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Title: Meiotic maturation failure in primary ovarian insufficiency: insights from a bovine model

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ABSTRACT
Purpose
Oocytes from women presenting Primary Ovarian Insufficiency (POI) generate viable embryos at a lower rate than non-POI women, but the mechanisms responsible for the lower oocyte quality remain elusive. Due to the scarcity of human oocytes for research, animal models provide a promising way forward. We aimed at investigating the molecular events characterizing final maturation in POI oocytes in a recently defined POI-like bovine model.
Methods
Single cell RNA-sequencing of bovine control and POI-like, GV and MII oocytes (n=5 per group) was performed. DEseq2 was used to identify differentially expressed genes. Further, a Gene Set Enrichment Analysis and a transcriptomic meta-analysis between bovine and human oocytes were performed.
Results
In control cows, we found 2223 differentially expressed genes between the GV and MII stages. Specifically, the affected genes were related to RNA processing and transport, protein synthesis, organelle remodeling and reorganization, and metabolism. The meta-analysis with a set of young human oocytes revealed 315 conserved genes through the GV-MII transition in cows and humans, mostly related to meiotic progression and cell cycle. Gene expression analysis between GV and MII of POI-like oocytes showed no differences in terms of differentially expressed genes, pointing towards a substantial failure to properly remodel the transcriptome in the POI model, and with the clustering analysis showing how the cow's genetic background had a higher impact than the oocyte's maturation stage.
Conclusion
Overall, we have identified and characterized a valuable animal model of POI, paving the way to identifying new molecular mechanisms involved in POI.

INTRODUCTION
The ovarian environment is highly dynamic throughout a woman's reproductive life. Alterations in its molecular regulation could cause reproductive dysfunctions, often resulting resulting in a reduced ovarian reserve and/or a lower oocyte competence [1]. A decrease in ovarian reserve and

oocyte quality is generally associated with advanced maternal age (AMA) in women [1,2]. However, some women show altered ovarian function and low antral follicle counts (AFC) before 35 years of age, accompanied by oocytes of diminished developmental competence. This condition, known as primary ovarian insufficiency (POI), affects 1-10% of women [3,4]. POI is associated with systemic symptoms such as amenorrhea, hypoestrogenism, high serum follicle-stimulating hormone (FSH) levels, low anti-Mullerian hormone (AMH) levels, and a reduced oestradiol concentration. Furthermore, POI is characterized by accelerated ovarian senescence, reduced follicle reserve, and hypofertility [5,6]. Oocytes from POI women present diminished developmental competence, hence the mechanisms responsible for this decrease in oocyte quality remain elusive.

The scarcity of oocytes available from POI women limits studies in human, and animal models are critical to understanding POI physiopathology, given the convergent phenotype of POI features in other mammalian species. A POI-like phenotype has been described in 5% of 4-8-year-old culled dairy cows [7-9]. These animals have small 57 ovaries (< 2-4 cm length on the major axis) and are characterized by a significant gram of ovary [10]. Macroscopically, POI-like cows present less than 10 antral follicles in both ovaries (Gandolfi et al, 1997; Tessaro et al., 2011, Lodde et al., 2021) and normal oocyte maturation rates but reduced developmental competence, with a blastocyst rate of around 6% blastocyst rate, 60% aneuploidy rates in MII oocytes (Luciano et al., 2013) and reduced embryo quality [7,11,12]. In addition, they present a distinctive hormonal profile with low AMH, reduction in oestradiol, and increase in progesterone levels in the follicular fluid, as much as women affected by POI [10].

Cow POI-like oocytes showed alterations in mitochondrial distribution and activity, histone modifications, DNA damage, glutathione content, and communication between the oocyte and the cumulus cells [7]. So far, however, an in-depth characterization of the transcriptome of POI-like cow oocytes is lacking. Given the similarities in gene expression profiles during oocyte maturation and embryo development between humans 71 and bovine [13], studying these oocytes in the bovine model could provide insights into POI in our species. We aim to define the transcriptional profile of POI-like oocytes in 73 cows to identify conserved alterations across species linked to this phenotype.

MATERIALS AND METHODS

Oocytes collection and culture

Bovine ovaries were recovered at a local abattoir (IT 2270M CE; Inalca S.p.A., Ospedaletto Lodigiano, LO, Italy) from adult 4-8-year-old Holstein cows subjected to routine veterinary inspection and according to specific health requirements. The ovaries were transported to the laboratory within 3 h in sterile saline (NaCl, 9 g/L), supplemented with penicillin 100 U/mL and streptomycin 0.1 mg/mL (pen/strep), at 26-28°C while subsequent procedures were performed at 35-38oC. Ovaries were classified into two categories, based on the number of medium antral follicles (2-6 mm) visible on the varian surface, as low AFC (Lo) when displaying < 10 follicles or high AFC (Hi) when presenting with ≥ 10 follicles, as previously described (Tessaro et al., 2011). Only ovaries isolated from cows having both Lo or Hi ovaries were used. The presence or absence a corpus luteum was not considered [11.14]. Cumulus–oocyte complexes (COCs) were retrieved from medium antral follicles with a 16-gauge needle mounted on an aspiration pump (COOK-IVF, Brisbane, QLD, Australia). COCs were washed in TCM-199 of supplemented with 20 mM HEPES buffer, 1790 U/l heparin, and 0.4% bovine serum albumin (BSA) (H-M199) and examined under a stereomicroscope. Only COCs medium 92 brown in color, with five or more complete layers of cumulus cells and oocytes with a finely granulated, homogenous ooplasm, were used. Twenty

cumulus-oocytes complexes (COC) from ten cows were collected as previously described [7]. COCs were denuded and either processed immediately at the GV stage or matured in vitro (IVM) to the MII stage. Groups of 10-14 COCs were matured for 24 h in TCM-199 supplemented with 0.68 mM L-glutamine, 25 mM NaHCO3, 0.4% fatty acid free BSA, 0.2 mM sodium pyruvate and 0.1 IU/mL recombinant human FSH (Gonal-F, Merck-Serono) in humidified air under 5% CO2 at 38.5oC. After IVM, all the COCs were denuded and processed. Only oocytes that extruded the first polar body (PB) were considered mature.

Single cell RNA sequencing

For single cell RNA sequencing, twenty oocytes from 10 cows were included. Oocytes were assigned to the study (POI-like, n=10) or control (CTRL, n=10) group based on AFC and ovary size (length on the major axis). POI-like oocytes were from ovarian pairs with AFC<10 and <2-4 cm in length. Each group included 5 GV and 5 MII oocytes. Each cow provided one GV and one IVM-MII oocyte (Supplementary Table S1). The cumulus cells were mechanically removed and the zona pellucida was digested with pronase (Roche Diagnostics, Basel, Switzerland). The zona-free oocytes were lysed individually in 20µL of Extraction Buffer (PicoPure RNA Isolation Kit, Thermo Fisher, Waltham, Massachusetts, USA), incubated at 42°C for 30 min and stored at -80°C. Total RNA extraction was performed following manufacturer's specifications (PicoPure RNA Isolation Kit, Thermo Fisher, Waltham, Massachusetts, USA).

Single oocyte cDNA libraries were constructed using the Ovation SoLo RNA-Seq System (NuGEN, TECAN, Männedorf, Switzerland). Sequencing of libraries was carried out using a NextSeq500 (Illumina, San Diego, California, USA) with 2x150 bp paired-end reads, with 30 million reads per sample. FastQC (Babraham Bioinformatics, Cambridge, UK) was used to perform the sequencing quality control.

Transcriptome analysis

After quality control of raw reads, the clean reads for each sample were aligned with bwamem2 to the BosTau8 bovine reference genome using Galaxy (https://usegalaxy.eu/, [15]) and bam files were generated. Reads belonging to whole genes or single exons were counted using the function SummarizeOverlaps of the Bioconductor/R package Genomic Features [16] DESeq2 [17] was then used to detect differentially expressed genes (DEGs) between experimental groups, and genes with an adjusted p-value <0.1 were considered significant.

Metanalysis of human public data

Bovine data from this study were further compared to human scRNA sequencing data obtained from oocytes at different maturation stages (GSE213267), from the Gene Expression Omnibus (GEO) at https://www.ncbi.nlm.nih.gov/geo. The STRING database was used to visualize protein interactions [18].

Gene set enrichment analysis

Gene set enrichment analysis (GSEA) was performed by WebGestalt [19] using the gene ontology (GO) database for biological function selecting the following parameters:Organism: btaurus; Enrichment Categories: geneontology Biological Process noRedundant; ID type: ensemble gene id; Minimum number of IDs in the category: 3. We then selected the top 20 pathways sorted by False Discovery Rate (FDR).

RESULTS

The transcriptome of bovine oocytes during meiotic maturation highlights processing of meiosis and female germ cell development related genes

First, the main molecular switches characterizing the GV-MII transition were analysed in The oocytes collected from control bovine ovaries. The principal component analysis (PCA) showed clear clustering of CTRL group oocytes based on their developmental stage, with a variance of 50% in the first and 17% in the second component (Figure 1A). The high variance was caused by one oocyte, as seen also when considering the top 50 variable genes (Figure 1B), and the score for PC1 was over 2SD of the average of all the scores. For this, we considered this oocyte an outlier and excluded it from further analyses, which were performed on 5 CTRL GV and 4 CTRL IVM MII. We identified 2223 DEGs between oocytes in IVM-MII vs. GV (Supplementary Table
156 S2, Figure, 1C), 1176 with increased abundance and 1047 with decreased abundance in
157 IVM-MII. Selecting for log2 Fold Change > |±1|, we found 38 DEGs with increased (7%)
158 and 511 with decreased (93%) abundance in IVM-MII (Figure 1D). GSEA highlighted
159 biological processes related to the development of the reproductive system, regulation of
160 hormonal levels, mRNA processing and meiotic cell cycle, protein transport, and localization (Figure 1E).

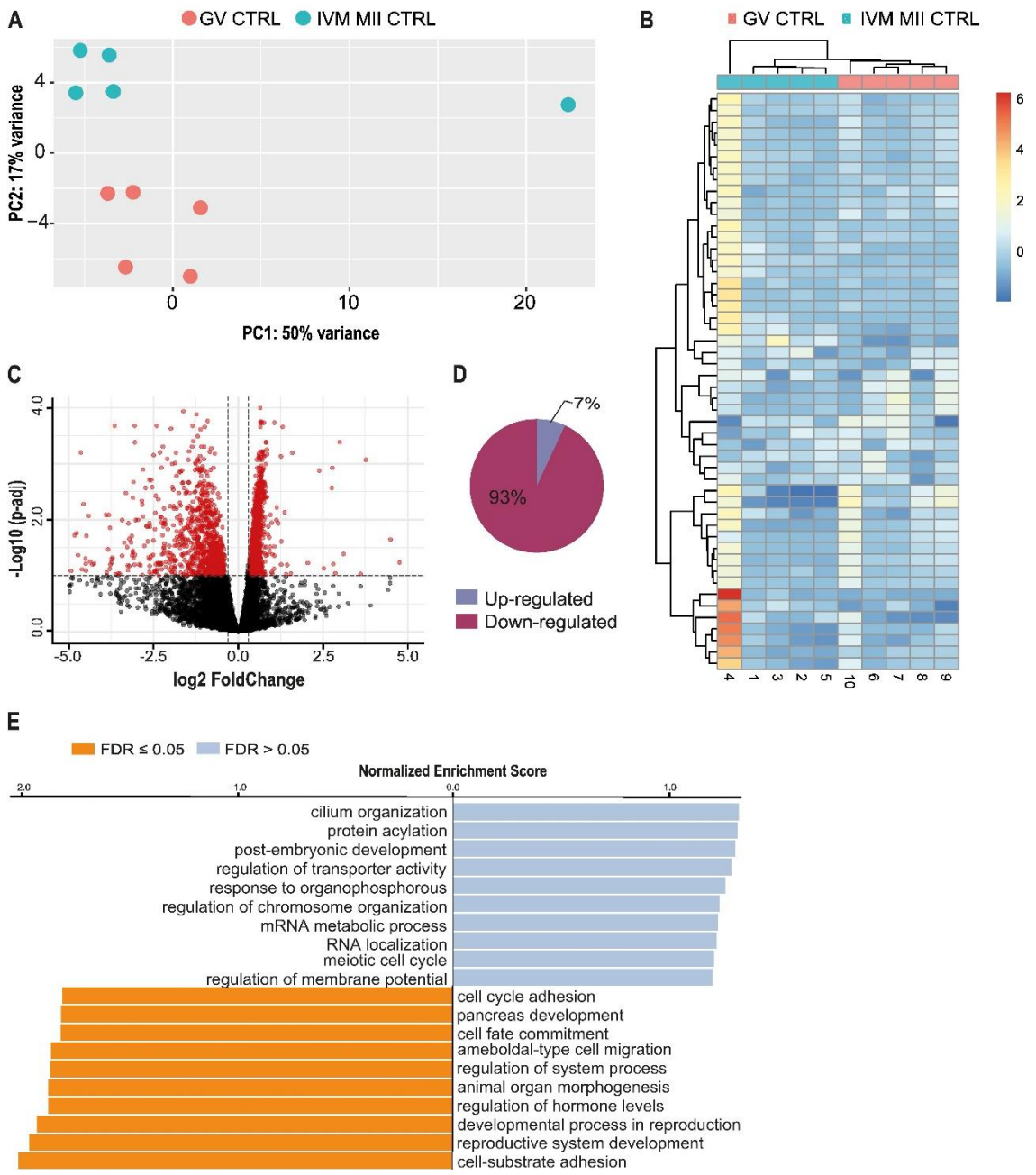

Figure 1 Transcriptomic changes through oocyte meiotic maturation of control bovine oocytes. (A) Principal component analysis (PCA) of GV and IVM-MII oocytes from young cows with normal ovarian physiology (CTRL group). (B) Heatmap of the top 50 differentially expressed genes (DEGs) between GV and IVM-MII oocytes; the shades of colours represent the counts per million. (C) Volcano plot of the DEGs found in the comparison IVM-MII versus GV in CTRL group. Red dots represent the genes with p.value<0.1 considered statistically significant (D) Proportion of up- and down-regulated genes in IVM-MII versus GV in CTRL group. (E) Gene Set Enrichment Analysis (GSEA) of the most affected pathways in the IVM-MII versus GV comparison in CTRL group. The affected pathways with FDR ≤ 0.05 are reported in orange and blue, while those with FDR > 0.05 are in light orange and light blue

Transcriptome convergence of human and bovine oocytes during meiosis

To evaluate the degree of similarity between human and bovine oocytes, we processed 178 data from a set of 10 GVs and 10 in vivo matured MII human oocytes from oocyte donors (GSE213267) [20]. We found 1675 DEGs between GV and in vivo matured MII oocytes, mostly related to translation initiation, protein transport, RNA metabolism, RNA transport, and splicing. We then compared them with the DEGs identified in bovine CTRL group IVM-MII vs GV and observed an overlap of 285 genes (Figure 2A, Supplementary Table S3). GSEA of the common DEGs highlighted RNA processing, cell cycle and oocyte meiosis pathways as the most affected biological processes across species (Figure 2B). Moreover, we found clusters of proteins with high level of interaction in pathways related to meiosis and chromosome segregation (Figure 2C), and mRNA processing and transport (Figure 2D).DEGs that were conserved between human and bovine include MAGOH (Mago Homolog, Exon Junction Complex Subunit), SREK1 (Splicing Regulatory Glutamic Acid And Lysine Rich Protein 1), YTHDC1 (YTH N6-Methyladenosine RNA Binding Protein C1), THOC1 (THO Complex Subunit 1), THOC7 (THO Complex Subunit 7), SRSF4 (Serine And Arginine Rich Splicing Factor 4), PAPOLG (Poly(A) Polymerase Gamma), PRPF18 (Pre-MRNA Processing Factor 18), involved in mRNA processing, transport and degradation, and FRG1 (FSHD Region Gene 1), RNF20 (Ring Finger Protein 20), KIF4A (Kinesin Family Member 4A), NIPBL (NIPBL Cohesin Loading Factor), CDC40 (Cell Division Cycle 40), TUBB (Tubulin Beta Class I), and SASS6 (SAS-6 Centriolar Assembly Protein), involved in cell cycle and sister chromatid segregation (Supplementary Figure 1).

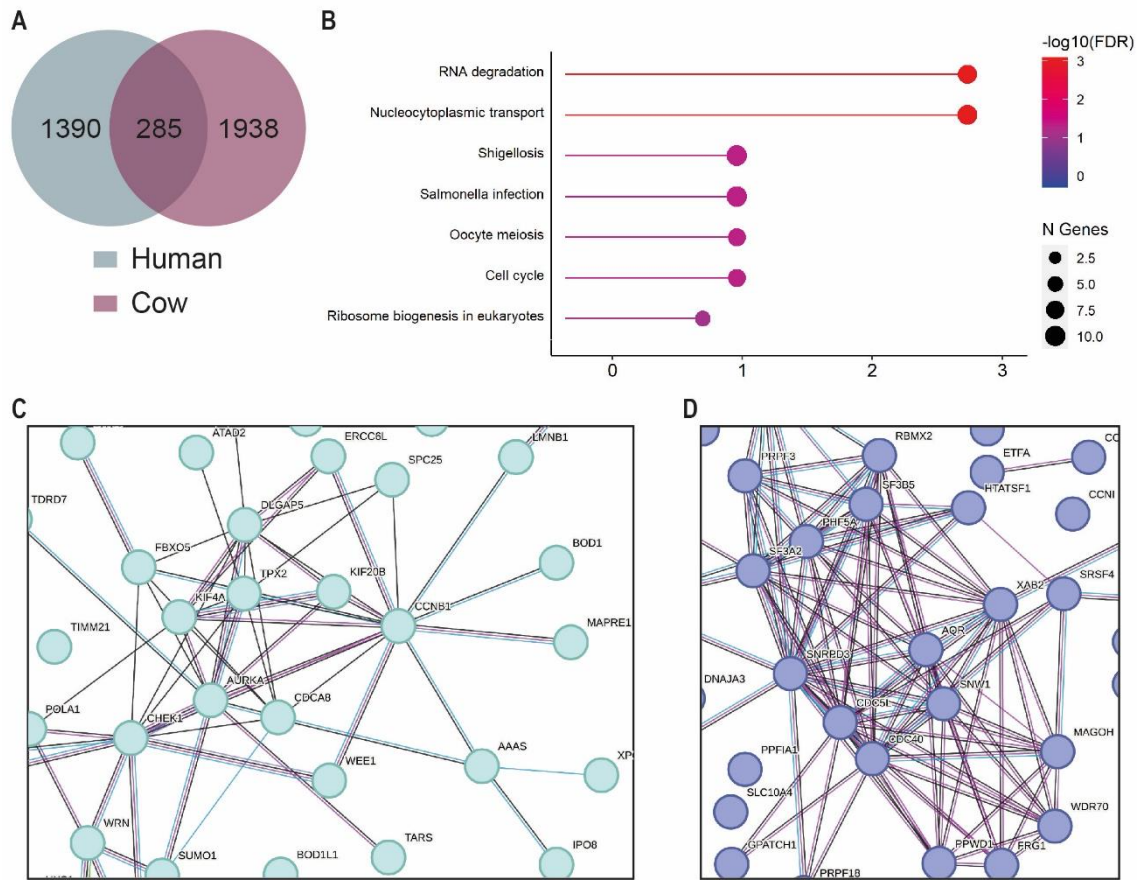

Figure 2 Human-Bovine cross-species comparison of the different genes expressed through meiosis. (A) Comparison between human (GSE213267) and bovine differentially expressed genes (DEGs) in mature versus immature oocytes. (B) Most significant biological processes involved in the transition from immature to mature oocytes in common DEGs between human and bovine. In the lollipop plot, the -log10 (False Discovery Rate, FDR) is represented in red to blue shades, while the dot of each pathway is proportional to the number of genes in the pathway. (C) Network interactions of DEGs involved in oocyte meiosis and (D) mRNA processing and transport

POI-like bovine oocytes fail to remodel their transcriptome during in vitro maturation

PCA showed a stronger clustering of the bovine POI-like samples by individual rather than by the meiotic stage of the oocyte (Figure 3A). In the PCA, a variance of 57% in the first and 11% in the second component was reported. Even when considering the top 50 variable genes, the separation of the oocytes continued to be associated with the individual animal rather than the meiotic stage (Figure 3B). Again, having seen that two samples from the same cow were further away from the bulk of other samples and that PC1 was accounting for 57% of the variance, with their scores over 2SD of the average, we considered these 2 oocytes as outliers and proceeded with 4 GVs versus 4 IVM-MII. The individual-of-origin clustering, rather than the meiotic stage-driven clustering of the samples, was very specific to the POI-like samples and not observed in the CTRL group,as shown in Fig. 1A.

The comparison of IVM-MII vs. GV oocytes yielded only 7 DEGs (Figure 3C, Supplementary Table S4), showing a high correspondence of the transcriptomes of GV and IVM-MII oocytes and indicating a failure to achieve a correct cytoplasmic maturation. The low number of genes did not allow us to identify specific biological processes affected in this comparison.

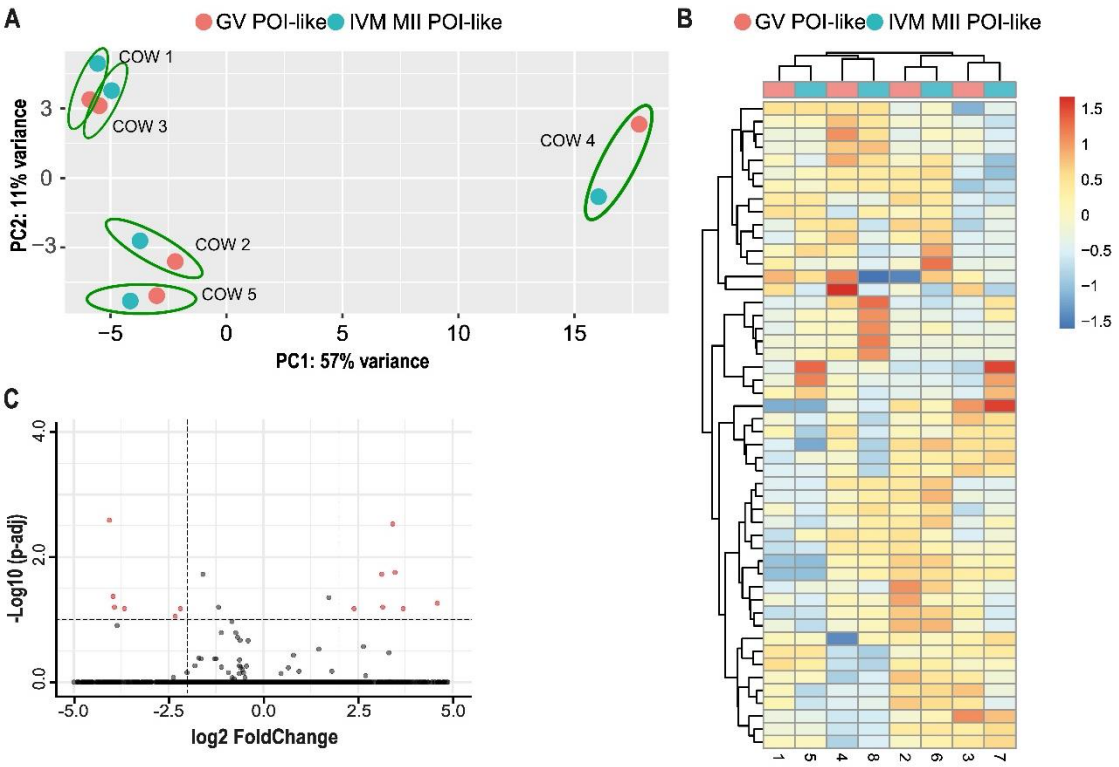

Figure 3 Transcriptional characterization of maturing oocytes from POI-like group. (A) Principal component analysis (PCA) of immature GV oocytes and IVM-MII oocytes in the POI-like group. Green circles indicate the oocytes coming from the same cow. (B) Heatmap of the top 50 differentially expressed genes (DEGs) between GV and IVM-MII oocytes. The shades of colours represent the counts per million. (C) Volcano plot of the DEGs found with p-adjusted<0.1 (red dots) in the comparison of IVM-MII versus GV 233 oocytes in the POI-like group.

Comparison between CTRL and POI-like DEGs highlight differences in meiosis-related transcript processing

To understand the characteristics of the low number of DEGs observed in the POI-like group, we compared CTRL and POI-like groups. When comparing the same meiotic stages between CTRL and POI-like oocytes, we found 134 and 166 DEGs in GV oocytes (Supplementary table S5) and IVM-MIIs oocytes (Supplementary table S6), respectively. DEGs in GV were mostly related to cell cycle, development and cell junctions (Figure 4A), with genes specifically related to cumulus-oocyte complex communication and microtubule organisation, such as GJA1, CEP120, NUP35, and NEK9. DEGs in IVM-MII oocytes were primarily involved in biological processes related to cell cycle and chromosome segregation (Figure 4B). When comparing these genes to the ones identified in the meiotic progression from GV to MII in controls, we observe that the vast majority of the POI-like vs CTRL DEGs were also found in IVM-MII vs GV of CTRLs (90 out of 135 in the GV comparison, and 140 on 166 in the MII comparison)(Figure 4C) with the fold changes strikingly similar to each other in absolute values but with opposite signs. Finally, when considering POI-like vs CTRLs MII, we observed that all the overlapping genes were less abundant in POI-like MIIs and therefore more abundant in CTRL MII when compared to GV, while none of the ones that decreased their abundance in CTRL MIIs during maturation was identified as differentially expressed in the POI-like MIIs.

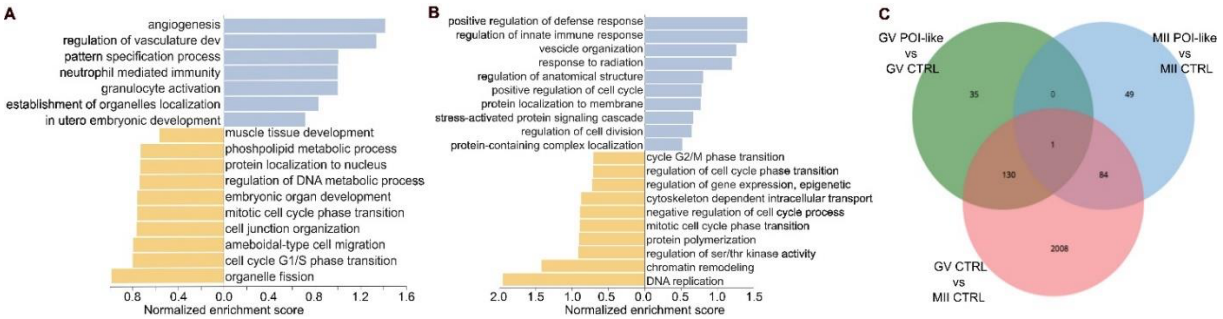

Figure 4 Transcriptomic differences between CTRL and POI-like oocytes at the same maturation stage, and their overlap with the physiological transcriptomic maturation in CRTL GV and MII. Gene set enrichment analysis of the most affected pathways in the comparison between (A) GV POI-like and GV CTRL and (B) IVM MII POI-like and IVM MII control. (C) Overlap of the DEGs found in POI-like versus CRTL oocytes and the DEGs found between GV and IVM MII in controls.

DISCUSSION

We identified changes in gene expression during the transition between GV and MII stages in oocytes from cows with normal reproductive physiology, mainly affecting genes related to reproductive system development, regulation of hormonal levels, mRNAprocessing and meiotic cell cycle, protein transport, and localization. A core set of genes specific to meiotic maturation was also found to be conserved in human maturing oocytes [20], reinforcing the notion that bovine oocytes can be considered a useful model for studying human oocyte molecular physiology. Interestingly, we observed significant defects in the nuclear/cytoplasmic maturation of o ocytes from cows with a POI-like phenotype; in this group, we could not detect transcript processing in phenotypically mature MII oocytes compared to the GV stage. This highlights a molecular defect that could contribute to the very low developmental competence of such oocytes. Further, when comparing the DEGs between the same stages of POI-like and CRTL IVM oocytes, we found that they mostly belong to genes that are processed in the GV to MII meiotic maturation, indicating that failure to undergo the final stages of oocyte growth and maturation could play a role in their

very low developmental competence. The low number of DEGs identified in these comparisons is likely due to the more variable nature of the POI-like oocytes, suggesting that further to maturation failure, other genetic components might participate in producing this phenotype.
Gene expression in in vitro maturing bovine oocytes has been investigated with different techniques, from pooled oocyte microarray analysis [21,22] to single oocyte sequencing with massive parallel sequencing [23]. Despite their technical differences, these studies consistently identify changes during the transition from GV to MII in the expression of genes involved in cell cycle regulation, like MAPK activity, translation initiation and transcription. In all studies, including ours, most transcripts are downregulated in MII, in concordance with other species like humans [20,24-26], monkeys [27] and mice [28], showing a remarkably conserved feature of oocyte transcriptional control across mammals. This indicates that mRNAs are either degraded due to utilization, the MII being transcriptionally silenced, or stabilized/degraded via polyadenylation/deadenylation.
Despite the studies being rather consistent, it has to be noted that the in vitro maturation process affects the gene expression profiles in processes related to metabolism and development[29], and therefore the transcriptome remodelling in in vivo mature oocytes might differ.
Our study evaluates the whole transcriptome with massive parallel sequencing, while 299 Reyes and colleagues focused exclusively on polyadenylated transcripts [23]. However, when comparing the results in terms of DEGs, we found 224 genes in common between ours and the study from Reyes, all related to cell cycle, spindle organisation and regulation of chromosome organisation, RNA degradation, progesterone-mediated oocyte maturation, and metabolism. These results confirm that the biological processes that are crucial to the success of the process are robust and maintained across reports in spite of significant technical variations.
The comparison with a dataset previously generated in our laboratory on a set of 20 human oocytes [20], collected and processed in the exact same way, allowed us to perform an accurate inter-species comparison and control for several confounding factors, such as library preparation and sequencing platform variability. We found, in fact, a significant overlap in DEGs between reproductive age women and CTRL bovine GV and MIIs, with RNA degradation, nucleo-cytoplasmic transport, and oocyte meiosis as themost involved biological processes. However, we also observed differences, as most genes remain distinct in the two systems, as confirmed independently in a recent meta-analysis [13].
Our findings on the POI-like model suggest the possibility that failure to execute a correct cytoplasmic maturation could cause the very low oocyte competence observed in these cows. We found few DEGs when comparing GVs and MIIs, to the point that the only clustering we found was due to the cow background, something not observed in the control group, which highlights the lack of differences between developmental stages. When comparing the same developmental stage between CTRL and POI-like, we found genes in line with the hypothesis that POI-like oocytes fail to complete nuclear and cytoplasmic maturation. Comparing MIIs, we observed DEGs related to chromosome 323 organization and spindle assembly pathways. This is analogous to what was observed in in vitro matured MII from aged patients in different studies [24,30]. In GVs, on the contrary, we found differences in genes related to different processes, including cumulus-oocyte complex communication and microtubule organisation, which is in contrast with the few to no differences between GV from aged and young women [24].
This might suggest that oocytes from POI-like cows share some common features with aged oocytes, but their maturation incompetence, not observed in aged oocytes, might be related to different causes already acting during folliculogenesis.
This hypothesis is supported by reports characterising the models: the cross-talk between the follicle and the oocyte, mainly achieved through transzonal projections (TZPs) that protrude into

the oocyte and transport a plethora of signals and molecules required for growth and meiotic maturation, is critical for cytoplasmic maturation [7,23]. In POI-like models, the transzonal network is less developed than the one from normal cows, with a
reduced number of TZPs. Further, over 50% of the gap junction-mediated communications are closed, compared to the normal condition, where most are functionally active [7,31]. Since all the hormonal receptors are in the cumulus cells, the failure to communicate with the oocyte could be responsible for the insensitivity to hormonal signalling, which could cause the uncoupling of nuclear and cytoplasmic maturation.
In conclusion, we have identified a valuable animal model to study the human condition of POI and we have defined the transcriptional profile of POI-like oocytes during in vitro maturation. Our study provides clues for the possible mechanism of oocyte quality decrease in POI through impaired cytoplasmic maturation.

DECLARATIONS

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Conflict of interest

The authors declare no financial or competing interests.

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