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Global transcriptome analysis of porcine oocytes in correlation with follicle size

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Abstract

Although our knowledge regarding oocyte quality and development has improved significantly, the molecular mechanisms that regulate and determine oocyte developmental competence are still unclear. Therefore, the objective of this study was to identify and analyze the transcriptome profiles of porcine oocytes derived from large or small follicles using RNA high-throughput sequencing technology. RNA libraries were constructed from oocytes of large (LO; 3–6 mm) or small (SO; 1.5–1.9 mm) ovarian follicles and then sequenced in an Illumina HiSeq4000. Transcriptome analysis showed a total of 14,557 genes were commonly detected in both oocyte groups. Genes related to the cell cycle, oocyte meiosis, and quality were among the top highly expressed genes in both groups. Differential expression analysis revealed 60 up- and 262 downregulated genes in the LO compared with the SO group. *BRCA2*, *GPLD1*, *ZP3*, *ND3*, and *ND4L* were among the highly abundant and highly significant differentially expressed genes (DEGs). The ontological classification of DEGs indicated that protein processing in endoplasmic reticulum was the top enriched pathway. In addition, biological processes related to cell growth and signaling, gene expression regulations, cytoskeleton, and extracellular matrix organization were among the highly enriched processes. In conclusion, this study provides new insights into the global transcriptome changes and the abundance of specific transcripts in porcine oocytes in correlation with follicle size.

KEYWORDS
follicular size, oocyte, porcine, RNAseq

1 | INTRODUCTION

In mammals, oocyte developmental competence is defined as the capability of the oocyte to resume and complete meiosis, to fertilize successfully, and to support the early stages of embryo development. During follicular development, the oocyte acquires its competence through a complex bidirectional communication with the surrounding follicular cells regulated by gap-junctions, cytokines, and growth factors (Monniaux, 2016). As the follicle grows and increases in size, the oocyte is increasing its diameter and progresses toward higher developmental capacity (Bagg, Nottle, Armstrong, & Grupen, 2007; Kauffold, Amer, Bergfeld, Weber, & Sobiraj, 2005). However, not only

the size but also the physiological stage of the follicle (growing, static, or regressing phases) is an important determining factor related to the oocyte developmental competence (Vassena, Mapletoft, Allodi, Singh, & Adams, 2003). During the oocyte growth, there is a global repression in the transcriptional activity that is associated with changes in the chromatin configurations from diffused nonsurrounded nucleolus (NSN) to condensed surrounded nucleolus (SN) structures and ends up with a global transcriptional silencing (Labrecque et al., 2015; Lodde et al., 2008). A significant decrease in transcriptional activity has been detected in pig oocytes collected from 2 mm follicles followed by a progressive silencing in oocytes from follicles larger than 3 mm (Bjerregaard et al., 2003). Therefore a sufficient amount of RNAs, proteins, and nutrients need to be accumulated, processed and appropriately stored within the oocyte to support further development and to ensure developmental competence (Biase et al., 2014; Sirard, Richard, Blondin, & Robert, 2006).

Transcriptome profiles of oocytes from various mammalian species including humans (Kocabas et al., 2006; Zhang et al., 2018), bovines (Reyes, Chitwood, & Ross, 2015) and pigs (Paczkowski et al., 2011) have been identified using different technologies. Differences in transcriptome abundance have been correlated with variations in the developmental competence of different oocyte models (reviewed by Labrecque & Sirard, 2014). A few studies have used follicle size as one of these models to compare RNA contents. Transcriptome analysis of bovine oocytes collected from four different follicle sizes identified important oocyte transcripts whose abundance is gradually modulated during folliculogenesis (Labrecque, Fournier, & Sirard, 2016). In humans, oocytes from primordial and primary follicles exhibited differences in the expression of genes involved in pathways that potentially regulate oocyte dormancy and activation (Ernst et al., 2017). Chromatin configuration has been used as a model for oocyte developmental competence, as NSN oocytes are not able to develop to the blastocyst stage (Zuccotti et al., 2002). In mature mouse oocytes, a group of 380 genes was identified as differentially expressed, with the majority being upregulated in NSN compared to SN (Zuccotti et al., 2008). Moreover, RNA sequencing technology revealed a higher number of DEGs between NSN and SN immature oocytes (Monti et al., 2013). These genes were involved in processes related to transcription, translation and cell division. In another model, brilliant cresyl blue (BCB) stain was widely used to characterize oocytes based on their potential developmental capacity (Catalá et al., 2011; Salviano, Collares, Becker, Rodrigues, & Rodrigues, 2016). Recently, Liu et al. (2018) used single-cell RNAseq technology to analyze the transcriptome profiles of porcine oocytes categorized by the BCB stain. A total of 155 genes related to oocyte meiosis, cell cycle, tight junction, and metabolic pathways were differentially expressed between BCB-negative and BCB-positive immature germinal vesicle (GV) stage oocytes. Although our knowledge regarding oocyte quality and development has improved significantly, the molecular mechanisms regulating and determining oocyte development are still unclear. Therefore the objective of this study was to identify and analyze the transcriptome profiles of porcine oocytes aspirated from follicles of different sizes as a model for different developmental stages using RNA high-throughput sequencing technology.

2 | RESULTS

2.1 | RNA sequencing data

Six RNA libraries were prepared from the oocytes of large (3–6 mm; LO) and small (1.5–1.9 mm; SO) follicle groups and sequenced using Illumina HiSeq4000. Approximately 376.4 million raw reads were sequenced from both groups. After adapter trimming and quality filtering, an average of 89% of the quality controlled (QC) reads was mapped to the *Sus scrofa* reference genome and 85% were uniquely mapped. A summary of the total number of reads and mapping results for each group is presented in Table S1. Principal component analysis (PCA) revealed that biological replicates for each group clustered together except for the third replicate of the LO group (Figure 1a). The same replicate showed a relatively lower number of raw reads (Table S1). Therefore, this replicate has been excluded from further analysis. To avoid the bias for low expression, genes with a minimum of 0.1 transcripts per million (TPM) in all replicates were considered to be expressed genes in this study. Boxplots of log10-TPM values for each replicate showed that the overall range and distribution of the TPM values were consistent among all replicates (Figure 1b). In total, 15,008 and 15,053 genes were detected in the LO and SO oocyte groups, respectively, with 14,557 genes mutually expressed in both groups (Figure 1c). A group of 930 genes represented the most highly expressed genes in both groups with a total count of reads more than 5000 and TPM more than 40 (Table S2). This group includes genes related to the cell cycle and oocyte meiosis and quality (*CCNB2*, *ESPL1*, *CPEB1*, *CUL1*, *CDC25B*, *CDC27*, *BMP15*, and *GDF9*). Of this list, a group of 18 genes was the most abundantly expressed in all oocyte samples including *ACCSL*, *DNMT1*, *FTL*, *BMP15*, and *H1FOO* (Table 1).

2.2 | Differentially expressed genes and ontological classification

Genes with adjusted $p < .05$ and fold change (FC) ≥ 1.5 were considered to be Differentially expressed genes (DEGs). A total of 322 DEGs (60 up- and 262 downregulated) were identified in the LO compared with the SO group, including 62 genes with unknown function (Table S3). The heat map and a volcano plot (Figure 2) illustrates the clusters and distribution of DEGs, respectively. The top 25 known DEGs identified in the LO compared with the SO group are listed in Table 2. The highly abundant and highly significant DEGs in the LO and SO groups are presented in Tables 3 and 4, respectively. Gene ontology (GO) enrichment analysis revealed that five pathways were significantly enriched in the DEGs (Figure 3a, Table S4). Protein processing in endoplasmic reticulum was the top enriched pathway, involving five DEGs (*EIF2AK1*, *XBP1*, *ERP29*, *STUB1*, *DERL3*) followed by glycosylphosphatidylinositol (GPI)-anchor biosynthesis pathway. The highly enriched biological processes included processes related to

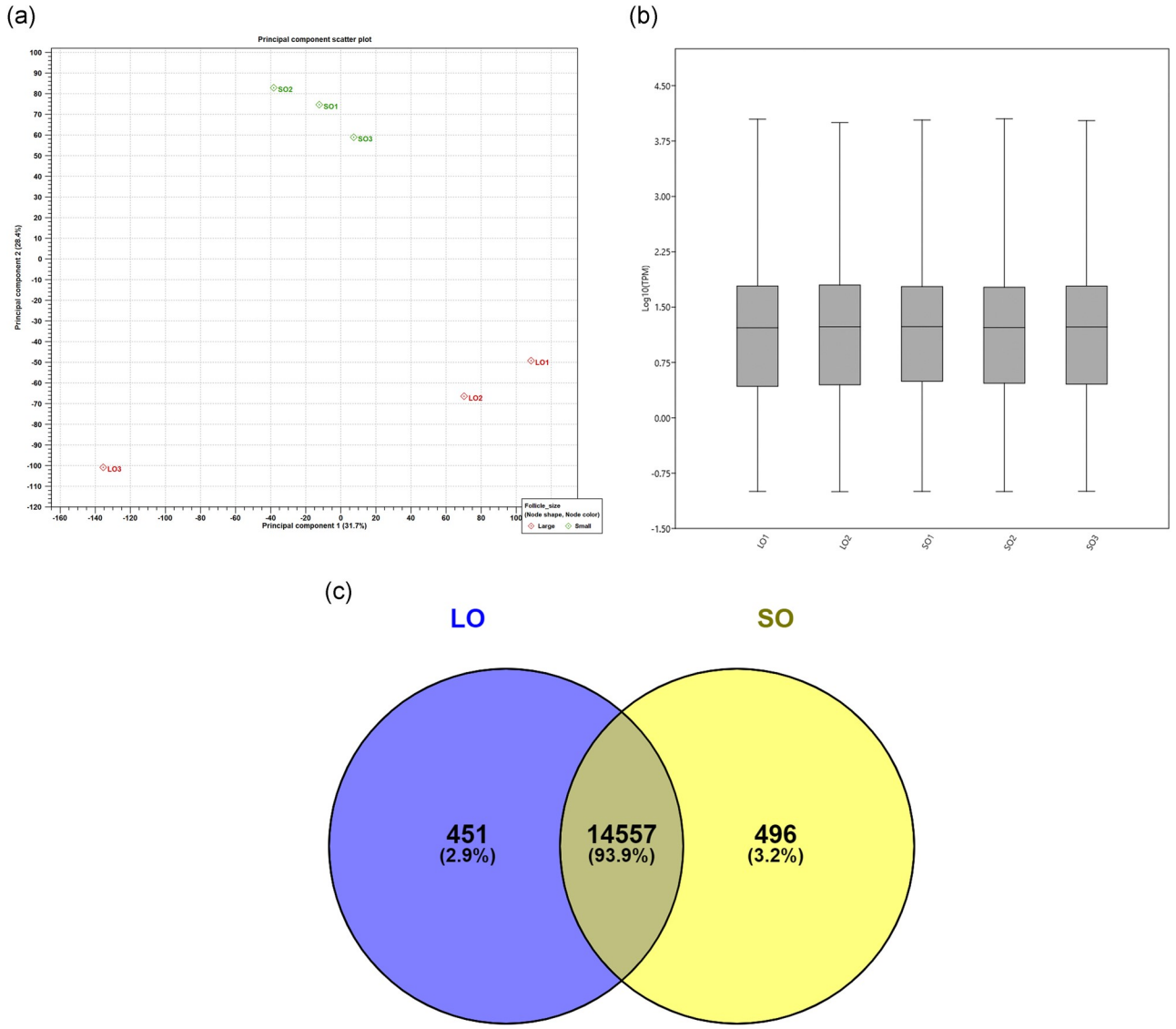

FIGURE 1 Transcriptome analysis of oocytes derived from large (LO) compared with small (SO) follicle groups. (a) Principal component analysis (PCA). LO1-LO3: large oocyte replicates, SO1-SO3: small oocyte replicates. (b) Boxplot for gene expression level (log10-TPM) showing the distribution and overall range of the TPM values in all replicates. (c) Venn diagram for commonly and exclusively expressed genes in both oocyte groups. TPM, transcripts per million [Color figure can be viewed at wileyonlinelibrary.com]

cell growth and signaling, gene expression regulations, and cytoskeleton organization (Figure 3a; Table S4). Molecular function analysis showed that the highly enriched functions included palmitoyl-CoA hydrolase activity, antioxidant activity and binding-related activities including extracellular matrix, insulin-like growth factor, sequence-specific DNA, and calcium ion binding (Figure 3b; Table S4). The extracellular space, exosome, region, and matrix were among the top significant enrichment cellular component terms (Figure 3b; Table S4). The interaction networking of DEGs commonly involved in highly enriched biological processes including extracellular matrix organization, insulin-like growth factor binding, microtubule cytoskeleton reorganization, and cell junction organization is presented in Figure 4. Comparison analysis with our previously published data (Gad et al., 2019) showed that a list of 99 DEGs between the LO and SO group was the same as the predicted target genes of the DE miRNAs in the same groups (Table S5). These genes are involved in different signaling pathways, gene expression regulation, and functions related to an extracellular matrix organization.

2.3 | Quantitative reverse transcription polymerase chain reaction validation

Gene expression comparison of the reverse transcription quantitative polymerase chain reaction (RT–qPCR) validation and the RNAseq results shows the same pattern of expression for the selected genes (Figure 5). Five out of the eight selected DEGs were statistically significant ($p < .05$).

TABLE 1 Topmost abundantly expressed genes in all samples of large (LO) and small (SO) oocyte groups

			TPM[a]	
Gene name	**Gene identifier**	**Gene description**	**LO**	**SO**
ACCSL	ENSSSCG00000013283	1-Aminocyclopropane-1-carboxylate synthase homolog (inactive) like	10537.01	10899.24
DNMT1	ENSSSCG00000013659	DNA methyltransferase 1	9353.13	7258.28
LOC100621706	ENSSSCG00000000296	Gametocyte-specific factor 1-like	7582.84	6537.09
FTL	ENSSSCG00000003153	Ferritin light chain	7581.07	9719.61
BMP15	ENSSSCG00000012310	Bone morphogenetic protein 15	7175.75	9892.26
ATP6	ENSSSCG00000018081	ATP synthase F0 subunit 6	6796.37	9048.97
H1FOO	ENSSSCG00000011591	Oocyte-specific H1 histone	6110.97	6661.85
COX1	ENSSSCG00000018075	Cytochrome c oxidase subunit I	5647.24	7297.51
ZP4	ENSSSCG00000010141	Zona pellucida glycoprotein 4	4980.68	6342.85
UBB	ENSSSCG00000018033	Ubiquitin B	4888.93	6154.17
ZP3	ENSSSCG00000007691	Zona pellucida glycoprotein 3	4825.26	7880.52
COX2	ENSSSCG00000018078	Cytochrome c oxidase subunit II	4259.47	6235.68
COX3	ENSSSCG00000018082	Cytochrome c oxidase subunit III	4130.24	5762.51
ZP2	ENSSSCG00000007851	Zona pellucida glycoprotein 2	4033.43	4766.38
WEE2	ENSSSCG00000016489	WEE1 homolog 2	3899.85	3241.41
PCNA	ENSSSCG00000030642	Proliferating cell nuclear antigen	3875.88	4145.74
DPPA5	ENSSSCG00000004283	Developmental pluripotency associated 5	3729.27	3978.80
KPNA7	ENSSSCG00000007607	Karyopherin subunit alpha 7	3724.96	4045.49

[a]Average value of transcripts per million (TPM) for each oocyte group.

3 | DISCUSSION

In our previous study (Gad et al., 2019), we reported significant differences in the developmental competence of porcine oocytes aspirated from large (3–6 mm) compared with small (1.5–1.9 mm) follicles. We also showed that oocytes collected from large follicles were characterized by a higher proportion of SN chromatin configuration than oocytes from smaller follicles which exhibited a higher percentage of the NSN configuration (Gad et al., 2019). In the current study, we aimed to identify and analyze the transcriptome profile of porcine oocytes derived from these categories of follicle size. In different mammalian species, it is well known that oocyte developmental competence is correlated with follicle size (Bagg et al., 2007), follicular growth stage (Humblot et al., 2005), and chromatin remodeling (Lodde, Modina, Galbusera, Franciosi, & Luciano, 2007). However, the molecular mechanisms regulating and determining oocyte development are not yet clear. Understanding such mechanisms will support our current IVF/IVC procedures and will improve the criteria used to assess the quality of oocytes and embryos. RNA sequencing is a recent widely used technology for the transcriptome profiling of different biological systems that offers many advantages over other technologies. However, when we consider oocytes, the interpretation of transcriptome and gene expression data is a challenge. This is particularly due to the special maternal RNA storage feature of oocytes together with the declined transcription activity and the low correlation between mRNA expression and protein contents in oocytes (Labrecque & Sirard, 2014; Sternlicht & Schultz, 1981). Therefore care should be taken when interpreting gene expression data with regard to related biological functions.

In pigs, oocyte maturation competence is acquired in follicles greater than 2 mm and is associated with a significant decrease in oocyte transcriptional activity (Bjerregaard et al., 2003; Motlik, Crozet, & Fulka, 1984). Transcription declines dramatically near the end of oocyte growth, coinciding with changes in the chromatin configurations from NSN to SN structures (De La Fuente, 2006). Some mRNAs must be appropriately stored to support late oogenesis stages and early embryonic development, while some others need to be translated to support oocyte growth (Biase et al., 2014; Clarke, 2012). In this study, we detected approximately 15,000 genes in large or small oocyte groups. More than 14,500 of these genes were mutually expressed, with the majority exhibiting no differences in expression abundance. In agreement with several other studies in different animal models, we found that genes related to oocyte meiosis and the cell cycle (including *CCNB1* and *CCNB2*), as well as genes known to be specific for oocytes such as *H1FOO*, *ZP2*, *ZP3*, *ZP4*, *GDF9*, and *BMP15*, were among the top highly abundant genes in both groups. For instance, cyclin B-related genes (*CCNB1* and *CCNB2*) have been reported to be transcribed and translated in early stages of mice GV oocytes with an important role in meiotic progression (Han et al., 2017). Moreover, we found that *DNMT1* was the second highly abundant gene in both oocyte groups. The expression of *DNMT1* was previously identified in different stages of GV oocytes in bovines (Lodde et al., 2009), humans (Huntriss et al., 2004), and mice (Ratnam et al., 2002). It is well known that global DNA methylation

TABLE 2 Top 25 up- and downregulated known genes in large (LO) compared with small (SO) oocyte groups

Gene name	Gene identifier	Gene description	Fold change	*p*-Adj.
ACOD1	ENSSSCG00000009469	Aconitate decarboxylase 1	16.4	.049
TNFSF11	ENSSSCG00000009429	TNF superfamily member 11	8.06	.033
OAZ3	ENSSSCG00000027091	Ornithine decarboxylase antizyme 3	6.18	.013
LOC110261313	ENSSSCG00000037257	Zinc finger protein 548-like	5.35	.006
AVPR1A	ENSSSCG00000033383	Arginine vasopressin receptor 1A	4.68	8.05E−04
PPM1F	ENSSSCG00000010079	Protein phosphatase, Mg2+/Mn2+ dependent 1F	−3.64	.002
GJA1	ENSSSCG00000004241	Gap junction protein alpha 1	−3.87	.01
ADAMTS5	ENSSSCG00000012027	ADAM metallopeptidase with thrombospondin type 1 motif 5	−3.88	5.48E−04
DCHS1	ENSSSCG00000029189	Dachsous cadherin-related 1	−3.97	.002
MAN2B2	ENSSSCG00000027178	Mannosidase alpha class 2B member 2	−4.07	.049
THBS1	ENSSSCG00000004789	Thrombospondin 1	−4.16	.024
IL17B	ENSSSCG00000014434	Interleukin 17B	−4.34	8.95E−05
FAM207A	ENSSSCG00000028571	Family with sequence similarity 207 member A	−4.75	.008
ITIH5	ENSSSCG00000011129	Inter-alpha-trypsin inhibitor heavy chain family member 5	−4.87	.018
LGALS3BP	ENSSSCG00000036383	Galectin 3 binding protein	−4.98	.043
GPRIN3	ENSSSCG00000039821	GPRIN family member 3	−5.03	.036
CLDN15	ENSSSCG00000038901	Claudin 15	−5.17	.014
IQCA1	ENSSSCG00000016321	IQ motif containing with AAA domain 1	−5.61	.017
PENK	ENSSSCG00000006243	Proenkephalin	−5.89	.01
ZNF213	ENSSSCG00000007967	Zinc finger protein 213	−5.97	.038
IGFBP2	ENSSSCG00000035392	Insulin like growth factor binding protein 2	−6.18	.024
SERPINE1	ENSSSCG00000025698	Serpin family E member 1	−6.26	.029
HYAL3	ENSSSCG00000011402	Hyaluronidase 3	−7.17	5.63E−03
RGS5	ENSSSCG00000037821	Regulator of G protein signaling 5	−7.41	7.83E−03
KCNJ14	ENSSSCG00000003135	Potassium voltage-gated channel subfamily J member 14	−7.71	.005

TABLE 3 Highly abundant and highly significant differentially expressed genes in large (LO) compared with small (SO) oocyte groups

Gene name	Gene identifier	Gene description	TPM[a]	Fold change	*p*-Adj.
BRCA2	ENSSSCG00000029039	BRCA2, DNA repair associated	248.43	1.53	7.95E−06
GPLD1	ENSSSCG00000001089	Glycosylphosphatidylinositol specific phospholipase D1	197.68	1.52	1.11E−05
FAT1	ENSSSCG00000007000	FAT atypical cadherin 1	147.44	1.55	3.07E−06
IFIT5	ENSSSCG00000010454	Interferon-induced protein with tetratricopeptide repeats 5	103.84	1.53	1.35E−05
RAI14	ENSSSCG00000016824	Retinoic acid induced 14	82.81	1.58	2.54E−06
HERC1	ENSSSCG00000004561	HECT and RLD domain containing E3 ubiquitin protein ligase family member 1	52.03	1.75	9.56E−10
DENND2C	ENSSSCG00000006755	DENN domain containing 2C	49.22	1.54	1.94E−05
ZFPM2	ENSSSCG00000006038	Zinc finger protein, FOG family member 2	42.73	1.54	1.04E−11
ALOX15B	ENSSSCG00000017978	Arachidonate 15-lipoxygenase B	34.79	1.77	5.6E−09
PCLO	ENSSSCG00000015401	Piccolo presynaptic cytomatrix protein	29.10	1.50	3.72E−05
PAK3	ENSSSCG00000025306	P21 (RAC1) activated kinase 3	24.95	1.60	3.68E−06
PTCH1	ENSSSCG00000027312	Patched 1	14.19	1.69	8.6E−08

[a]Average value of transcripts per million (TPM) for LO oocyte group.

TABLE 4 Highly abundant and highly significant differentially expressed genes in small (SO) compared with large (LO) oocyte groups

Gene name	Gene identifier	Gene description	TPM[a]	Fold change	*p*-Adj.
ZP3	ENSSSCG00000007691	Zona pellucida glycoprotein 3	7880.52	1.65	5.88E−08
ND3	ENSSSCG00000018084	NADH dehydrogenase subunit 3	1518.86	1.69	.001
ND4L	ENSSSCG00000018086	NADH dehydrogenase subunit 4 L	660.03	1.58	.011
TMEM247	ENSSSCG00000008442	Transmembrane protein 247	484.00	3.19	.014
RBMXL2	ENSSSCG00000035683	RBMX like 2	394.92	1.81	.001
RPS14	ENSSSCG00000031370	Ribosomal protein S14	300.46	1.57	4.28E−06
NTN1	ENSSSCG00000017993	Netrin 1	240.29	2.06	.006
SLC8B1	ENSSSCG00000009880	Solute carrier family 8 member B1	219.08	1.57	<1.11E−16
RETREG3	ENSSSCG00000017395	Reticulophagy regulator family member 3	212.71	1.81	1.07E−10
PKP4	ENSSSCG00000015878	Plakophilin 4	188.06	1.81	.002
DPEP1	ENSSSCG00000021971	Dipeptidase 1	185.51	1.67	<1.11E−16
RPLP1	ENSSSCG00000004970	Ribosomal protein lateral stalk subunit P1	176.58	1.66	6.78E-08
RABAC1	ENSSSCG00000003046	Rab acceptor 1	149.39	1.54	1.8E−05
RPLP2	ENSSSCG00000012842	Ribosomal protein lateral stalk subunit P2	148.24	1.54	1.57E−05
POMGNT2	ENSSSCG00000011293	Protein O-linked mannose N-acetylglucosaminyltransferase 2	145.26	1.67	7.26E−06
TYSND1	ENSSSCG00000010259	Trypsin domain containing 1	137.16	2.02	.012
LOC100510930	ENSSSCG00000010099	Tubulin alpha-3 chain	116.73	1.52	2.13E−05
RNASEH2C	ENSSSCG00000012979	Ribonuclease H2 subunit C	108.84	1.61	1.71E−04
PSAP	ENSSSCG00000010281	Prosaposin	104.07	1.79	4.64E−10
POFUT2	ENSSSCG00000012088	Protein O-fucosyltransferase 2	89.32	1.71	1.67E−08
IGFBP7	ENSSSCG00000008913	Insulin like growth factor binding protein 7	86.99	1.57	2.77E−06
TMEM129	ENSSSCG00000008678	Transmembrane protein 129	84.97	1.60	<1.11E−16
APOE	ENSSSCG00000003088	Apolipoprotein E	78.86	1.96	5.38E−12
SELENOW	ENSSSCG00000034313	Selenoprotein W	69.24	1.79	.021
MFSD12	ENSSSCG00000026169	Major facilitator superfamily domain containing 12	65.34	1.71	<1.11E−16

[a]Average value of transcripts per million (TPM) for SO oocyte group.

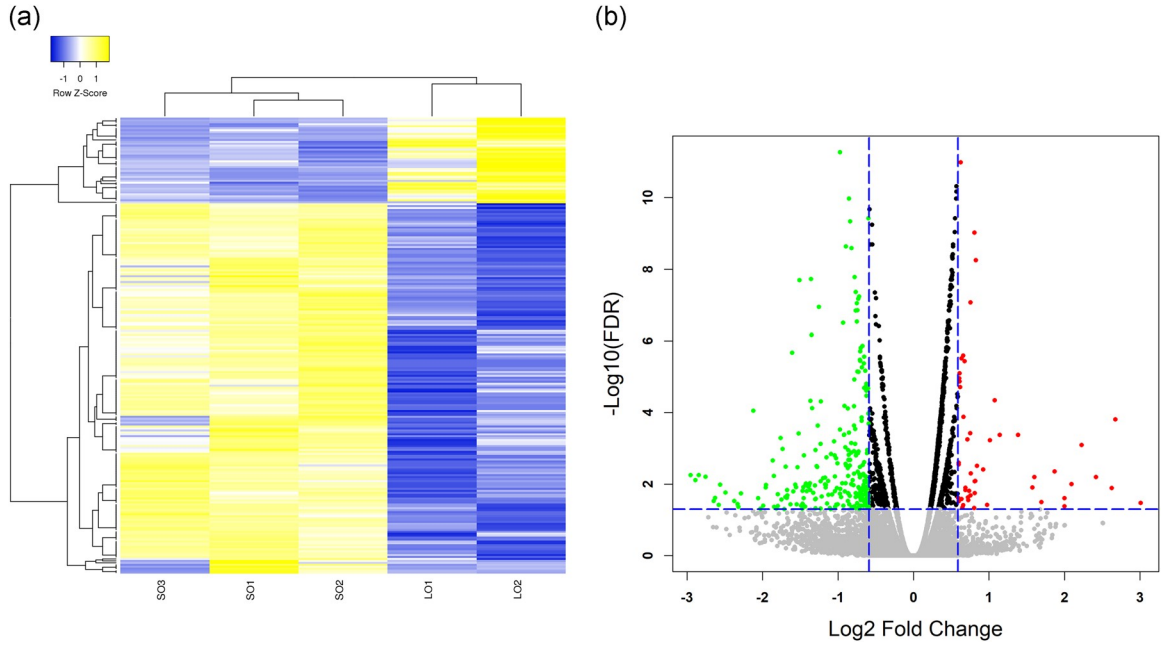

FIGURE 2 Differential expression analysis of oocytes derived from large (LO) compared with small (SO) follicle groups. (a) Heat map and hierarchical clustering of differentially expressed genes. LO1-LO2: large oocyte replicates, SO1-SO3: small oocyte replicates. (b) Volcano plot of expressed genes. Up- and downregulated genes in the LO compared to the SO group are labeled with red and green points, respectively [Color figure can be viewed at wileyonlinelibrary.com]

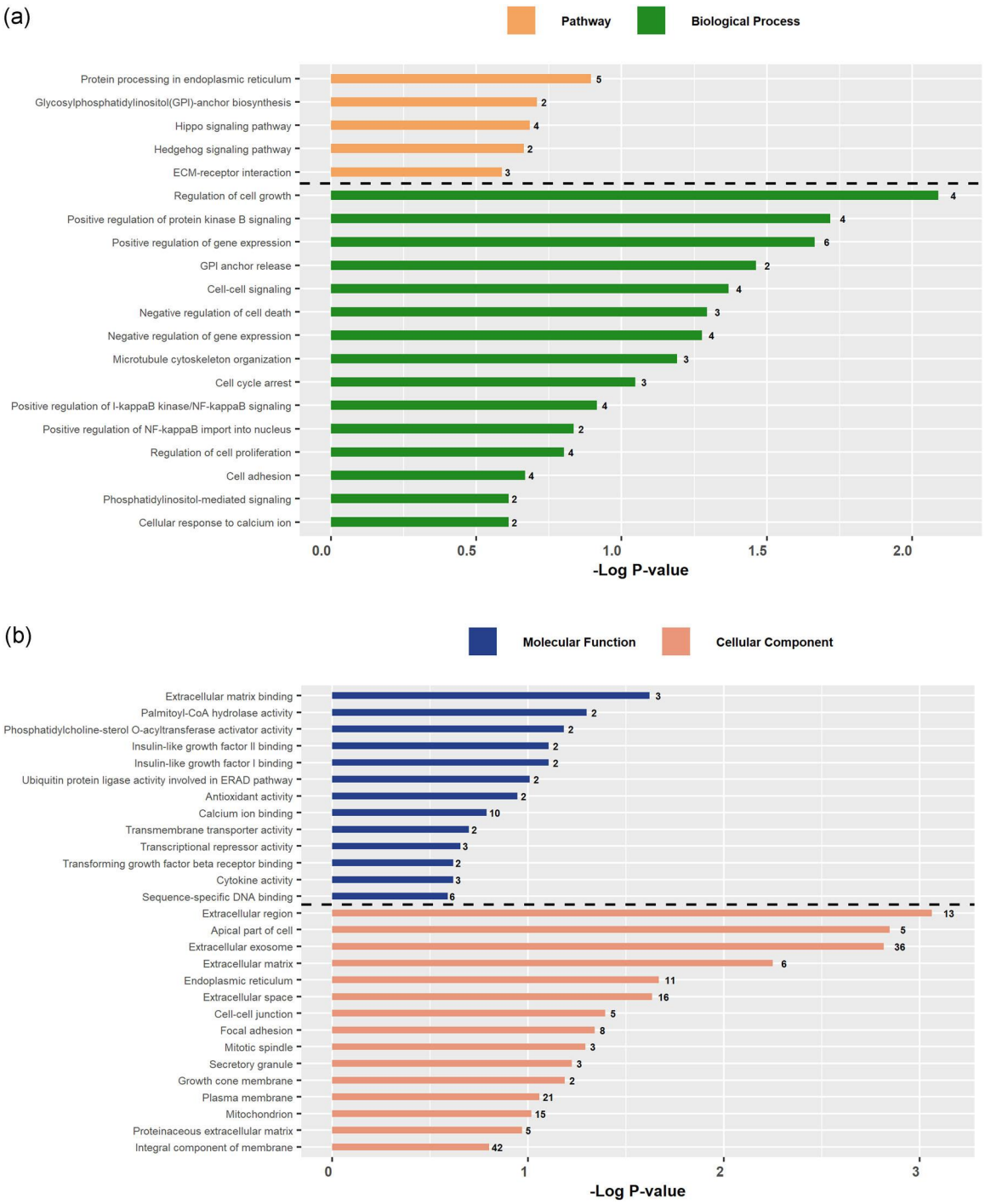

FIGURE 3 Ontological classification of differentially expressed genes. (a) Top Kyoto Encyclopedia of Genes and Genomes pathways and biological processes. (b) Top molecular functions and cellular components. The number of genes involved in each term is shown to the right of the column [Color figure can be viewed at wileyonlinelibrary.com]

progressively increases in growing GV oocytes (Saitou, Kagiwada, & Kurimoto, 2012). Therefore, a higher expression of *DNMT1* to maintain the DNA methylation status of GV oocytes is expected.

Differential expression analysis revealed a relatively lower number of DEGs between the two-oocyte groups. Changes in the expression patterns could be due to the accumulation, degradation or utilization of the mRNAs through the translation process during oocyte development (Schultz, Stein, & Svoboda, 2018). The down-regulation of the majority of DEGs in the LO group could be related to the global decrease in transcription activity which occurs in large more than in small oocytes. In line with this argument, decreases in the expression of specific gene clusters have been reported in relation to oocyte growth (Pan, O'Brien, Wigglesworth, Eppig, & Schultz, 2005). The same study suggested that oocyte competence is

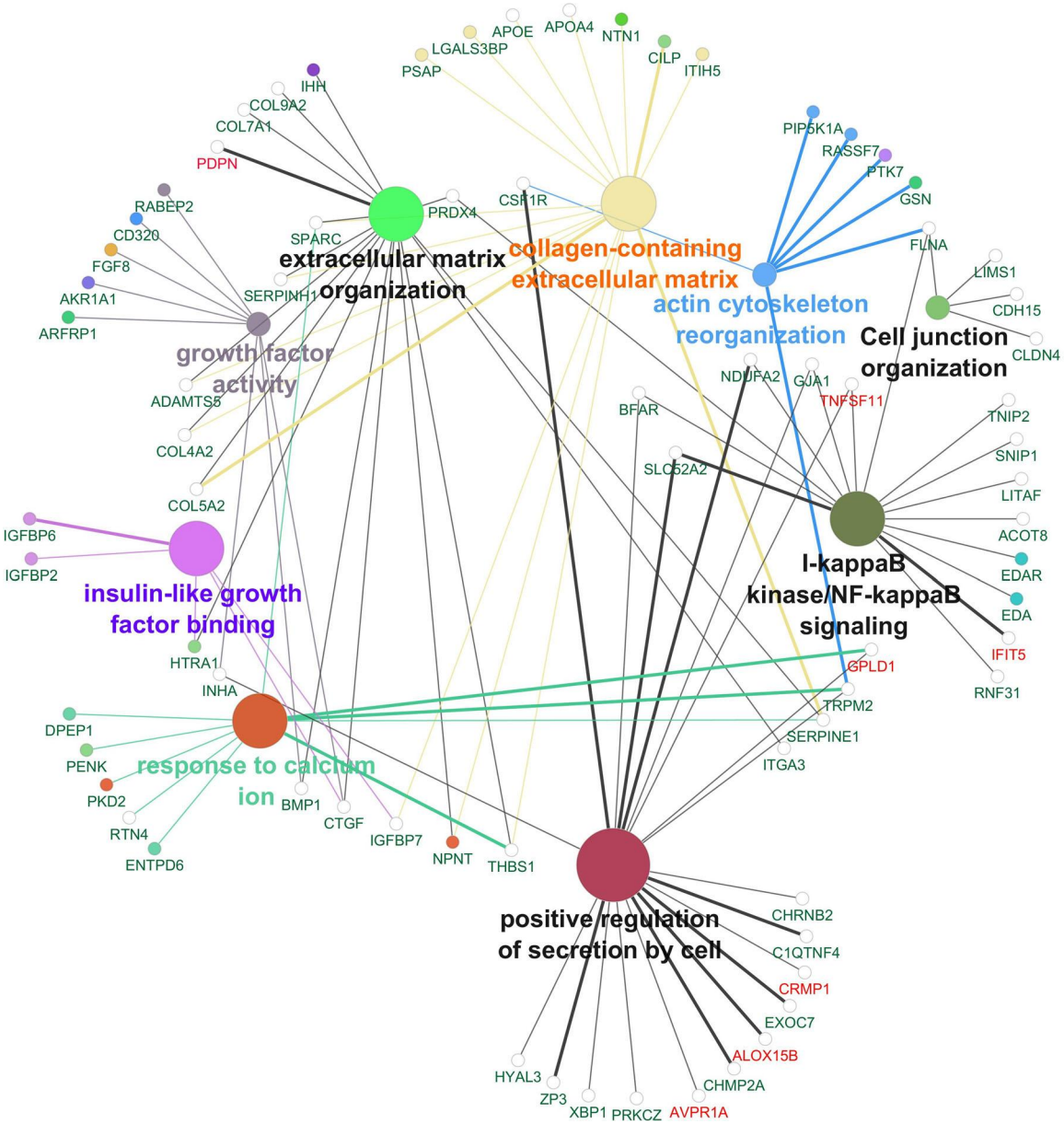

FIGURE 4 Interaction networking of differentially expressed genes commonly involved in highly enriched biological processes. The network shows upregulated (red) and downregulated (green) genes in the large oocytes compared with the small oocytes group [Color figure can be viewed at wileyonlinelibrary.com]

not the result of the expression of highly limited genes, instead, it is an output of different expression spectra throughout the life history of the oocyte. This could explain the upregulation of some specific genes in the LO group, despite the expected reduction in the transcription activity of this group. These genes could be candidates for oocyte developmental competence, as they are expressed or accumulated more in the oocytes from large follicles (Table 3). Among this list were *BRCA2* and *GPLD1*. The role of *BRCA2* was initially discovered to be related to breast and ovarian cancer susceptibility (Hall et al., 2009; Xu et al., 1997). It has been also reported that the BRCA2 protein plays a role in DNA repair and maintaining genome stability (Chen et al., 1998). A knock-out mice model provides evidence for a key role of *BRCA2* in gametogenesis. In this model, oocyte meiotic competence and oogenesis progress was compromised in the absence of *BRCA2* (Sharan et al., 2004). In human oocytes, a more than 15-fold reduction in *BRCA2* transcription level was observed directly after fertilization (Wells et al., 2005), suggesting its important role in oocyte meiotic competence. *GPLD1* is a well-characterized mammalian GPI- specific phospholipase (Davitz et al., 1987). It is involved in the GPI-anchor biosynthesis pathway and plays a key role in releasing membrane-bound GPI anchored proteins (AP) from the cell surface during various cellular processes including differentiation, adhesion, and survival (Verghese, Gutknecht, & Caughey, 2006). Several GPI-AP are involved in the

FIGURE 5 Comparison of the RT–qPCR validation and the RNAseq results for the selected DEGs in the LO compared with the SO group. *Statistical significance ($p < .05$). DEG, differentially expressed genes; RT-qPCR, reverse transcription quantitative polymerase chain reaction

fertilization process by facilitating the interaction and fusion of the sperm–oocyte extracellular matrix (Fujihara & Ikawa, 2016). Moreover, the transcriptome profiling of human GV oocytes identified *GPLD1* as one of the highly and uniquely expressed genes in oocytes compared to embryonic stem cells (Zhang et al., 2007), suggesting a unique role in oocyte development to ensure a successful maturation and/or fertilization process.

On the other hand, we identified a group of genes that were highly abundant and significantly upregulated in the SO group (Table 4). An example of these genes is *ZP3* that encodes for one of the four sulfated glycoproteins which build the oocyte zona pellucida in pigs (Rath et al., 2006). It has been previously reported that *ZP1* and *ZP3* transcripts are accumulated as the oocyte begins to grow and decline in the later stages of oocyte growth (Epifano, Liang, Familari, Moos, & Dean, 1995). The same pattern has been observed in mouse oocytes, as the *ZP3* transcript increases gradually from primordial to small antral follicles then decreases in the large antral follicles (Pan et al., 2005). Moreover, in porcine oocytes, the ZP3 protein level has been reported to be significantly higher in oocytes isolated from large compared to small follicles (Antosik et al., 2010). In agreement with our findings, these results could suggest that *ZP3* is accumulated in the early growing oocyte, then it is utilized and accumulated as a protein in the fully grown oocytes to build the zona pellucida and support oocyte development. Other transcripts that showed the same pattern were *ND3* and *ND4L*. Both genes are encoded in the mitochondrial genome and involved in the oxidative phosphorylation pathway and ATP synthesis. The expression of mitochondrial encoded genes, including *ND3*, were found to be positively correlated with maternal aging and subsequently with a lower developmental competence of mouse oocytes (Hamatani et al., 2004). It is well known that mitochondrial activity, distribution, and ATP synthesis are highly associated with the further developmental competence of mammalian oocytes (Van Blerkom, 2011). However, the regulation of energy metabolism in relation to mitochondrial activity during oocyte development is still unclear. On the basis of chromatin configurations, genes related to the oxidative phosphorylation pathway were highly expressed in SN compared with NSN immature oocytes (Ma et al., 2013), however, MII stage oocytes exhibited the opposite pattern (Zuccotti et al., 2008). A reduction in oxidative phosphorylation activity has been reported in bovine oocytes during the transition from the GV to the MII stage (Sugimura et al., 2012) with downregulation of the pathway-related genes (Su et al., 2007). These studies suggested that the decreased rate of energy production in the MII oocyte reflects its quiescent state in terms of energy consumption. This could be the same relative state between fully grown and growing oocytes. In agreement with this explanation, Torner et al. (2008) reported that in bovines, highly competent oocytes exhibited a lower mitochondrial activity than oocytes with a lower developmental competence. The same study reported an upregulation of mitochondrial-related transcripts in BCB- compared to BCB + oocytes. They speculated that the upregulation of such transcripts accompanied by higher mitochondrial activity could be due to the higher demand of growing oocytes for ATP compared with fully grown oocytes to support cytoplasmic maturation processes. However, more studies including enzymatic activities and protein analysis are needed to fully understand energy metabolism during oocyte growth.

In the current study, genes involved in the regulation and reorganization of microtubule cytoskeleton-related functions were among the DEGs. Oocyte meiosis, in contrast to somatic cell mitotic division, is characterized by the asymmetric nature of cell division, in which meiotic spindles migrate near to the oocyte cortex and segregate homologous chromosomes to generate the haploid oocyte and a small polar body (Clift & Schuh, 2013). This asymmetric meiotic division is highly controlled by actin and microtubules cytoskeleton distribution and remodeling during oocyte development (Wang, Abeydeera, Prather, & Day, 2000). Abnormalities of the actin cytoskeleton have been reported to be associated with meiotic incompetence in porcine and bovine oocytes (Somfai et al., 2011). This may indicate the importance of these genes as potentially involved in the attainment of oocyte development. Another interesting group of genes involved in several biological and molecular functions is the IGF-binding protein (*IGFBP*)-related genes. In this study *IGFBP2*, *6*, and *7* exhibited a downregulation in the LO compared with the SO group. It is well known that IGF-binding proteins regulate the bioavailability of IGFs to their corresponding cells (Jones & Clemmons, 1995). The expression level of *IGFBPs* is correlated with the follicular development and selection of the dominant follicle in relation to the availability of IGFs during the development (Mazerbourg & Monget, 2018). In other mammalian species, the intrafollicular level of IGFBP-2 is dramatically reduced as the follicle develops from a diameter of 1–2 mm to the preovulatory stage, however, it increases in the atretic follicles (Armstrong et al., 1998; Gérard & Monget, 1998; Mazerbourg, Bondy, Zhou, & Monget, 2003). At the oocyte level, IGFBP-2 mRNA and protein expression were found to be decreased during oocyte maturation in bovines (Nuttinck et al., 2004). These results suggested that the reduction of

IGFBP-related genes could be a way to ensure higher IGF availability, which is essential for follicular and oocyte growth and development.

In conclusion, this study provides new insights into the global transcriptome changes and the abundance of specific transcripts in porcine oocytes in correlation with follicle size. This knowledge could assist in answering the fundamental question: What is the mRNA composition of a competent oocyte? However, different research models with meta-analysis approaches are still needed to unravel the full answer to this question.

4 | MATERIAL AND METHODS

4.1 | Follicle categorization and oocyte collection

Prepubertal gilts ovaries were collected from a local abattoir and transported to the lab in a thermo-flask within 2 hr of their slaughter. Ovaries were washed three times with saline solution. Cumulus oocyte complexes (COCs) were aspirated with a 20-Gauge needle attached to a 10 ml syringe from large follicles (3–6 mm) or with a 23-Gauge needle attached to a 5 ml syringe from small follicles (1.5–1.9 mm). The follicle diameter was measured as previously reported (Ireland, Murphee, & Coulson, 1980). COCs with at least three layers of cumulus cells and an evenly granulated ooplasm were used in this study. For the transcriptome analysis experiment, COCs for each group were vortexed for approximately 2 min in phosphate-buffered saline (PBS) to remove all the cumulus cells. Denuded oocytes were then washed in PBS and stored at −80°C until RNA extraction.

4.2 | Total RNA isolation and quality control

Total RNA was isolated from denuded oocytes of the two different groups (three replicates each, $n = 360$) using an AllPrep DNA/RNA Micro Kit (Qiagen) according to the manufacturer's instructions. The RNA quality and integrity were assessed with a NanoDrop 8000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), respectively. Samples with RNA Integrity Number (RIN) > 7 were used in this study.

4.3 | Library preparation and RNA sequencing

The RNA libraries were prepared using a NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB). Briefly, 20 ng of total RNA underwent rRNA depletion and DNase digestion using a NEBNext rRNA Depletion Kit (NEB) according to the manufacturer's protocol. A nucleoMag NGS Clean-up and Size Select Kit (MACHEREY-NAGEL) was used for sample purification and library size selection. RNA samples were purified, fragmented at 94°C for 15 min, and primed with random primers. Samples were converted into double-stranded cDNA, purified, and adapters were ligated to the 3′ and 5′ ends. The cDNA samples were amplified by PCR (14 cycles) using indexing forward primers and a universal reverse primer. After PCR amplification, RNA libraries were purified and quality control was performed using an Agilent High-Sensitivity DNA Kit in an Agilent 2100 Bioanalyzer. On the basis of the quality of the inserts and the concentration measurements, the libraries were pooled in equimolar ratios. The library pool was quantified using a KAPA Library Quantification Kit for Illumina (Roche, Pleasanton, CA) and then sequenced in a HiSeq4000 (Illumina, Inc. San Diego, CA) according to the manufacturer's instructions in a single-end read with a read length of 100 bases. Raw data were demultiplexed and FASTQ files for each sample were generated using the software bcl2fastq (Illumina Inc.).

4.4 | RNAseq data analysis

Data were analyzed using the software CLC Genomics Workbench, version 12.0, from Qiagen (www.qiagenbioinformatics.com). After importing FASTQ files, raw sequencing reads were trimmed based on quality score (Q-score > 20 was considered high-quality data), removing adapter sequences and discarding reads with length less than 15 nt. The raw FASTQ files and processed CSV files have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE129994. Sequence reads were mapped and annotated to the *Sus scrofa* reference genome with genes and transcripts (release Sscrofa11.1, ftp://ftp.ensembl.org/pub/release-95/fasta/sus_scrofa/) using the RNA-Seq Analysis tool of CLC Genomics Workbench v. 12.0. Then, expression values of read count and TPM were calculated for each gene. Data were normalized using the trimmed mean of M-values normalization method (TMM normalization; Robinson & Oshlack, 2010). Expression analysis was done using the Differential Expression In Two Groups tool of CLC Genomics Workbench v. 12.0 based on a negative binomial Generalized Linear Model (GLM) function. Differentially expressed genes (DEGs) were filtered based on the fold change (FC ≥ 1.5) and *p*-adjusted value (FDR < 0.05) (Benjamini & Hochberg, 1995).

4.5 | Ontological classification of DEGs

The DEGs were submitted to the Database for Annotation, Visualization, and Integrated Discovery (DAVID) Bioinformatics web-tool v.6.8 (http://david.abcc.ncifcrf.gov/) for ontological classification enrichment analysis (Huang, Sherman, & Lempicki, 2009) using all detected genes as a background. Significant pathways were characterized by the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Ogata et al., 1999). Interaction networking between DEGs as well as the identified pathways and the annotated functions were visualized using the software Cytoscape v.3.7.1 (http://www.cytoscape.org/; Shannon et al., 2003) with the ClueGO plugin v.2.5.4 (http://apps.cytoscape.org/apps/cluego; Bindea et al., 2009).

4.6 | Validation of DEGs using RT-qPCR

Total RNA was extracted from four independent biological replicates for each of the oocyte groups ($n = 400$) using an AllPrep DNA/RNA

Micro Kit (Qiagen) according to the manufacturer's instructions. The RNA quality and integrity were assessed as mentioned above. Based on the RNAseq data analysis, eight DEGs were randomly selected for RT-qPCR validation. The primers were designed using the software Beacon Designer v. 8.21 (http://www.premierbiosoft.com/index.html) and listed in the supplementary table S6. The one-step RT-qPCR was conducted in a RotorGene 3000 cycler (Corbett Research, Mortlake, Austria) using the QIAGEN OneStep RT-PCR Kit (Qiagen, Germany) in a 20 µl reaction mixture containing 4 µl 5× reaction buffer, 0.8 µl dNTP mix (10 nM stoch), 0.4 µl forward and reverse primers (20 nM stock), 0.125 µl RNasine (20 U/ml stock, Promega), 0.8 µl enzyme mix, 0.8 µl EvaGreen (Biotium, CA), 2 µl RNA, and nuclease-free water. Reaction conditions were as follows: reverse transcription at 50 °C for 30 min, initial denaturation at 95°C for 15 min, followed by PCR cycles consisting of denaturation at 94°C for 15 s, annealing at temperature specific for each set of primers (Table S6) for 15 s and extension at 72°C for 20 s; and final extension at 72°C for 5 min. Fluorescence data were acquired at the end of each extension step. Products were verified by melting analysis and gel electrophoresis on 1.5% agarose gel with MidoriGreen Direct (Nippon Genetics, Dueren, Germany). Comparative analysis software (Corbett Research) was used for gene expression analyses after normalization to the geometric mean of *H1FOO* and *TUBA1B* mRNA abundance as internal control genes. Statistical analysis of mRNA expression was performed using Student's *t*-test and $p < .05$ were considered to be statistically significant.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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