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In vivo and *in vitro* matured bovine oocytes present a distinct pattern of single-cell gene expression

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Summary

Oocyte gene expression is a well controlled event that promotes gamete competence to undergo maturation, fertilization, and to support early embryo development, directly affecting reproductive outcomes. Considering that *in vivo* controlled ovarian stimulation or *in vitro* maturation (IVM) for the acquisition of mature oocytes has distinct implications for gene expression, we sought to evaluate the effects of these procedures on the expression of competence-related genes in single-cell oocytes. Healthy Nelore cows of reproductive age were synchronized to harvest *in vivo* matured oocytes; ovaries from slaughtered animals were used to obtain cumulus–oocyte complexes that were *in vitro* matured. Single-cell gene expression was performed using TaqMan Low-Density Arrays and 42 genes were evaluated. *In silico* analysis of protein interactions and Gene Ontology (GO) analysis was performed. Reduced gene expression was observed for 24 targets in IVM oocytes when compared with those of *in vivo* matured oocytes ($P < 0.05$). Differences ranged from 1.5-fold to 4.8-fold higher in *in vivo* oocytes and the *BMP15* (5.28), *GDF9* (6.23), *NOBOX* (7.25), *HSPA8* (7.85) and *MSX1* (11.00) showed the greatest fold increases. The strongest score of functional interactions was observed between the *CDC20* and *CKS2*, with the differentially expressed gene *CDC20* being the main marker behind GO enrichment. IVM negatively affected the expression of important genes related to oocyte competency, and showed higher expression levels in *in vivo* matured oocytes. *In vivo* controlled ovarian stimulation may be a better strategy to achieve proper oocyte competence and increase the success of assisted reproductive technologies.

Introduction

The success of fertilization and embryonic development is highly influenced by appropriate oocyte maturation (Keefe *et al.*, 2015). During folliculogenesis, crosstalk between somatic and germ cells, global chromatin remodelling, intense RNA synthesis and storage, and protein translation, progressively promote nuclear and cytoplasmic maturation of the oocyte (Susor *et al.*, 2015; Dumdie *et al.*, 2018). The acquisition of oocyte competence is characterized by the ability to complete the maturation into metaphase II (MII) oocyte, achieving its potential to be fertilized, and perform the first cleavages during early embryo development, before embryonic genome activation (EGA) (De La Fuente *et al.*, 2004; Evsikov and de Evsikova, 2009). In reproductive treatments, oocyte competence is acquired by *in vitro* manipulation during assisted reproductive technologies (ARTs) that may lead to maturation arrest and chromosomal abnormalities in the oocytes (MacLennan *et al.*, 2015). Moreover, in combination with the impaired fertility and the *in vitro* culture of preimplantation embryos, ARTs have been associated with altered epigenetic reprogramming in newborns (Hattori *et al.*, 2019).

In vivo maturation of oocytes through controlled ovarian stimulation (COS) is a widely used strategy to obtain a great number of matured oocytes in human reproductive treatment. Conversely, the *in vitro* maturation (IVM) of germinal vesicles or metaphase I (MI) oocytes, is mainly used for *in vitro* production of domestic animals, such as bovine and ovine, to obtain MII oocytes to be *in vitro* fertilized. In humans, IVM might be used to assist health-risk patients with polycystic ovary syndrome (PCOS) with an increased risk of ovarian hyperstimulation syndrome (OHSS) and during cancer treatment in fertility preservation programmes (De Vos *et al.*,

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2014; Langbeen *et al*., 2015; Sánchez *et al*., 2015). These technologies have different implications for oocyte competence, zona pellucida (ZP) formation, cumulus cell interaction (Walker and Biase, 2020), as well as for transcriptional and translational regulation during meiotic maturation, an essential event for oocyte-to-zygote transition as, in the early stages of embryo development, these proceed without transcription and the embryo depends exclusively on post-transcriptional regulation of maternal transcripts (Jansova *et al*., 2018).

Well controlled gene expression during oocyte competence is essential for maturation, fertilization, and embryo development. Environmentally induced epigenetic changes during ARTs are related to aberrant reprogramming of the oocyte transcriptome (Gao *et al*., 2017; Yu *et al*., 2020), leading to an increase in the incidence of developmental disorders and birth defects in both humans and animals (Gomes *et al*., 2007; Duranthon and Chavatte-Palmer, 2018; Luke *et al*., 2020). In the bovine model, it has been shown that nuclear maturation is not always accompanied by cytoplasmatic maturation during IVM. Different transcript abundance was observed in cumulus cells from different maturation methods in bovine (Watson, 2007; Tesfaye *et al*., 2009). Looking for better outcomes, oestrus synchronization with exogenous hormones, optimum medium for oocyte maturation and embryo culture, laser-assisted hatching and, cryopreservation procedures have improved the *in vitro* production of bovine embryos (Ferré *et al*., 2020).

COS and IVM are important techniques to achieve MII oocytes for reproductive treatment and *in vitro* production of embryos. Nevertheless, these technologies affect oocyte transcriptome differently and the results of ARTs as reduced fertility, poor embryo quality, and higher miscarriage rates (Heinzmann *et al*., 2015; Adona *et al*., 2016). However, studies are controversial on whether the IVM procedure or the ART itself is responsible for some of the unfavourable reproductive outcomes such as the higher prevalence of imprinting disorders and other genetic abnormalities. According to Buckett *et al.* (2007), when comparing reproductive results in cycles of IVF, ICSI or IVM, ART pregnancies, in general, are associated with an increased risk of congenital abnormality and, when compared with IVF and ICSI, IVM is not associated with any additional risk. In another study from the same group the miscarriage rates were higher in IVM cycles than in conventional ART procedures (IVF and ICSI). However, this seems to be related more to the patient population with PCOS, once there were similar miscarriage rates among IVM PCOS pregnancies and IVF/ICSI PCOS pregnancies, which were higher than in the no PCOS couples submitted to IVF/ICSI (Buckett *et al*., 2008).

As the oocytes are responsible for initiating embryo development, altered transcriptional activity may lead to impaired embryo development and many diseases in the offspring. Considering the importance of transcriptional levels during oocyte maturation, we evaluated the expression of genes related to meiotic competence in *in vivo* and *in vitro* matured single-cell oocytes from bovine, to provide a better understanding of the molecular pathways involved in the oocyte maturation process for future clinical applications.

Materials and methods

Ethics statement

The present study was approved by the Bioethics Commission on Animal Experiments of the Ribeirão Preto Medical School, University of São Paulo (protocol number 073/2012), which complies with the ethical principles of animal research.

Estrous synchronization and in vivo *maturation of the oocyte*

In total, 52 healthy Nelore cows of reproductive age (3–6 years old) had their ovarian follicular growth wave synchronized to obtain *in vivo* matured oocytes throughout COS. The emergence of a new follicular wave was induced and synchronized among the cows by follicular ablation (Day 0) and insertion of an intravaginal progesterone (1 g) releasing device (Sincrogest®, Ourofino Saúde Animal, São Paulo, Brazil). On the sixth day, prostaglandin analogous was administered intramuscularly (500 µg of sodium cloprostenol; Sincrocio®, Ourofino Saúde Animal, São Paulo, Brazil) and, after 12 h, the intravaginal progesterone was removed. Ovulation was induced on the eighth day with the gonadotropin-releasing hormone (GnRH) analogue buserelin acetate (10 µg) (Sincroforte®, Ourofino Saúde Animal, São Paulo, Brazil) and, after 25 h, before natural ovulation, ovum pick-up (OPU) was performed.

To collect the oocytes, an epidural anaesthetic of 2% lidocaine hydrochloride (3–4 ml) was given immediately before follicle aspiration to block eventual pain during the procedure and facilitate the transrectal ovarian manipulation. Follicle aspiration was performed using an ultrasound device (MyLab30 Vet Gold, Esaote, Genova, Italy), equipped with a micro-convex 7.5 MHz transducer connected to a needle-guide system and vacuum pump (Diapump; FANEM, São Paulo). A single lumen 19-gauge 60-cm long sterile needle and a vacuum pressure of 100 mmHg was used during the follicle puncture on each session by the same operator. Periovulatory follicles (>11 mm in diameter) were punctured and the follicular fluid was collected in a 50-ml Falcon tube containing 10 ml of Dulbecco's phosphate-buffered saline (DPBS) supplemented with 50 IU/ml heparin (Hepamax-S) and kept at a temperature of 37°C in a bath. The contents of the tube were placed in a Petri plate and searched for oocytes under a stereomicroscope (Stemi 1000/2000/2000-C, Carl Zeiss, Germany). Only those oocytes with a visible first polar body [metaphase II (MII) oocytes] were selected (20 oocytes from different animals). The oocytes were denuded in a maturation medium supplemented with 0.2% hyaluronidase (Sigma-Aldrich, USA), washed in phosphate-buffered saline (PBS) and stored individually in RNA*later*®/PBS (1:3) for gene expression analysis.

In vitro *maturation of oocytes*

Bovine ovaries obtained in a slaughterhouse were transported to the laboratory in 0.9% physiological saline solution supplemented with 0.05 g/L streptomycin at 35–37°C. In total, 20 ovaries were used for oocyte selection and *in vitro* maturation. Viable follicles measuring between 2 and 8 mm in diameter were aspirated using 18G needles adapted to 20 ml syringes. Cumulus–oocyte complexes (COCs) were recovered from the follicular fluid of ovaries and evaluated under a stereomicroscope. Oocytes with a homogeneous cytoplasm and sufficient surrounding cumulus cells (three layers or more) (category I and II) were selected (Leibfried and First, 1979). COCs from the same cow (both ovaries) were randomly assigned to *in vitro* maturation (IVM) in HEPES-buffered tissue culture medium-199 (TCM-199, Gibco/BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS; Invitrogen Gibco/BRL), 0.2 M sodium pyruvate, 100 IU/ml penicillin G, 100 µg/ml streptomycin, 0.5 µg/ml follicle-stimulating hormone (FSH; Folltropin-Bioniche, Canada), 5 µg/ml luteinizing hormone (LH; Lutropin-Bioniche, Canada), and 1 µg/ml 17β-estradiol.

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Maturation was carried out throughout 22–24 h, at 38.5ºC, with high humidity and 5% CO_2. After IVM, the oocytes were denuded in a maturation medium supplemented with 0.2% hyaluronidase (Sigma-Aldrich, USA), under a stereomicroscope and only those oocytes with a visible first polar body (MII oocytes) were selected. These were washed in PBS and stored individually in RNA*later*®/PBS (1:3) for gene expression analysis.

Gene expression analysis

In total, 42 genes related to oocyte development and competence were selected according to previous studies (Table 1). Total RNA from each sample (single oocyte) was extracted using the RNeasy® Micro kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions. All samples were treated with an RNase-Free DNase Set (Qiagen, Valencia, California, USA) and RNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). Complementary DNA (cDNA) was synthesized using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, California, USA) according to the manufacturer's protocol. Oocyte cDNA was preamplified using a TaqMan PreAmp Master Mix kit (Applied Biosystems, Foster City, California, USA) following the manufacturer's recommendations with modifications as follows: 1× TaqMan PreAmp Master Mix, 0.05× of each assay in a pooled assay mix (0.2×), and 62.5 ng of cDNA in 20 µl of the final volume. Preamplification cycles were 95°C for 15 min and 20 cycles of 95°C for 15 s and 60°C for 4 min. The resulting material was diluted 20× and stored at −20°C.

Gene expression analyses were performed using TaqMan Low-Density Arrays (TLDA) (ThermoFisher Scientific, Massachusetts, USA). A custom TLDA was manufactured into the 384-well cards that included 48 genes (format 48 – part no. 4342253). In total, 42 target genes were evaluated and the following reference genes *GAPDH*, *ACTB*, *H2AFZ*, *PPIA* and *GUSB* were selected based on our previous study (Caetano *et al*., 2019); 18S RNA was used as an internal control for amplification. Quantification was performed using 5 ml of preamplified cDNA, 45 ml of nuclease-free water (1:20) and 50 ml of TaqMan Universal PCR Master Mix (ThermoFisher Scientific, Massachusetts, USA). The mixture was transferred into a TLDA card and centrifuged for 2 min at 1000 *g* to distribute the samples into each well. Quantification was performed using the ViiA-7 Real time PCR system (Applied Biosystems) and the thermal cycling conditions were 2 min at 50ºC and 10 min at 95ºC, followed by 40 cycles of 15s at 95ºC and 1 min at 60ºC. Ten random *in vivo* matured oocytes were pooled and used as a reference sample for gene expression normalization. Relative quantification (RQ) of gene expression was obtained using the $2^{-\Delta\Delta Cq}$ method (where Cq is the cycle of quantification) (Livak and Schmittgen, 2001). Fold changes are reported by the number of times that each gene was expressed *in vivo* compared with the *in vitro* matured oocytes (*in vivo*/IVM).

In silico *analysis of protein–protein interaction and pathways*

Protein–protein interactions between the analyzed targets were predicted using the STRING database (https://string-db.org, version 11.0) (Szklarczyk *et al*., 2017). Network interactions were built with a score of 0.4 or more, using multiple proteins, selecting *Bos taurus* as an organism and an initial input of 42 proteins. A comprehensive analysis of the function and pathways of the differentially expressed genes was obtained using the PANTHER Classification System (www.pantherdb.org, version 16.0) (Mi *et al*., 2019), based on Gene Ontology (GO) terms and the UniProtKB protein data bank (www.uniprot.org). Based on the main biological process and molecular function observed on PANTHER, a Venn diagram was created using the *Venn diagram* R package with the differentially targets and the three main pathways related.

Statistical analysis

The values are presented in the median and interquartile ranges. The gene expression analysis was performed using the non-parametric univariate Mann–Whitney test to compare the distribution of two independent groups. A heatmap showing differential expression levels between *in vitro* and *in vivo* matured oocytes generated from the median values of gene expression using the *heatmap.2* function from the R *gplots* package (Warnes *et al*., 2015). The Spearman's correlation coefficient was used to correlate the gene expression of *in vivo* matured oocytes and the age of the cows. All results were obtained using SAS 9.0 software (SAS Institute), with the level of significance set at 5% ($P < 0.05$).

Results

Of the 42 genes analyzed in *in vitro* and *in vivo* matured oocytes, expression of the following *GLYCAM-1*, *HSPA1A*, *LUM*, *PLAU*, *PTGFR* and *SPARC* genes was not detected in the single-cell gene expression analysis. Therefore, 35 were evaluated and an individual variability within the same group (*in vivo* or *in vitro*) was observed in the relative gene expression, as *MAPK13* was expressed only in a few samples, including 29.41% ($n = 5$) for IVM and 31.58% ($n = 6$) for *in vivo* matured groups. Although the RQ value was decreased in the IVM group (0.162 ± 0.22) when compared with the *in vivo* matured group (3.28 ± 3.67), statistical analysis was not performed.

From 35 genes related to oocyte growth and competence, 24 showed reduced expression levels in IVM oocytes when compared with the *in vivo* maturated ones (Table 2). The expression levels of *ANXA2*, *ATP5A1*, *BCAP31*, *DDR1*, *F11R*, *GJA1*, *RPS15*, *SERPINE*, *TMIGD1*, *TXN* and *ZDHHC16* did not differ between the groups analyzed (Table 2). Median values of normalized relative gene expression were used to generate a heatmap for differential gene expression of the 35 genes analyzed (Figure 1), showing a distinct gene expression pattern observed between *in vitro* and *in vivo* matured oocytes.

Among the differentially expressed genes, most of them showed a fold change varying from 1.5 and 2.8 (*IGF2R*, *RPS6KB1*, *CLU*, *PSEN1*, *GOT1*, *DNMT3B*, *TCF4* and *PLIN2*) and from 3.0 to 4.8 (*GTF2F1*, *CDC20*, *CD97*, *PPA1*, *CETN3*, *CKS2*, *BTG1*, *EEF1A1*, *HK1*, *ARL6IP6* and *ZP2*) times higher in the *in vivo* than *in vitro* matured oocytes. However, the genes displaying the greatest fold change were *BMP15* (5.28), *GDF9* (6.23), *NOBOX* (7.25) and *HSPA8* (7.85) and *MSX1* (11.00), which showed higher fold differences between the studied groups (Figure 2). Considering age influence on gene expression, Spearman correlation analysis showed a strong negative correlation between *ANXA2* ($r^2 = -0.91$; $P < 0.01$) gene expression and age, and a moderate negative correlation considering the *CD97* gene ($r^2 = -0.59$; $P < 0.04$) (Supporting information Table S1). However, *ANXA2* was not differentially expressed in the studied groups.

Based on the targets evaluated, a functional network of protein interactions was performed using the STRING database (https://string-db.org, version 11.0). The network of interactions considered a score of 0.4 or more, using multiple proteins, selecting

Table 1. Genes selected, TaqMan assay and molecular function

Official name	GenBank	TaqMan assay	Name/Function
ANXA2	NM_174716.1	Bt03215891_g1	Calcium-dependent phospholipid-binding protein (cell signalling)
ARL6IP6	NM_001034281.2	Bt03225773_m1	Regulation of protein biosynthesis
ATP5A1	NM_174684.2	Bt03259558_g1	ATP Synthase F1 Subunit Alpha/Carrier molecule
BCAP31	NM_001014941.1	Bt03218742_m1	Stress adaptation, apoptosis and immune response, cell adhesion
BMP15	NM_001031752.1	Bt03286494_u1	Intracellular signalling, signal transduction
BTG1	NM_173999.3	Bt03230931_m1	Transcription regulation, adhesion molecule, zona pellucida protein
CD97	NM_176661.1	Bt03224521_m1	Apoptosis and cellular death
CDC20	NM_001082436.2	Bt03255241_m1	Control of cell cycle, meiosis regulation
CETN3	NM_001075976.2	Bt03248118_m1	Calcium binding, cytoskeleton organization, cell cycle
CKS2	NM_001113319.2	Bt03276410_m1	Control of cell cycle, meiosis regulation
CLU	NM_173902.2	Bt03211827_m1	Apoptosis
DDR1	NM_001076012.2	Bt03269593_m1	Protein metabolism, integrant of cell membrane
DNMT3B	NM_181813.2	Bt03259818_m1	Transcription regulation
EEF1A1	NM_174535.1	Bt03223795_g1	Protein synthesis
F11R	NM_174095.5	Bt03230949_m1	Adhesion molecule, zona pellucida protein
GDF9	NM_174681.2	Bt03223996_m1	Growth factor
GJA1	NM_174068.2	Bt03244351_m1	Cell communication
GLYCAM1	NM_174828.2	Bt03216430_m1	Adhesion molecule, zona pellucida protein
GOT1	NM_177502.2	Bt03217248_m1	Cell metabolism
GTF2F1	NM_001015527.1	Bt03210588_m1	Transcription regulation
HK1	NM_001012668.1	Bt03210444_m1	Metabolism
HSPA1A	NM_174550.1	Bt03292232_gH	Protein framework, stress response
HSPA8	NM_174345.4	Bt03231003_g1	Protein framework, stress response, cell cycle regulation, RNA processing
IGFR2	NM_174352.2	Bt03223452_m1	Cell membrane integrant, glycoprotein binding
LUM	NM_173934.1	Bt03211921_m1	Signal transduction, cell communication
MAPK13	NM_001014947.1	Bt03218761_m1	Cell cycle, transcription regulation, stress response
MSX1	NM_174798.2	Bt03216303_m1	Transcription regulation, cell cycle and apoptosis
NOBOX	HQ589330.1	Bt04347738_m1	DNA binding
PLAU	NM_174147.2	Bt03212959_m1	Regulation of cell proliferation, stress response
PLIN2	NM_173980.2	Bt03212182_m1	Cell component, lipid stock
PPA1	NM_001075118.1	Bt02645262_m1	Metabolism
PSEN1	NM_174721.2	Bt03215936_m1	Apoptosis, cell signalling
PTGFR	NM_181025.3	Bt03292638_s1	Signal transduction, apoptosis
RPS15	NM_001024541.2	Bt03220160_m1	Regulation of protein biosynthesis, metabolism, RNA binding, translate, RNA processing
RPS6KB1	NM_205816.1	Bt00923436_m1	Transcription regulation, apoptosis regulation, cell cycle
SERPINE1	NM_174137.2	Bt03212915_m1	Protein metabolism, immune system and defence
SPARC	NM_174464.2	Bt03214620_m1	Cell proliferation and differentiation, signal transduction
TCF4	NM_001034621.2	Bt03221445_m1	Transcription regulation
TMIGD1	NM_001035036.2	Bt03227977_m1	Surface receptor and transmembrane protein
TXN	NM_173968.3	Bt03222878_m1	Intracellular signalling, transcription regulation, cell metabolism
ZDHHC16	NM_001024482.2	Bt01198586_m1	Apoptosis
ZP2	NM_173973.2	Bt03212146_m1	Adhesion molecule, zona pellucida protein
*H2AFZ**	NM_174809.2	Bt03216348_g1	DNA binding, chromatin packing

(*Continued*)

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Table 1. (*Continued*)

Official name	GenBank	TaqMan assay	Name/Function
*GAPDH**	NM_001034034.2	Bt03210913_g1	Carbohydrate metabolism, oxyreduction
*ACTB**	NM_173979.3	Bt03279174_g1	Cell transport, cytoskeleton protein, cell division
*PPIA**	NM_178320.2	Bt03224615_g1	Protein metabolism, nuclear transport
*GUSB**	NM_001083436	Bt03256165_m1	Carbohydrate metabolism

*Reference genes.

Bos taurus as an organism. The input of 36 proteins and the scores obtained for each interaction are summarized in Table S2. The strongest interaction was observed between CDC20 and CKS2 (score 0.991) followed by DNMT3B and H2AFZ (score 0.937), CLU and SERPINE1 (score 0.915) and HSPA8 and IGF2R (score 0.906) (Figure 3 and Table S2). The network had more interactions than expected for a random set of proteins of similar size, drawn from the bovine genome [protein interactions (PPI) enrichment *P*-value 0.00625]. This enrichment indicated that the proteins are biologically connected. The main five functional enrichments in the interacting proteins network based on the lowest false discovery rate are presented in Table S3.

Considering the differentially expressed genes in *in vivo* and *in vitro* matured oocytes a GO term enrichment was carried out using PANTHER GO-Slim. These genes participated in specific biological processes and molecular functions related to cell growth, differentiation, metabolism and signalling, as well as binding proteins, transcriptional and catalytic activities (Figure 4a,b). The most representative pathways are presented in the Venn diagram. For biological processes, genes *BMP15*, *CDC20*, *GDF9*, *GTF2F1*, *HK1*, *NOBOX*, *PSEN1* and *RPS6KB1* were involved in all 'Cellular Processes', 'Biological Regulation' and 'Metabolic Processes' (Figure 4c). For molecular function, *BMP15*, *GDF9*, *NOBOX*, *GTF2F1*, *CDC20* and *CKS2* participated in all representative GO functional enrichment: 'binding', 'catalytic activity' and 'molecular function regulator' (Figure 4d). *CDC20* seems to be the main gene behind this functional enrichment.

Discussion

In this study *in vivo* and *in vitro* matured oocytes showed a different pattern of gene expression, in which reduced transcription levels of several genes involved in oocyte metabolism, competence, cell signalling, and apoptosis were observed in IVM oocytes. Differently, in the study by Jones *et al.* (2008) more than 2000 genes were identified as expressed at more than two-fold higher levels in oocytes matured *in vitro* than those matured *in vivo* (Jones *et al.*, 2008), with 162 genes with overexpression levels of more than 10-fold higher in the *in vitro* matured oocytes. These results may be due to dysregulation in either transcription or post-transcriptional regulation, leading to changes during early embryo development. When comparing our list of upregulated genes with Jones' list there was no overlap, although the processes with which the genes have been associated are similar (transcription regulation, cell metabolism, intracellular signalling and others).

Oocyte maturation and competence is a crucial period for female gametogenesis, with changes in the nucleus and cytoplasm, increase in chromatin accessibility and transcriptional activity, and production of cellular components necessary for the maintenance of the embryo prior to EGA. Proper oocyte maturation affects fertilization rates and embryo development, directly impacting reproductive outcomes (Landim-Alvarenga and Maziero, 2014; Gilchrist *et al.*, 2016). Although IVM oocytes can promote the first stages of embryo development, a higher rate of miscarriage was observed in IVM cycles when compared with COS (De Vos *et al.*, 2021). Once removed from the context of the follicle, the meiosis process was spontaneously resumed and, in many mammals, the majority of oocytes achieved the MII stages, although in humans this is less frequent (Edwards, 1965). In addition, nuclear maturation is not necessarily accompanied by cytoplasmatic maturation (De Vos *et al.*, 2021).

Oogenesis is a coordinated and complex biological process in which the primordial germ cells undergo differentiation and the germinal vesicle (GV) breaks down and resumes cellular division to the matured MII oocytes able to be fertilized (Yu *et al.*, 2020). In the growing oocyte, increased transcriptional activity is required for the oocyte-specific process, growth and maturation, and stored RNA is needed for early embryo development (Lonergan *et al.*, 2003; Gandolfi *et al.*, 2005; Sirard *et al.*, 2006; Gennari Verruma *et al.*, 2021). The regulation of gene expression is controlled in the oocyte after the GV phase, almost exclusively by mRNA translation and post-translational modifications in the synthesized proteins (Susor *et al.*, 2015; Dumdie *et al.*, 2018). During the differentiation of primordial germ cells and gametogenesis, a genome-wide epigenetic reprogramming is responsible for the establishment of oocyte-specific gene expression patterns, as well as maternal genomic imprinting, essential for reproductive success and proper embryo development (Fassnacht and Ciosk, 2017).

Aberrant epigenetic reprogramming and developmental disorders are frequently related to ART. Hattori and colleagues (2019) showed, in an epidemiologic study in Japan, an increase in the incidence of imprinting disorders such as Beckwith–Wiedemann (BWS), Silver–Russel (SRS) and Prader–Willy (PWS) syndromes in ART-conceived children. Nevertheless, the authors recruited a great number of participants with all four imprinting disorders and did not access the causes of infertility and the indication for treatment, which is also related to aberrant imprinting reprogramming (Kopca and Tulay, 2021). The ESHRE Capri Workshop Group showed that newborns spontaneously conceived by subfertile couples present 29% of congenital abnormalities, and the risk is further increased to 34% with ART. Probably the increased risk of birth defects may be more related to infertility than ART itself (ESHRE Capri Workshop Group, 2014). Although many chromosomal anomalies and mutations have evolved to implantation failure or spontaneous miscarriages, IVF, especially ICSI may favour the transmission of these anomalies by artificially enhancing fertility (Wang *et al.*, 2012). However, after more than 40 years of ARTs, up to 2019 over 8 million children have been born using IVF (Fauser, 2019), and the benefit of treatment is greater than the changes expected.

In the oocytes, epigenetic reprogramming starts after sexual maturation during oocyte growth that is arrested in the diplotene

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Table 2. Relative quantification of all genes analyzed on oocytes matured *in vitro* and *in vivo*

Gene	Group	*n*	Minimum	1st quartile	Median	3rd quartile	Maximum	*P*-value*
ANXA2	*In vitro*	13	0.08	0.31	0.49	0.81	4.57	0.95
	In vivo	11	0.12	0.21	0.90	1.05	1.81	
ARL6IP6	*In vitro*	16	0.02	0.11	0.29	0.59	1.39	**<0.01**
	In vivo	19	0.47	0.79	1.18	1.61	3.82	
ATP5A1	*In vitro*	17	0.12	0.55	0.95	1.18	2.49	0.39
	In vivo	19	0.37	0.85	1.13	1.37	1.89	
BCAP31	*In vitro*	17	0.08	0.21	0.38	0.84	1.97	0.12
	In vivo	18	0.10	0.26	0.82	1.47	4.50	
BMP15	*In vitro*	17	0.01	0.11	0.18	0.58	2.91	**<0.01**
	In vivo	19	0.07	0.51	0.95	1.38	1.68	
BTG1	*In vitro*	17	0.03	0.13	0.26	0.41	1.92	**<0.01**
	In vivo	19	0.30	0.70	0.93	1.10	2.23	
CD97	*In vitro*	17	0.01	0.07	0.21	0.41	1.83	**<0.01**
	In vivo	18	0.00	0.37	0.66	1.31	2.44	
CDC20	*In vitro*	17	0.05	0.20	0.34	0.76	3.30	**<0.01**
	In vivo	19	0.43	0.87	1.06	1.39	3.51	
CETN3	*In vitro*	17	0.05	0.08	0.27	0.71	3.45	**0.01**
	In vivo	19	0.20	0.48	0.95	1.44	7.66	
CKS2	*In vitro*	17	0.00	0.06	0.23	0.78	1.98	**<0.01**
	In vivo	19	0.15	0.56	0.82	1.52	3.53	
CLU	*In vitro*	17	0.09	0.22	0.47	0.58	4.78	**<0.01**
	In vivo	19	0.45	0.58	0.90	1.78	2.46	
DDR1	*In vitro*	15	0.01	0.18	0.46	1.31	3.12	0.07
	In vivo	16	0.07	0.58	1.43	2.65	7.37	
DNMT3B	*In vitro*	17	0.09	0.26	0.48	0.82	1.29	**<0.01**
	In vivo	19	0.30	0.74	1.10	1.64	2.16	
EEF1A1	*In vitro*	17	0.05	0.22	0.30	0.79	2.18	**<0.01**
	In vivo	19	0.37	0.77	1.09	1.37	2.94	
F11R	*In vitro*	13	0.01	0.12	0.45	1.90	6.58	0.17
	In vivo	13	0.01	1.05	1.69	2.43	4.44	
GDF9	*In vitro*	17	0.00	0.07	0.13	0.50	3.12	**<0.01**
	In vivo	19	0.02	0.44	0.81	1.50	3.27	
GJA1	*In vitro*	16	0.02	0.41	0.68	2.20	5.04	0.30
	In vivo	19	0.33	0.88	1.24	1.72	2.82	
GOT1	*In vitro*	13	0.09	0.25	0.50	0.65	1.23	**<0.01**
	In vivo	19	0.25	0.79	1.08	1.33	3.24	
GTF2F1	*In vitro*	17	0.06	0.14	0.36	0.51	1.26	**<0.01**
	In vivo	19	0.40	0.67	1.11	1.46	3.51	
HK1	*In vitro*	17	0.05	0.11	0.30	0.33	3.25	**<0.01**
	In vivo	19	0.03	0.66	1.10	2.78	6.41	
HSPA8	*In vitro*	17	0.00	0.05	0.13	0.28	1.44	**<0.01**
	In vivo	19	0.02	0.27	1.02	1.48	4.27	

(*Continued*)

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Table 2. (*Continued*)

Gene	Group	*n*	Minimum	1st quartile	Median	3rd quartile	Maximum	*P*-value*
IGF2R	*In vitro*	16	0.08	0.26	0.60	0.84	2.06	**0.02**
	In vivo	19	0.10	0.63	0.93	1.39	2.44	
MSX1	*In vitro*	17	0.00	0.04	0.08	0.27	4.50	**<0.01**
	In vivo	19	0.01	0.23	0.88	2.19	4.01	
NOBOX	*In vitro*	17	0.00	0.03	0.12	0.52	2.50	**<0.01**
	In vivo	19	0.01	0.18	0.87	2.76	4.40	
PLIN2	*In vitro*	17	0.09	0.17	0.36	0.61	1.81	**<0.01**
	In vivo	19	0.57	0.66	1.00	1.38	2.45	
PPA1	*In vitro*	17	0.02	0.11	0.30	0.52	1.73	**<0.01**
	In vivo	19	0.13	0.62	0.98	1.27	2.70	
PSEN1	*In vitro*	16	0.03	0.33	0.51	0.90	2.64	**0.01**
	In vivo	19	0.27	0.54	0.99	1.42	2.44	
RPS15	*In vitro*	17	0.00	0.09	0.67	3.39	5.39	0.49
	In vivo	19	0.10	0.54	0.61	2.20	5.14	
RPS6KB1	*In vitro*	16	0.00	0.13	0.45	0.60	1.09	**<0.01**
	In vivo	17	0.07	0.49	0.77	2.45	7.48	
SERPINE1	*In vitro*	14	0.00	0.56	1.28	3.07	8.07	0.92
	In vivo	16	0.21	0.59	1.04	2.40	3.94	
TCF4	*In vitro*	17	0.08	0.29	0.47	0.66	3.07	**<0.01**
	In vivo	19	0.28	0.85	1.20	2.36	2.95	
TMIGD1	*In vitro*	13	0.00	0.07	0.33	0.93	5.45	0.64
	In vivo	11	0.00	0.30	0.56	0.80	1.86	
TXN	*In vitro*	17	0.07	0.24	0.49	1.65	3.58	0.07
	In vivo	19	0.41	0.65	1.13	2.15	3.14	
ZDHHC16	*In vitro*	14	0.03	0.18	0.51	1.56	1.87	0.23
	In vivo	18	0.09	0.46	0.88	1.74	2.35	
ZP2	*In vitro*	17	0.01	0.08	0.19	0.66	3.22	**<0.01**
	In vivo	18	0.08	0.60	0.91	1.38	1.68	

*In bold: $P < 0.05$.

stage from meiosis, with the establishment of gametic markers and maternal genomic imprinting (Reik *et al.*, 2001; Lucifero *et al.*, 2002; Hajkova *et al.*, 2002). As induced maturation of oocytes is necessary for fertility treatment, maternal epigenetic reprogramming can be affected by environmental changes during ARTs, which directly affects the cell epigenetic landscape, as well as the imprinting acquisition necessary for proper embryo development (Sato *et al.*, 2007; Owen and Segars, 2009). As maternal imprinting is completed late in the female epigenome, the time of fertilization seems to be a critical step for imprinting disorders. One cannot forget that the male factor may also be responsible for imprinting disorders. Differently from the oocyte, imprinting acquisition in the male genome is established early in diploid gonocytes before the meiosis stage (Lucifero *et al.*, 2002). Tang *et al.* (2018) showed that idiopathic male infertility is associated with aberrant methylation in imprinted loci, with loss of imprinting in the paternally methylated H19DMR and maternally methylated GNAS and DIRAS3 DMR in spermatozoa. In a study of genome-wide mutation, the authors found that the father's age at conception was related to a diversity mutation rate of single nucleotide polymorphisms (SNP) (Kong *et al.*, 2012).

In the bovine model, IVM is the most used procedure to obtain MII oocytes during *in vitro* production of embryos (IVP), with an oocyte maturation rate of 80% (Luciano and Sirard, 2018). As observed in the present study, despite this success, IVM oocytes presented a reduced gene expression when compared with *in vivo* matured oocytes, especially for the genes related to metabolism (*EEF1A1*, *GOT1* and *PPA1*), apoptosis (*CD97*, *CLU* and *PSEN1*), cell cycle (*CDC20*, *CETN3* and *CKC2*) and *de novo* DNA methylation (*DNMT3b*). Mouse oocytes appear to be capable of maintaining their epigenetics marks during IVM (Anckaert *et al.*, 2013). However, Borghol *et al.* (2006) demonstrated that in humans this could be different and that IVM may lead to aberrant DNA methylation status on H19/IGF2 DMR. In addition, patients with PCOS have

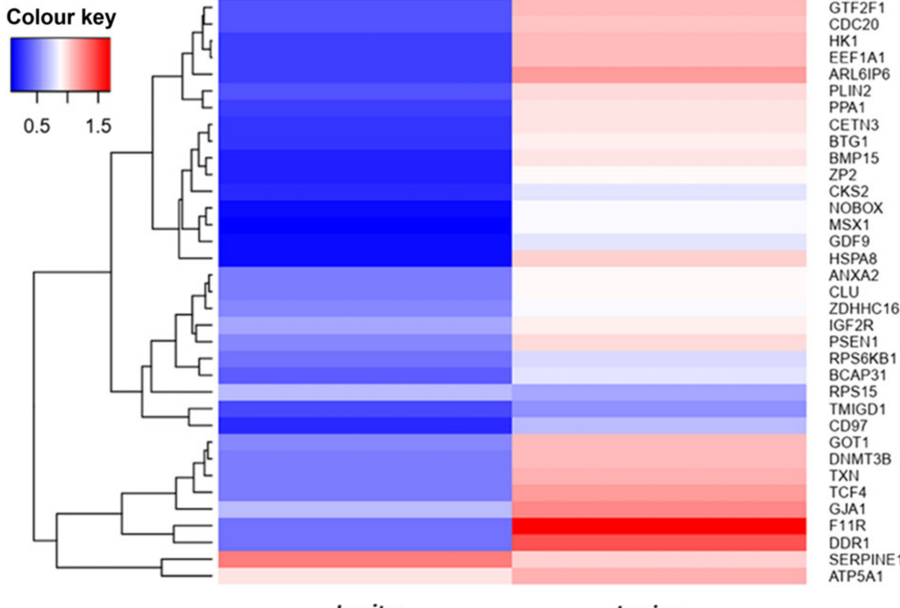

Figure 1. Heatmap showing differential expression of 35 genes between *in vitro* and *in vivo* matured oocytes. Red shading indicates higher expression levels and blue shading indicates lower expression levels. The colour key indicates the intensity associated with the normalized gene expression median values.

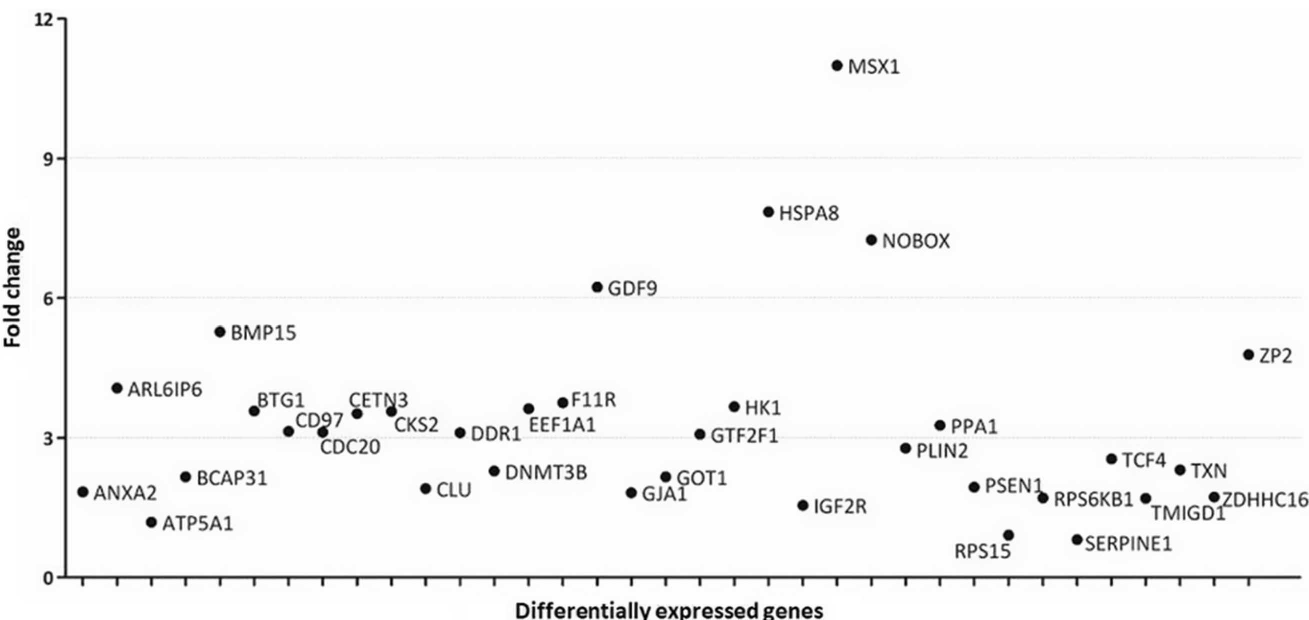

Figure 2. Fold change of gene expression differences in *in vivo/in vitro* matured bovine oocytes.

shown higher arrest and lower embryo production after oocyte IVM when compared with patients who had undergone COS treatment (Walls *et al.*, 2015). Although IVM is not common in humans, the establishment of a better culture medium to obtain MII oocytes with minimal stimulation represents a safe alternative for women with health problems, such as PCOS, poor responders, oocyte maturation defects and fertility preservation in cancer treatment (Li *et al.*, 2011; De Vos *et al.*, 2014; Walls *et al.*, 2015; Vuong *et al.*, 2019). Using low hormone concentrations, followed by proper IVM medium to obtain mature oocytes, important results have been reported in promoting the completion of nuclear and cytoplasmic maturation of cumulus–oocyte complexes, with an increase in the quality of 3-day preimplantation embryos (Sánchez *et al.*, 2015, 2019).

Controlled gene expression regulation is fundamental for oocyte maturation, cumulus cell expansion, first polar body (PB) extrusion and cytoplasmatic changes, and characteristics of meiotic maturation in a fertilizable oocyte (Biase *et al.*, 2014). The *CDC20* gene co-activated by anaphase-promoting complex/cyclosome (APC/C) is an essential regulator of the cell cycle, playing an important role in the transition from meiosis I to meiosis II, influencing the first PB extrusion and chromosomal segregation (Jin *et al.*, 2010; Yang *et al.*, 2014a). In this study, there was a clear reduction in *CDC20* expression *in vitro* compared with the *in vivo* matured oocytes. Changes in *CDC20* expression or genomic mutations are related to aneuploid gametes, female infertility and chromosomal abnormalities in the embryo (Yang *et al.*, 2014a; Zhao *et al.*, 2020). Similar to *CDC20*, *CETN3* and *CKS2* are both

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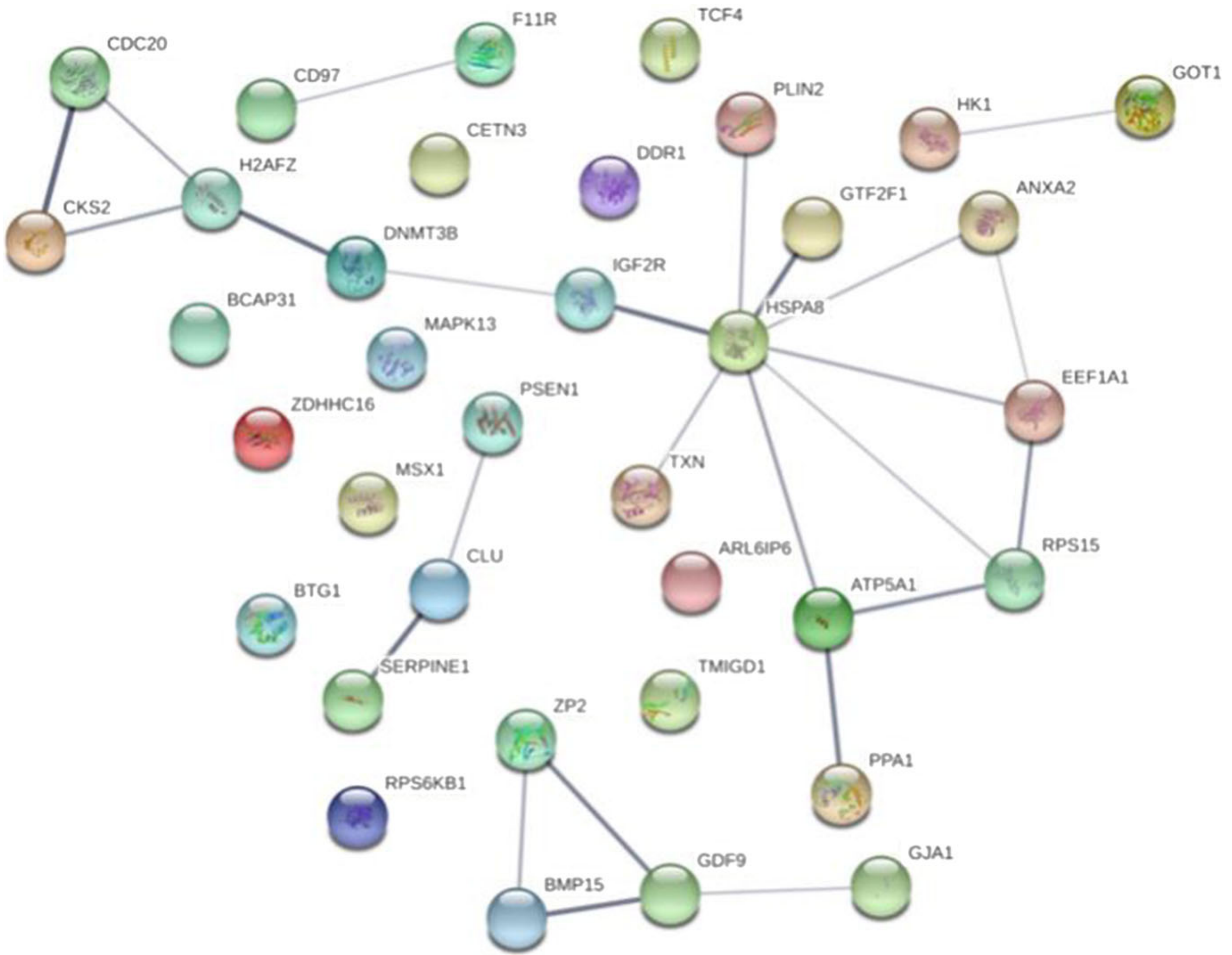

Figure 3. Protein–protein interactions (PPI) network of 36 genes evaluated in single-cell gene expression. Stronger interactions are shown in the thickest lines and lower interactions are shown in the thinnest lines.

cell cycle regulators, with an essential role in centrosome duplication, segregation and genomic stability (Giotti *et al.*, 2019); these two genes were also expressed less in the *in vitro* matured oocytes.

Most genes presented a large fold difference, varying from 1.5 to 4 times higher in the *in vivo* matured oocytes. Interestingly, *BMP15*, *GDF9*, *NOBOX*, *HSPA8* and *MSX1* showed the highest fold changes, from 5 to 11 times higher in the *in vivo* matured oocytes. *BMP15* and *GDF9* members of the TGF-β superfamily are essential in the process of folliculogenesis and oogenesis, through endocrine and paracrine signalling that promotes cell differentiation (De Resende *et al.*, 2012). These genes are co-expressed in human oocytes, and the reduced expression is related to reproductive disorders (Wei *et al.*, 2011, 2014), impaired oocyte quality and developmental competence, altered cumulus cells function and embryo development. *NOBOX*, *HSPA8* and *MSX1* genes are important modulators of cell fate and differentiation (Yang *et al.*, 2014b). *NOBOX* and *MSX1* homeobox transcription factors regulate oocyte-specific genes, with increased expression throughout oocyte growth, especially in MII oocytes (Le Bouffant *et al.*, 2011; Belli *et al.*, 2013). *HSPA8* regulates mRNA stability and the transcriptional level in mature oocytes (Marei *et al.*, 2019).

The evaluated genes participate in an intricate network of interactions that indicates a biological connection. The proteins with the higher scores of interactions showed reduced transcriptional levels and some of them had higher fold differences including *CDC20* (3.12), *CKS2* (3.57), *HSPA8* (7.85) and *IGF2R* (1.55). *IGF2R* is an imprinted maternally expressed gene, with implications in the large offspring syndrome (LOS), a fetal overgrowth alteration similar to BWS in humans due to loss of imprinting (LOI) in a specific locus that could be induced by ARTs (Chen *et al.*, 2015). A recent study reported that LOS can occur spontaneously, and the epigenetic changes observed are similar to those conceived by ARTs (Li *et al.*, 2022). PANTHER GO analysis of functional enrichment showed important biological processes and molecular functions, in which the main genes involved were *BMP15*, *CDC20*, *GDF9*, *GTF2F1*, *HK1*, *NOBOX*, *PSEN1* and *RPS6KB1* for Biological Process, and *BMP15*, *GDF9*, *NOBOX*, *GTF2F1*, *CDC20* and *CKS2* genes for Molecular Function. However, *CDC20*, due to its key role in cell cycle control, seems to be an important molecular marker for oocyte growth and development.

Fewer studies have evaluated single-cell oocyte gene expression due to the low amount of material. Although gene expression analysis and MII oocyte production were carefully designed, this study has some limitations. First, we did not evaluate the embryo production rate after IVM or COS. Rizos and colleagues (2002)

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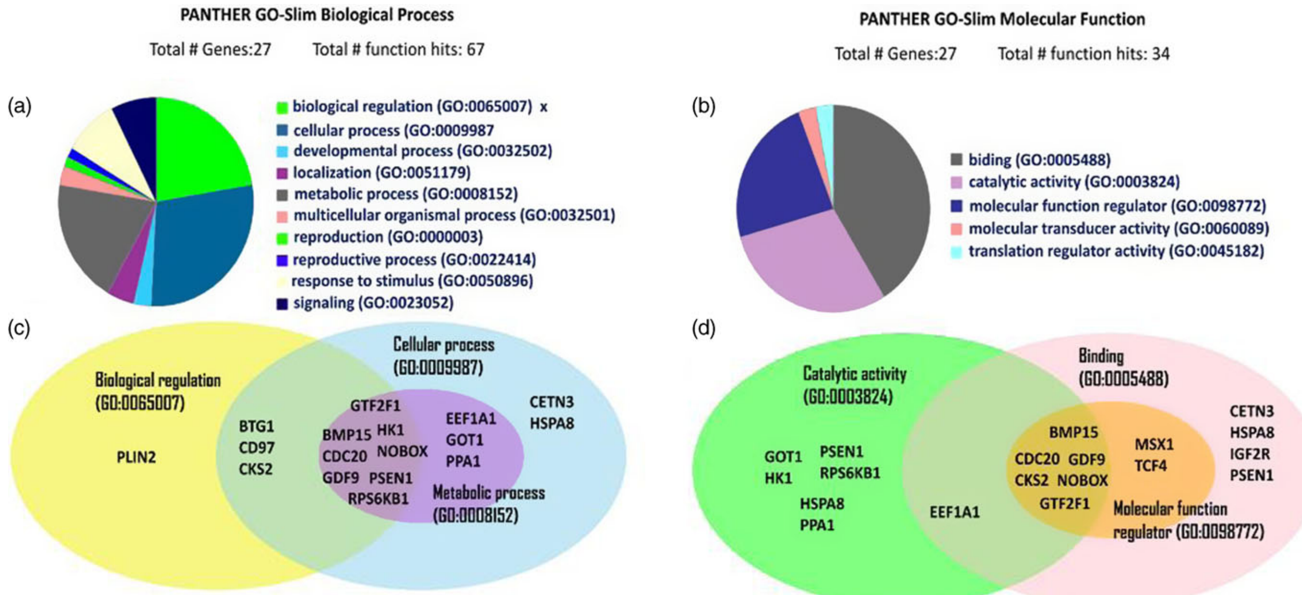

Figure 4. PANTHER-Slim Gene Ontology analysis of functional enrichment of the differentially expressed genes in *in vivo* and *in vitro* matured bovine oocytes displaying Biological Process (a) and Molecular Function (b) and a Venn diagram showing the genes related to the most representative biological process (c) and molecular function (d).

showed that *in vivo* matured oocytes present a higher embryo production rate at day 7 when compared with blastocysts from IVM oocytes. Also, we did not evaluate protein levels to confirm the changes in gene expression. However, a recent study using single-cell proteomics showed that IVM human oocytes are heterogeneous in terms of proteome level, and 45 differentially expressed proteins were observed in comparison with *in vivo* matured oocytes, with most of them (40) overlapping with mRNA levels, including TRIM28 maternal effect protein (Guo *et al.*, 2022). This altered gene expression may also be related to previous impaired fertility, however we did not measure mRNA levels in GV oocytes. Finally, we used bovine oocytes instead of human oocytes, mainly because of the difficulty in obtaining *in vivo* matured oocytes from women undergoing IVF, once these gametes are available for embryo production and transfer, and rarely donated by our patients. However, animal models are often used to better understand the molecular mechanisms involved in human gametogenesis, fertilization, early embryo development, and the molecular mechanisms that regulate these processes, such as epigenetic reprogramming and developmental disorders. Regarding the implications for human reproductive studies, bovine material has many physiological and genetic similarities, including the overgrowth syndrome related to ARTs (Adams *et al.*, 2012; Chen *et al.*, 2013; Abedal-Majed and Cupp, 2019).

In conclusion, IVM of bovine oocytes presented reduced expression of important genes related to oocyte competency and fertilization when compared with *in vivo* matured ones, suggesting that, although nuclear maturation is achieved, alterations in the transcript level may contribute to the impaired reproductive outcomes. These downregulated genes participate in several biological processes and molecular functions, including cell cycle control, transcriptional and catalytic activity, signal transduction, and oocyte growth and differentiation. Furthermore, our results pointed out that *CDC20* might be an important molecular marker for oocyte quality for clinical application, but further studies in human oocytes are necessary to confirm this hypothesis. Despite these changes, important results have been achieved using IVM to obtain preimplantation embryos in both humans and animals. However, transcriptional changes in matured oocytes give an important overview of the processes during maturation and mRNA storage to support embryo development, as the time of translational activation of maternal mRNAs is spatially and temporally regulated after fertilization (Esencan *et al.*, 2019). However further studies, including the epigenetic control of gene expression and protein levels in both IVM and COS MII oocytes may increase our knowledge of the mechanisms behind the altered gene expression and the implications for ART in genomic reprogramming during oogenesis and early development.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0967199422000478

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Declaration of interest. We have no conflict of interest in this study.

Ethics statement. The present study was approved by the Bioethics Commission on Animal Experiments of the Ribeirão Preto Medical School,

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University of São Paulo (protocol number 073/2012), which complies with the ethical principles of animal research.

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