maus.

In Maus *Nanog*-KO Embryonen konnte die fehlende SOX17 Expression durch das Hinzufügen von exogenem FGF4 und Heparin wieder hergestellt werden. Im Gegensatz dazu konnte ich zeigen, dass bovine *NANOG*-KO Embryonen eine unveränderte Expression von SOX17 zeigten, wohingegen Kontrollembryonen mit FGF4 Zugabe eine ubiquitäre SOX17 Expression im ICM aufwiesen und eine NANOG-Expression fehlte. Ich schlussfolgere, dass der NANOG vermittelte FGF/MEK Signalweg für die HB Entstehung in Rinderembryonen nicht benötigt wird. Ein anderer und bisher unbekannter Signalweg muss für die Initiierung des HB in bovinen Embryonen während der zweiten Zelldifferenzierung verantwortlich sein.

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