Similarities between bovine and human germline development revealed by single-cell RNA sequencing

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Abstract

The germ cell lineage ensures the creation of new individuals and perpetuates the genetic information across generations. Primordial germ cells are pioneers of gametes and exist transiently during development until they differentiate into oogonia in females, or spermatogonia in males. Little is known about the molecular characteristics of primordial germ cells in cattle. By performing single-cell RNA-sequencing, quantitative real-time PCR, and immunofluorescence analyses of fetal gonads between 40 and 90 days of fetal age, we evaluated the molecular signatures of bovine germ cells at the initial stages of gonadal development. Our results indicate that at 50 days of fetal age, bovine primordial germ cells were in the early stages of development, expressing genes of early primordial germ cells, including transcriptional regulators of human germline specification (e.g. *SOX17*, *TFAP2C*, and *PRDM1*). Bovine and human primordial germ cells also share expression of *KIT*, *EPCAM*, *ITGA6*, and *PDPN* genes coding for membrane-bound proteins, and an asynchronous pattern of differentiation. Additionally, the expression of members of Notch, Nodal/Activin, and BMP signaling cascades in the bovine fetal ovary, suggests that these pathways are involved in the interaction between germ cells and their niche. Results of this study provide insights into the mechanisms involved in the development of bovine primordial germ cells and put in evidence similarities between the bovine and human germline.

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Introduction

The specification of primordial germ cells (PGCs), the progenitors of sperm and eggs, occurs around the onset of gastrulation by the inductive signaling of bone morphogenetic proteins (BMP) on a group of cells that are initially destined toward a somatic mesodermal fate (Lawson *et al.* 1999, Saitou *et al.* 2002, Kobayashi *et al.* 2017, Chen *et al.* 2019). Even though the signaling pathways that operate during PGC specification are conserved across mammals (Nikolic *et al.* 2016), the origin of the signaling molecules, the key transcriptional regulators, and their hierarchy are divergent between species (Kojima *et al.* 2017).

In mice, the period of PGC development lasts around 7 days. This process is initiated by BMP4 signaling on WNT-primed cells, which activates PRDM1 (also known as BLIMP1) and PRDM14. Together with TFAP2C, a direct target of PRDM1, these genes act in combination to induce PGC fate (Ohinata *et al.* 2005, Yamaji *et al.* 2008). The PRDM1-PRDM14-TFAP2C tripartite genetic network acts to repress the somatic mesodermal program, induce re-expression of pluripotency genes, initiate expression of PGC genes, and prompt genome-wide epigenetic reprogramming (Saitou *et al.* 2002, Yabuta *et al.* 2006, Seki *et al.* 2007); all key events involved in the specification of PGCs. After specification, PGCs

follow guidance cues that will ultimately lead them to the genital anlage. Upon colonization of the undifferentiated developing gonad, PGCs undergo synchronous mitotic divisions with incomplete cytokinesis, forming nests of interconnected germ cells (Pepling & Spradling 1998). The PGC development period ends when female PGCs enter meiotic prophase or male PGCs enter mitotic quiescence (McLaren & Southee 1997). In the ovary, meiosis initiation is triggered by retinoic acid signaling which in the testis is antagonized by the cytochrome P450 activity of Sertoli cells (Bowles *et al.* 2006, Koubova *et al.* 2006).

In humans, the period of PGC development lasts around 7 weeks (from week 2 to week 9 of fetal development) (Tang *et al.* 2015). Activation of BMP signaling leads to upregulation of PRDM1, which in primates and pigs, is triggered by SOX17, a key regulator of PGC specification (Irie *et al.* 2015, Kobayashi *et al.* 2017, Kojima *et al.* 2017). BMP signaling initially upregulates TFAP2C independently from SOX17, which in combination with PRDM1 establish the gene expression program of human PGCs (Kojima *et al.* 2017). Upon specification, human PGCs migrate and colonize the genital ridge to extensively proliferate until they enter meiosis asynchronously in females (Anderson *et al.* 2007) or enter mitotic quiescence and undergo meiosis after puberty in males (Tang *et al.* 2015).

Despite the advances in mice and humans, PGC specification and development in livestock species are largely unknown. Similar to humans, the period of bovine PGC development lasts around 6 weeks (from week 2 to week 8 of fetal development) (Lavoie *et al.* 1994, Wrobel & Süß 1998). At 40 days of fetal development, the undifferentiated bovine gonad undergoes sexual differentiation, and then female germ cells initiate meiosis between 70 and 80 days of fetal age (Erickson 1966). At the molecular level, bovine PGCs are characterized by nonspecific alkaline phosphatase (AP) activity (Wrobel & Süß 1998), expression of well-conserved germline markers such as the tyrosine-kinase receptor KIT (also known as cKIT) (Kritzenberger & Wrobel 2004), and the RNA-binding proteins NANOS3 (Ideta *et al.* 2016), DDX4 (also known as VASA) (Pennetier *et al.* 2004, Bartholomew & Parks 2007, Luo *et al.* 2013) and DAZL (Hummitzsch *et al.* 2013). However, the molecular signatures that characterize the bovine germline during early gonadal development have not been elucidated, due in part to the challenges associated with studying PGCs. During their initial development, PGCs are embedded in the developing embryo in small numbers (Barton *et al.* 2016, Cantú & Laird 2017) and even though human and mice PGCs can be purified by cell sorting, antibodies to successfully target and isolate bovine PGCs are not commercially available. To overcome this limitation and elucidate the molecular signatures that characterize the bovine germline during early gonadal development, we performed single-cell RNA-sequencing (scRNA-seq), quantitative real-time PCR (RT-qPCR), and immunofluorescence analyses of fetal gonads between 40 and 90 days of age. At day 50 bovine PGCs were in early stages of development and shared with the human germline expression of transcriptional regulators and surface markers, and an asynchronous progression of differentiation. Bovine PGCs and their somatic counterparts expressed members of the BMP, Activin/Nodal, and Notch signaling cascades, suggesting that these pathways may have roles in coordinating the events of gonad development in cattle.

Materials and methods

Tissue collection and processing

Bovine fetuses were obtained from a local abattoir and transported on ice to the laboratory within 3 h of retrieval. Fetuses were inspected for normal development and the crown-rump length (CRL) was measured to estimate gestational age (DesCôteaux *et al.* 2009). Gonads were identified based on their location within the abdominal cavity, anatomy, and relationship with neighboring organs (mesonephros and/or kidneys). Tissues retrieved for histology were fixed in 4% paraformaldehyde for 24 h at 4°C and step-wise dehydrated through an ethanol gradient and processed in a VIP Tissue Tech processor (Sakura Finetek). Tissue was then embedded in paraffin, sectioned at 5 µm thickness and stained

with hematoxylin and eosin (H&E). Gonads collected for immunostaining analyses were fixed in 4% paraformaldehyde for 5 h at 4°C, washed and transferred to 30% sucrose until embedding in Tissue-Tek optimal cutting temperature (OCT) compound (4883, Sakura Finetek). Cryoblocks were sectioned at 10 µm thickness and tissue sections were stored at −20°C until staining. Gonads retrieved for scRNA-seq were washed in ice-cold PBS and slow frozen in DMEM containing 20% FBS and 10% DMSO using a freezing device (5100-0001, Thermo Scientific). Fetal ovaries and testes harvested for quantitative RT-PCR were snap-frozen in liquid nitrogen and stored at −80°C until RNA was extracted. Samples utilized in this study are listed in Table 1.

Immunofluorescence analysis

Cryosections were washed to remove the OCT compound before unmasking for 10 min in a steamer in 10 mM sodium citrate buffer, pH 6.0. Blocking was performed by incubating the tissue sections with 0.3 mM glycine and 10% normal donkey serum for 1 h, at room temperature. Tissue sections were then incubated overnight at 4°C with the following primary antibodies: anti-PRDM1 (1:50; 14-5963-80, Invitrogen), anti-OCT4 (1:500; sc-8628, Santa Cruz Biotechnology), anti-DAZL (1:500; Ab34139, Abcam), anti-DDX4 (1:500; Ab13840, Abcam). Secondary antibody incubation was performed for 1 h at room temperature and Hoechst 33342 was used for counterstaining. Tissue sections were mounted using ProLong Gold Antifade (P36934, Invitrogen) and images captured using

Table 1 List of bovine fetuses harvested for this study.

CRL (cm)	Sex	Estimated fetal age (days)	Analysis
2	N/A	40	H&E
2	Male	40	IMF
2.5	Male	45	IMF
3	Female	45–50	IMF
3	Male	45–50	RT-qPCR
3.5	Female	50	AP
3.5	Female	50	RT-qPCR
4	Female	50	scRNA-seq
4	Female	50	scRNA-seq
4.5	Female	50	H&E
4.5	Female	50	RT-qPCR
5.5	Female	50–60	RT-qPCR
5.5	Male	50–60	RT-qPCR
6	Female	60	IMF
6.3	Male	60	IMF & AP
8	Male	60–70	RT-qPCR
8.3	Female	60–70	RT-qPCR
11	Female	80	RT-qPCR
11	Male	80	RT-qPCR
12	Male	80	IMF & AP
14	Female	90	RT-qPCR
14	Male	90	RT-qPCR
15.5	Female	90	IMF
16	Female	90	IMF

AP, detection of alkaline phosphatase activity; CRL, crown-rump length; H&E, hematoxylin and eosin staining; IMF, immunofluorescence staining; N/A, not available; RT-qPCR, quantitative real-time PCR; scRNA-seq: single-cell RNA-sequencing.

a Leica TCS SP8STED 3X laser-scanning confocal microscope. Image processing was performed using ImageJ (v2.0.0-rc-69/1.52n). Immunostaining of mesonephros was utilized as negative control when germline-specific proteins were detected.

Alkaline phosphatase staining

Detection of AP activity was performed using the Alkaline Phosphatase Staining Kit II (00-0055; Stem-gent) adapting the manufacturer's protocol for tissue sections. Briefly, cryosections were washed in PBS supplemented with 0.05% Tween 20 and incubated with freshly prepared AP Substrate Solution (provided with the kit) in the dark at room temperature for 15 min. The reaction was stopped by washing tissue sections in PBS and stained sections were mounted in ClearMount mounting medium (MMC0112, American MasterTech). Imaging was performed using a Q Imaging camera on an Olympus BH2 microscope.

Sex determination by polymerase chain reaction

Fetuses less than 50 days of fetal age (4 cm CRL) were sexed by PCR targeting the DEAD box helicase 3 gene (*DDX3X/DDX3Y*), which allows discrimination between X and Y chromosomes based on amplicon size (Gokulakrishnan *et al.* 2012). DNA extraction was performed using the DNeasy Blood and Tissue Kit (69504, Qiagen) according to manufacturer's protocol. DNA was quantified using a NanoDrop 2000C Spectrophotometer (Thermo Scientific) and amplified using the Go Taq Hot Start Green Master Mix. Amplicons were observed by agarose gel electrophoresis stained with ethidium bromide. Genomic DNA from adult testes and ovaries were used as controls.

cDNA preparation and quantitative RT-PCR

Total RNA was extracted using the RNeasy Mini Kit (74104, Qiagen) and DNase treated with the RNase-Free DNase Set (79254, Qiagen). After extraction, RNA was quantified using the Qubit RNA BR Assay Kit (Q10211, Invitrogen) and reverse transcribed using SuperScript III Reverse Transcriptase (18080044, Invitrogen) according to the manufacturer's protocols.

Evaluation of gene expression was performed using PowerUp SYBR Green Master Mix (A25742, Applied Biosystems) on

a QuantStudio 3 Real-Time PCR System (A28137, Applied Biosystems). The Primer Quest tool (Integrated DNA Technologies) was used to design primers (Table 2) spanning an exon–exon junction. Samples were run in technical duplicates and relative expression was calculated by the delta Ct method, normalizing values to the HMBS housekeeping gene. cDNA from adult testes and ovaries were used as controls.

Fetal gonad dissociation for single cell RNA-sequencing

Slow-frozen gonads from two independent replicates of approximately 50 days of fetal age (4 cm CRL) were removed from liquid nitrogen and thawed at 37°C in a water bath. Gonads were then rinsed in ice-cold PBS, minced into ~0.1 mm pieces and incubated in Collagenase Type IV (800 U/mL) (LS004186, Worthington) supplemented with DNase I (6 U/mL) (04536282001, Roche Applied Science) at 37°C in a prewarmed orbital shaker for 10 min at 150 rpm. After dissociation, the cell suspension was filtered and dead cells were removed by magnetic bead sorting using a Dead Cell Removal Kit (13009010, Miltenyi) following the manufacturer's protocol. Viable cells resuspended in 0.4% BSA were submitted on ice to the UC Davis DNA Technologies & Expression Analysis Core for library preparation and sequencing.

10× Genomics library preparation and sequencing

Cells were captured using a 10× Chromium controller targeting 10,000 cells per sample. Libraries were prepared using the Chromium Single Cell 3' v2 chemistry for sample 1 and v3 chemistry for sample 2 according to the manufacturer's protocol. Sequencing was performed in an Illumina HiSeq 4000 platform as 150 base paired-end.

Mapping, cell quality control and downstream analyses

Demultiplexing of raw reads and mapping to the bovine reference genome (ARS-UCD1.2.96) was performed using the Cell Ranger software (v3.0.2). Reads were filtered and counted through the Cell Ranger Count pipeline. The R package Seurat (v3.1.0.9003) was used for sample integration, quality control,

Table 2 Sequence of primer sets used for qPCR.

Gene	Forward primer sequence	Reverse primer sequence	Tm (°C)	Amplicon size (bp)
<i>DDX4</i>	GAAGGTGATAGCTCTGGTTTCT	GTCTTGATAACCGCCTCTCTT	62	99
<i>HMBS</i>	CTTCACCATGGAGCTGTCT	TAGTTCCTACCACACTCTTCTCT	62	116
<i>NANOS3</i>	TGTGCAGGTTCCAAAGGT	GTCTCCTTAGGCAGAAAGTTGAG	62	81
<i>PRDM1</i>	CCACATGAATGCCAGGTTTG	TGCACTGGTAAGGTTTCTCTC	62	97
<i>PRDM14</i>	GGACAAGGGTGACAGGAAAT	TCCCGCATGTAGAACAACCTTG	62	128
<i>POU5F1</i>	AACGAGAATCTGCAGGAGATATG	TCTCACTCGGTTCTCGATACT	62	87
<i>SOX2</i>	CATTAACGGCACACTGCCCC	TGAAAATGTCTCCCCCGCCC	62	76
<i>SOX17</i>	AAGATGCTGGGCAAGTCG	CGGTACTTGTAGTTGGGATGG	62	116
<i>TFAP2C</i>	CGACATGGCACACCAGAT	GGAAATAGGACCTTTGCGAATAAC	62	94
<i>DDX3</i>	AGGAAGCCAGGAAAGTAA	CATCCACGTTCTAAGTCT	58	184 and 208

Tm, melting temperature.

and secondary analyses (Stuart *et al.* 2019). Quality check of datasets and cell filtering was based on 4 criteria: number of detected genes, number of unique molecular identifiers, percentage of mitochondrial genes, and percentage of ribosomal genes. Cells that passed the selection criteria (cells containing 200 to 6000 reads and <5% of mitochondrial genes) were used for downstream analysis (Supplementary Fig. 1A, see section on [supplementary materials](#) given at the end of this article). Integration and normalization of the two replicates was performed by identifying ‘anchors’ across datasets. Dimensionality reduction was performed by uniform manifold approximation and projection (UMAP) and differential gene expression was performed using non-parametric Wilcoxon rank sum test, which is part of the standard Seurat pipeline. Functional clustering of gene ontology (GO) term enrichment of differentially expressed genes was performed using DAVID (Huang *et al.* 2009). The integration of bovine and human data sets was performed using the human UMI count data from <http://github.com/zorrodong/germcell> (Li *et al.* 2017). Only female cells expressing 2000 to 10,000 genes and 100,000 to 1,100,000 transcripts were kept for integration, resulting in 970 total cells. Integration of the bovine and human datasets was also performed by the identification of ‘anchors’ using R package Seurat (v3.1.5.9003) (Stuart *et al.* 2019) and variations due cell cycle heterogeneity were regressed out. The identification of candidate surface marker genes was performed using the computational framework COMET (Delaney *et al.* 2019) using their stand-alone software package.

Data availability

Accession numbers for the 10×X Genomics single cell RNA-seq data that support the findings of this study are openly available at <https://www.ncbi.nlm.nih.gov/geo/>, GEO accession: GSE162952. Code availability of the custom scripts used can be found on GitHub at https://github.com/deliasoto/Bovine_scRNA-seq.

Results

Morphology and alkaline phosphatase activity of bovine fetal gonads

We evaluated the anatomical relationship of the developing gonad with the mesonephros between 40 and 50 days of fetal age (2–4.5 cm CRL) by H&E staining. At 40 days of fetal age (Supplementary Fig. 2A and B), the gonads were identified by their ventral location to the mesonephros, one of the biggest organs in the abdominal cavity at this stage. Around 10 days later (Supplementary Fig. 2C and D), gonads had tripled in size and grown out from the mesonephros. Similar to other mammals, bovine PGCs possess temporary AP activity that is lost upon differentiation (Gropp & Ohno 1966, Lavoie *et al.* 1994). We detected nonspecific AP activity throughout the fetal gonad in ovaries and testes (Supplementary Fig. 2E, F and G). In ovaries at 50

days of fetal age (3.5 cm CRL), AP positive cells were detected throughout the whole organ, but the surface epithelium seemed to be AP negative. In testes, positive cells in the seminiferous tubules progressively lost AP activity between 60 and 80 days of development (6–12 cm CRL), whereas somatic cells in the interstitium remained AP positive. Thus, in female and male fetal gonads, germ cells were masked by the positive AP reaction of the surrounding cells, confirming the lack of specificity of AP staining that others have described (Gropp & Ohno 1966, Lavoie *et al.* 1994, Wrobel & Süß 1998). Even though bovine fetal gonads can be easily identified after 40 days of development, AP activity is not an appropriate germ cell marker as it is unspecific and germ cells lose AP expression over time.

Single-cell RNA-sequencing of 50 days old bovine fetal ovaries

Since specific surface markers and antibodies to sort the different cell populations of the bovine fetal gonad are not available, we performed scRNA-seq on dissociated fetal gonads to capture the molecular signatures of the bovine germline and its niche. A total of 19,499 cells from two independent replicates of approximately 50 days of fetal age (4 cm CRL) were sequenced obtaining 564,587,528 sequence reads, with 64,039 and 22,332 mean reads per cell in sample 1 and 2, respectively (Supplementary Fig. 1A). After filtering out cell doublets and low-quality cells, 15,548 cells were used for clustering. We identified 11 cell clusters with all clusters being represented in each replicate (Supplementary Fig. 1B). Cluster identity was assigned based on the expression of well-conserved marker genes (Fig. 1A and B) and functional GO term enrichment (Supplementary Table 1). Cells allocated to cluster 5 were identified as PGCs based on the expression of pluripotency and germline-specific genes such as *NANOG*, *OCT4* (also known as *POU5F1*), *NANOS3*, and *EPCAM* (Fig. 1B and C). PGCs represented 10.8% of the cells present in the fetal gonad. Clusters 0, 1, and 2 expressed genes associated with stromal (*NR5A1* and *COL1A2*) and epithelial (*KRT19* and *MSLN*) cell populations of the fetal ovary (Jameson *et al.* 2012, Mork *et al.* 2012, Hummitzsch *et al.* 2013), and respectively represented 51.7, 20.1, 17.5% of the cells restricted to the somatic and germ cell compartment. GO analysis indicated that clusters 0, 1, and 2 showed specific enrichment for terms such as collagen and extracellular matrix, translation and cell–cell adhesion, and cell proliferation, respectively (Supplementary Table 1). Thus, we identified clusters 0, 1, and 2 as stromal cells, quiescent epithelial cells and proliferative epithelial cells, respectively (Fig. 1B and C). Clusters 3, 4, 6, 7, 8, 9, and 10 represented 29.7% of the total cells analyzed and corresponded to endothelial cells, erythrocytes,

Figure 1 Cell populations detected by scRNA-seq in the bovine fetal ovary at 50 days of fetal age. (A) Heatmap of differentially expressed genes in each cell cluster. Genes used for cluster identification are colored in yellow. (B) Dot plot of the average level of expression (dot color) and percentage of positive cells (dot size) expressing specific marker genes in each cell cluster. (C) Identity of cell populations in UMAP projection.

monocytes/macrophages, reticulocytes, fibroblasts, platelets, and lymphocytes, respectively (Fig. 1C). This analysis demonstrated that different cell populations, including PGCs, can be identified in the bovine fetal gonad by scRNA-seq.

Evaluation of the gene expression profile of the somatic compartment (clusters 0, 1 and 2) (Supplementary Fig. 3) showed high expression of genes involved in the development of the genital ridge (*WT1*, *GATA4*, *SF1*, *EMX2* and *LHX9*) (Luo *et al.* 1994, Miyamoto *et al.* 1997, Birk *et al.* 2000, Hammes *et al.* 2001, Kusaka *et al.* 2010, Hu *et al.* 2013). On the other hand, genes associated with pre-granulosa cells (*LGR5*, *RSP01*, *WNT4*, *FOXL2* and *FST*) (Jorgez *et al.* 2004, Mork *et al.* 2012, Rastetter *et al.* 2014, Zheng *et al.* 2014) presented only low expression in around 5.5% of the cells. These results indicate that the somatic cells of 50 days old (4 cm CRL) bovine fetal gonads are largely undifferentiated and starting the transition toward supportive cells.

Molecular profiling of the bovine germline during early fetal development

scRNA-seq data indicated that at 50 days of fetal age (4 cm CRL) most of the cells in the PGC cluster (98.7%) expressed at least one marker of pluripotency including *OCT4* (72.5%), *NANOG* (95.2%), and *SALL4* (47.2%), or at least one marker of early PGCs development (95.5%) including *NANOS3* (80.2%), *KIT* (69%), and *AP* (57.6%). A smaller subset of cells (21.1%) expressed genes that characterize pre-meiotic late-stage PGCs including *DAZL* (6.3%), *DDX4* (16%), and *STRA8* (0.5%) (Fig. 2A and Table 3). Marker co-expression analysis showed that 76% of the cells allocated to the PGC cluster shared expression of pluripotency and early markers, with 21% also co-expressing late PGCs markers (Fig. 2B), indicating that most PGCs in day 50 fetuses are still in early stages of differentiation, with a subset of cells transitioning toward a more advanced stage.

To confirm the early stage of bovine PGCs at 50 days of fetal age (4 cm CRL) and assess the developmental

Figure 2 Gene expression profile of bovine PGCs. (A) Level of expression of pluripotency, early and late markers of PGC development in cells of the bovine gonad. (B) Venn diagram of the co-expression of pluripotency, early, and late markers of PGCs development in bovine PGCs. (C) Relative expression of *OCT4*, *NANOS3* and *DDX4* in fetal gonads between 45 and 90 days of fetal age and adult testes and ovaries. Numbers in fetal samples represent crown-rump length in cm.

timeline of the bovine germline, we analyzed expression of well-known markers of early and late PGC at different stages of development by RT-qPCR and immunofluorescence (Figs 2C and 3). Detection of *OCT4*, *NANOS3*, and *DDX4* in fetal gonads by RT-qPCR (Fig. 2C) corroborated the findings from scRNA-seq. *OCT4* and *NANOS3* presented a similar pattern of expression, characterized by a progressive increase during the first 2–3 months of gonad development, likely reflecting PGC proliferation. *OCT4* and *NANOS3* expression decreased after this period (Fig. 2C). *NANOS3* was not detected in adult ovaries, but was expressed in the adult testes, which is consistent with its role during spermatogenesis (Lolicato *et al.* 2008). Expression of *DDX4* increased after 50 days of development (4 cm CRL) reaching its highest expression in the adult testis (Fig. 2C). Immunofluorescence analyses (Figs 3 and 6) indicated that *PRDM1* and *OCT4* were equally expressed in female and male PGCs early in development (40–70 days or 2–7 cm CRL). Upon progression of differentiation, *PRDM1* and *OCT4* were first downregulated in males while in females their expression persisted in germ

cells located at the ovarian cortex. *DAZL* protein was first detected at 60 days (6 cm CRL) of development and rapidly became broadly expressed in the germline, especially in males. *DAZL* detection persisted at 80–90 days (12–16 cm CRL), in spermatogonia and oogonia inside the seminiferous tubules and ovigerous cords, respectively. *DDX4* was only detected in the fetal gonads after day 80 of fetal development (12 cm CRL), following *DAZL* upregulation. Overall, RT-qPCR and immunofluorescence analyses confirmed scRNA-seq results indicating that at day 50 of fetal age (4 cm CRL), bovine PGCs are mostly in early stages of development.

Similarities between bovine and human germline development

Given the early developmental stage of day 50 bovine PGCs, we evaluated the presence of known transcriptional regulators involved in human germline commitment. The signaling cascade that triggers specification of the human germline has been well studied. *PRDM1*, *PRDM14*, and *TFAP2C* form a key

Table 3 Gene expression profile of female bovine PGCs at 50 days of fetal age.

Genes	PGC cluster	
	Number of positive cells	Percentage of positive cells
Pluripotency		
<i>OCT4</i>	854	72.5
<i>NANOG</i>	1121	95.2
<i>SALL4</i>	556	47.2
<i>KLF4</i>	128	10.9
<i>LIN28A</i>	59	5.0
<i>DPPA3</i>	0	0.0
<i>SOX2</i>	0	0.0
Early PGCs		
<i>PRDM1</i>	794	67.4
<i>PRDM14</i>	44	3.7
<i>TFAP2C</i>	1006	85.4
<i>SOX17</i>	1116	94.7
<i>SOX15</i>	1008	85.6
<i>NANOS3</i>	945	80.2
<i>ALPL</i>	679	57.6
<i>DND1</i>	3	0.3
Late PGCs		
<i>DDX4</i>	188	16.0
<i>DAZL</i>	74	6.3
<i>PIWIL2</i>	23	2.0
Meiosis		
<i>STRA8</i>	6	0.5
<i>SYCP1</i>	5	0.4
<i>SYCP2</i>	261	22.2
<i>SYCP3</i>	479	40.7
<i>REC8</i>	29	2.5
<i>STAG3</i>	9	0.8
Oocyte		
<i>NOBOX</i>	1	0.1
<i>FIGLA</i>	1	0.1
<i>ZP3</i>	57	4.8
<i>GDF9</i>	4	0.3
Surface proteins		
<i>KIT</i>	813	69.0
<i>EPCAM</i>	1133	96.2
<i>PDPN</i>	1110	94.2
<i>ITGA6</i>	409	34.7

transcriptional network that commands specification of the germline in different species (Ohinata *et al.* 2005, Yamaji *et al.* 2008, Kobayashi *et al.* 2017, Sybirna *et al.* 2020). Specifically in humans, the SRY-Box proteins SOX17 and SOX15 also play an important role in germline development (Guo *et al.* 2015, Tang *et al.* 2015, Kobayashi *et al.* 2017, Kojima *et al.* 2017, Chen *et al.* 2018, 2019). Bovine PGCs expressed *PRDM1*, *TFAP2C*, *SOX17*, and *SOX15* transcription factors (Fig. 4A). *PRDM14* was only detected in a few cells, which is consistent with observations in human PGCs (Guo *et al.* 2015, Irie *et al.* 2015, Tang *et al.* 2015, Kobayashi *et al.* 2017) and *SOX2*, which is only re-activated in mouse PGCs (Western *et al.* 2005, Perrett *et al.* 2008, Campolo *et al.* 2013), was not detected. We confirmed expression of *PRDM1*, *TFAP2C*, *SOX17*, *SOX2*, and *PRDM14* by RT-qPCR in fetal gonads (Fig. 4B). Levels

of *PRDM1*, *TFAP2C*, and *SOX17* increased until days 60–70 of development (approximately 8 cm CRL) and then decreased steadily. Interestingly, *PRDM14* shared a similar pattern of expression with *PRDM1*, *TFAP2C*, and *SOX17*, especially in fetal ovaries. Expression of *SOX2* was almost undetectable in all the fetal samples evaluated, confirming results of scRNA-seq analysis.

Integration of our dataset with a publicly available single-cell transcriptome of human fetal gonadal cells (FGCs) of 5–26 weeks of development (Li *et al.* 2017), showed that 50 days old bovine PGCs clustered together with early human germ cells (Mitotic) (Fig. 5A). Bovine PGCs and human mitotic FGCs had a similar gene expression profile, especially for genes involved in germline specification and pluripotency, and genes that characterize early and late PGC development (Fig. 5B). Our scRNA-seq results also revealed that bovine PGCs expressed genes coding for the surface proteins *EPCAM* (96.2%), *PDPN* (94.2%), *KIT* (69%), and *ITGA6* (34.7%) (Table 3), which are also present in human FGCs (Fig. 5C) (Sasaki *et al.* 2016, Chen *et al.* 2018). These findings further confirm the similar germline-specific molecular profiles between these two species.

We performed prediction of marker panels by COMET which ranks single or multiple marker genes for identification of cell populations of interest. In line with previous results, *EPCAM*, *KIT* and *PDPN* appeared among the top ranked markers to identify bovine PGCs (Supplementary Fig. 4A and Supplementary Table 3). Interestingly *CD9*, which has a role during fertilization (Umeda *et al.* 2020) and is present in 99.7% of the cells in the PGC cluster and ranked the fifth best marker for PGC identification and isolation. Reliable approaches for the isolation of gonadal somatic cells could be possible using single marker genes (Supplementary Fig. 4B) or by positive and negative selection of marker pairs (Supplementary Fig. 4C).

Exploring the distribution of OCT4 and DAZL in whole ovaries from 40- to 90 day-old fetuses (Fig. 6), we found a progressive loss of OCT4 expression from the center of the ovary outwards to the periphery, while DAZL expression persisted during differentiation, as others have described (Hummitzsch *et al.* 2013). At 40 days of age, few OCT4-positive cells were detected in the ovary. OCT4-positive cells dramatically increased 20 days later and at day 90 OCT4 was only expressed in cells confined to the vicinity of the surface epithelium. Expression of DAZL was first detected at 60 days of age in cells of the medulla and ovarian cortex. DAZL-positive cells localized in the same pattern of cells expressing OCT4; in fact, all the DAZL-positive cells detected at 60 days co-expressed OCT4. Overall, the expression pattern of germline transcriptional regulators, germline-specific, pluripotency, and cell surface markers, and the asynchronous pattern of differentiation detected in the bovine germline, revealed important similarities in the development of bovine and human germ cells.

Figure 3 Expression of well-conserved germ cell markers in bovine fetal gonads. Immunofluorescence for PRDM1, OCT4, DAZL, and DDX4 in gonads from 40 to 90 days of fetal age. Nuclear staining was performed using Hoechst 33342. Scale bar 20 μ m. OCT4 and PRDM1 detection was confined to the nuclei of PGCs, and DAZL and DDX4 to the cytoplasm. This is in agreement with their roles as transcriptional regulators and RNA-binding proteins, respectively.

Expression of mediators of different signaling pathways in the bovine fetal ovary

Our data confirmed that bovine PGCs express the *KIT* receptor (Kritzenberger & Wrobel 2004), while gonadal somatic cells expressed the *KIT* ligand (*KITLG*, also known as *SCF*) (Fig. 7), consistent with previous findings (reviewed by Driancourt *et al.* 2000).

It has been suggested that bone morphogenetic protein (BMP), Nodal, and Notch signaling cascades have roles in the initial development of the human germline (Li *et al.* 2017). We identified expression of *BMP2* and *BMP4* ligands in different cell populations, particularly in PGCs (Fig. 7). *BMP4* was more broadly expressed than *BMP2*, and it was among the top differentially expressed genes detected in the PGC cluster (Supplementary Table 2). Receptors (*ACVR1*, *BMPRI1A*) and effector (*SMAD5*) of BMP signaling were detected in several cell groups at different levels of expression. Notably, most of the cells expressing *ACVR1*, *BMPRI1A* or *SMAD5* corresponded to stromal cells, epithelial cells, and PGCs.

The *Nodal* ligand was detected only in a few PGCs, but receptors (*ACVR1C*, *ACVR2A*) and nuclear effectors (*SMAD2* and *SMAD3*) of the Nodal cascade, were present in several cell clusters with a higher number of positive cells in the stromal, epithelial, and PGC clusters. Since Nodal and Activin are both members of the TGF β superfamily of signaling molecules, and signal through

the same mediators (reviewed by Pauklin & Vallier 2015), we evaluated the expression of the subunits *INHBA*, *INHBA*, and *INHBB*) (Fig. 7) that form Activins (A, B, and AB), as well as Inhibins (A, B, and AB). We detected expression of the three Inhibin subunits in our dataset, with specific enrichment in stromal cells and epithelial cells.

Members of notch signaling members were found at varying levels of expression in many of the cell populations analyzed (Fig. 7). The Notch ligand *DLL3* was mostly detected in PGCs, and the receptors *NOTCH2*, *NOTCH3*, the effector *RBPI* (also known as *CBF1*), which cooperates with Notch intracellular domain to promote transcription, and the target gene *HES1*, were abundant in stromal and/or epithelial cells. The consistent expression of mediators of KIT, BMP, Nodal/Activin, and Notch signaling in PGCs and supporting somatic cells of the bovine fetal gonad, suggest that these pathways may be involved in the interaction between bovine PGCs and their niche.

Discussion

Obtaining a pure population of PGCs for molecular profiling is challenging. Previous means used to identify bovine PGCs included AP activity, *KIT* expression, detection of specific lectins, and histological features. AP activity and *KIT* expression are unspecific methods

Figure 4 Expression of transcriptional regulators of germline development in bovine PGCs. (A) Violin plots of common (*PRDM1*, *PRDM14* and *TFAP2C*), and species-specific genes with a function in human (*SOX15* and *SOX17*) germline development. (B) Relative expression of *PRDM1*, *TFAP2C*, *SOX17*, *SOX2*, and *PRDM14* in fetal gonads between 45 and 90 days of fetal age, and adult testes and ovaries. Numbers in fetal samples represent crown-rump length in cm.

to identify germ cells, as they are also present in hematopoietic cells and gonadal somatic cells (Ohno & Gropp 1965, Lavoie *et al.* 1994, Kritzenberger & Wrobel 2004). Similar to other species, bovine PGCs have a characteristic morphology that allows their identification (Leichthammer *et al.* 1990, Wrobel & Süß 1998); however, identification of cells by morphology is impractical, considering the time required for tissue dissociation and manual cell selection. Targeting specific lectins on bovine PGCs does not offer a great advantage either, as glycan-binding antibodies have weak affinity for their target, leading to poor sensitivity (Haab 2012). DDX4 is a germline-specific marker frequently used for sorting human germ cells, but since the expression of a membrane-bound isoform of DDX4 in pre-meiotic germ cells is debatable, the use of commercially available anti-DDX4 antibodies for sorting purposes has shown conflicting outcomes (Woods & Tilly 2013, Zarate-Garcia *et al.* 2016, Wagner *et al.* 2020). Therefore, by performing scRNA-seq, we overcame the limiting step of cell sorting

and obtained the transcriptional landscape of bovine PGCs and of different cell groups within the fetal ovary.

The relatively undifferentiated state of bovine PGCs captured by scRNA-seq and the positive expression of master regulators of human germline commitment suggest that the signaling network governing bovine PGC specification might be similar to what has been described in humans. Our scRNA-seq data indicated that at 50 days of fetal age, the transcriptional profile of bovine PGCs is characterized by the expression of *PRDM1*, *TFAP2C*, *SOX17*, *SOX15*, *SALL4*, *LIN28A*, *KLF4*, and *ITGA6* (Table 3), with *SOX17* being one of the top differentially expressed genes (Supplementary Table 2), and lacking *SOX2* expression (Fig. 5B). Therefore, bovine PGCs seem to possess a transcriptional network similar to human PGCs. It has been postulated that *SOX17* exerts its critical function in the development of the human germline by regulating gene expression as a binding partner of OCT4 (Tang *et al.* 2016). Expression of *SOX17* and *SOX2* are mutually exclusive in human

Figure 5 Similarities between bovine and human PGCs transcriptional profile. (A) Integration of scRNA-seq data from bovine (50 days) and human (5–26 weeks) fetal germ cells at different stages of differentiation (Li *et al.* 2017). (B) Comparison of the expression of germline-specific genes between bovine PGCs and human fetal germ cells (Li *et al.* 2017). (C) Expression of *EPCAM*, *KIT*, *PDPN*, and *ITGA6* genes coding for membrane-bound proteins in bovine PGCs and human fetal germ cells (Li *et al.* 2017). FGCs, fetal germ cells; RA, retinoic acid.

PGCs and downregulation of SOX2 is required for PGC-like cell differentiation *in vitro* (Lin *et al.* 2014). OCT4 switches binding partners from SOX2 to SOX17, and regulates the expression of endoderm genes during mouse primitive endoderm differentiation (Aksoy *et al.* 2013). Interestingly, targets of the SOX17-OCT4 partnership are PRDM1 and SALL4 (Aksoy *et al.* 2013), which are both also expressed in bovine PGCs (Table 3). Therefore, our data support the notion that there might be a correspondence between embryonic structure and usage of transcription factors, and that SOX17 may be critical in determining PGC fate in species that develop as bilaminar discs, such as pigs, primates and cattle (Kobayashi & Surani 2018). It will be of interest in the future to evaluate the requirement for SOX17 in the development of the bovine germline.

Unlike PRDM1, which seems to have a conserved role in PGC fate across mammals, PRDM14 function seems to be less explicit. In mouse PGCs, PRDM14 is involved in repressing the somatic program, upregulating germline-specific genes, and promoting epigenetic reprogramming (Saitou *et al.* 2002, Yabuta *et al.* 2006, Seki *et al.* 2007). Instead in human PGCs the role of PRDM14 is less understood. Since in fetal gonads PRDM14 has a cytoplasmic location (Irie *et al.* 2015) and its knockdown did not affect PGC-like cell specification (Sugawa *et al.* 2015) it has been suggested that PRDM14 is dispensable for human PGC fate. However, a recent report put in evidence the crucial role of PRDM14 in the

differentiation of human PGC-like cells by inducing a rapid and comprehensive loss of endogenous PRDM14 protein (Sybirna *et al.* 2020). Thus, we compared the expression profile of PRDM14 in bovine and human PGCs and interestingly, only a small group of bovine PGCs expressed PRDM14. This difference could be attributed to a different role of PRDM14 in bovine PGC fate, or possibly to the different scRNA-seq platform used between datasets.

The transcriptome of bovine PGCs revealed the expression of genes coding for membrane-bound proteins that could be of utility for cell sorting. Besides the KIT receptor (Kritzenberger & Wrobel 2004), bovine PGCs also expressed PDPN, EPCAM, and ITGA6. Even though these markers do not seem to be specific to the germline, they may be a good alternative to the also unspecific KIT receptor, especially if they are targeted simultaneously. KIT, AP, PDPN, EPCAM, and ITGA6 have also been used to sort or quantify gonadal and *in vitro* generated germ cells in humans (Guo *et al.* 2015, Irie *et al.* 2015, Sasaki *et al.* 2015, Kobayashi *et al.* 2017, Li *et al.* 2017, Yokobayashi *et al.* 2017, Chen *et al.* 2018).

Different signaling pathways have roles in regulating the delicate interaction between PGCs and gonadal somatic cells. BMP signaling has a well-known role in determining germline fate and regulating follicular development (reviewed by Rossi *et al.* 2016). BMP4 and BMP7, secreted from theca and granulosa cells, are important for the primordial to primary follicle

Figure 6 Expression of germ cell markers in whole bovine ovaries. Immunofluorescence for OCT4 and DAZL in the developing ovary. Histological sections were imaged at 20x and pictures were merged to inspect a complete section of the ovary. Nuclear staining was performed using Hoechst 33342. Scale bars 100 µm.

transition (Lee *et al.* 2001, Nilsson & Skinner 2003). Granulosa cells of human fetal ovaries are characterized by having high expression of BMP2, which possibly plays a role during the mitosis-meiosis transition of germ cells (Li *et al.* 2017). In the bovine fetal gonad, we detected BMP2 and BMP4 ligands in germ cells, and BMP7 highly expressed in a small group of cells in the somatic compartment. Receptors and mediators of BMP signaling were present in stromal cells, epithelial cells, and PGCs. These results suggest that BMP signaling operates two-ways between PGCs and the somatic compartment in the bovine fetal ovary. Human PGCs might induce differentiation of other germ cells through activation of the Nodal/Activin signaling pathway (Li *et al.* 2017). Our results indicate that Nodal/Activin signaling may also be active in different cell populations of the fetal ovary, but mainly in stromal cells, epithelial cells, and PGCs. We hypothesize that neither Activins nor Nodal ligands are the main drivers of activation of the cascade, but rather, that Inhibins are the ligands that trigger activation of Activin/Nodal pathway in the bovine fetal ovary. Recently the role of the Notch

pathway in the development of the mammalian ovary has begun to be elucidated (Vanorny & Mayo 2017). Notch activation in somatic cells of the mouse fetal ovary is required for germ cell nest breakdown, follicle assembly (Xu & Gridley 2013, Vanorny *et al.* 2014), and meiosis entry (Feng *et al.* 2014). Our scRNA-seq results show high expression of the *DLL3* ligand in PGCs, and expression of the *NOTCH2* receptor and the target gene *HES1* in somatic cells, in agreement with findings in the human fetal gonad (Li *et al.* 2017). Therefore, these results suggest that Notch signaling plays an important role during gonad development, which is conserved among the mouse, human, and cow.

Another difference between mouse and human germ cells development is the pattern by which differentiation proceeds. In the mouse fetal ovary, meiosis progresses in a rostro-caudal wave (Bowles *et al.* 2006, Koubova *et al.* 2006) while in the human fetal ovary meiosis occurs radially (Anderson *et al.* 2007). Interestingly, our data indicate similarities between the bovine and human germline in terms of spatio-temporal differentiation. In fetal ovaries, we

Figure 7 Gene expression of members of different signaling pathways in the bovine fetal ovary. Dot plot of the average level of expression (dot color) and percentage of positive cells in each cell cluster (dot size). Genes in black represent ligands. Genes in purple represent receptors, effectors, or targets of each signaling cascade.

detected a progressive downregulation of OCT4 from the center to the periphery. Germ cells in seminiferous tubules also progressively lost expression of OCT4, and only few OCT4-positive cells remained among DAZL/DDX4-positive cells at day 80 of development. This is in agreement with characterizations of the developing human testis, where differentiation is asynchronous and germ cells expressing early and late markers coexist inside the same seminiferous tubule (Anderson *et al.* 2007, Sohni *et al.* 2019). These results may indicate that the progression of bovine PGC differentiation is asynchronous and that germ cells at different stages of differentiation coexist in the fetal gonad, similar to what has been reported for the human ovary (Anderson *et al.* 2007).

Conclusions

Results from this work offer new insights into the mechanisms that govern gonadal and germline development in cattle. The data we report here is a useful resource to understand the different cell compartments of the developing gonad, the reciprocal relationship between germ cells and their niche, and the factors that may affect germ cell development. Further, results from this study may provide the basis for developing approaches for *in vitro* differentiation of bovine gametes from pluripotent stem cells. The similarities we found in the transcriptional profile of human and bovine PGCs confirm that mouse biology can only be partially extrapolated to other mammalian species and indicate that cattle may be a suitable model to study the specification and commitment of PGCs in humans.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-20-0313>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

D A S was involved in the conception and design, performing experiments, data collection, data analysis, bioinformatics, data interpretation, and manuscript writing. P J R contributed to the conception and design, financial support, data interpretation, and manuscript writing.

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