REPRODUCTION
RESEARCH

Genes of cellular components of morphogenesis in porcine oocytes before and after IVM

Joanna Budna[1], Artur Bryja[2], Piotr Celichowski[1], Rotem Kahan[2], Wiesława Kranc[2], Sylwia Ciesiółka[1], Marta Rybska[3], Sylwia Borys[2], Michal Jeseta[4], Dorota Bukowska[3], Paweł Antosik[3], Klaus P Brüssow[2], Małgorzata Bruska[2], Michał Nowicki[1], Maciej Zabel[1,5] and Bartosz Kempisty[1,2,4]

[1]Department of Histology and Embryology, Poznan University of Medical Sciences, Poznan, Poland, [2]Department of Anatomy, Poznan University of Medical Sciences, Poznan, Poland, [3]Institute of Veterinary Sciences, Poznan University of Life Sciences, Poznan, Poland, [4]Department of Obstetrics and Gynecology, University Hospital and Masaryk University, Brno, Czech Republic and [5]Department of Histology and Embryology, Wroclaw Medical University, Wroclaw, Poland

Correspondence should be addressed to B Kempisty; Email: bkempisty@ump.edu.pl

Abstract

Proper oocyte maturation in mammals produces an oocyte capable of monospermic fertilization and embryo preimplantation. The cumulus-oocyte complexes (COCs), surrounding an oocyte, play a significant role in oocyte maturation. During this process, when the COCs undergo cumulus expansion wherein tightly compact cumulus cells (CCs) form a dispersed structure, permanent biochemical and molecular modifications occur in the maturing oocytes, indicating that the gene expression between immature and mature oocytes differs significantly. This study focuses on the genes responsible for the cellular components of morphogenesis within the developing oocyte. Brilliant cresyl blue (BCB) was used to determine the developmental capability of porcine oocytes. The immature oocytes (GV stage) were compared with matured oocytes (MII stage), using microarray and qRT-PCR analysis to track changes in the genetic expression profile of transcriptome genes. The data showed substantial upregulation of genes influencing oocyte's morphology, cellular migration and adhesion, intracellular communication, as well as plasticity of nervous system. Conversely, downregulation involved genes related to microtubule reorganization, regulation of adhesion, proliferation, migration and cell differentiation processes in oocytes. This suggests that most genes recruited in morphogenesis in porcine oocyte *in vitro,* may have cellular maturational capability, since they have a higher level of expression before the oocyte's matured form. It shows the process of oocyte maturation and developmental capacity is orchestrated by significant cellular modifications during morphogenesis.
Reproduction (2017) **154** 535–545

Introduction

The nuclear and cytoplasmic maturation of the cumulus-oocyte complex (COC) is crucial in acquiring an oocyte capable of fertilization and embryonic development. Both of these maturational stages involve several morphological and molecular modifications to the growing oocyte that includes structural and ultrastructural changes within cellular organelles, and/or activation of transcriptome, proteome and metabolome (Eppig *et al.* 2002, Hulinska *et al.* 2011, Dunlop & Anderson 2014). It is well known that cytoplasmic maturation includes events enabling completing of nuclear maturation, fertilization, as well as early embryogenesis. Maturation rates are approximately 90% (94.6% – Galeati *et al.* 2016, and 86.3%–92.8% – Jin *et al.* 2016). A fully matured and competent oocyte has a molecular profile and cellular organization that differs significantly from the immature gamete. Successful maturation of the COC *in vitro* is improved by *in vitro* culture (IVC) supplements, however, the percentage of fully grown cells that reach metaphase II (MII) stage after IVM is still unsatisfactory in several mammalian species such as canine and porcine. Therefore, recognizing factors that contribute to COC maturation *in vitro* would improve success of IVM and is of current research interest. Moreover, determining genes involved in reaching MII stage in oocytes could lead to molecular diagnostics of gametes with decreased maturational capability or low fertilization potential.

Morphological modifications within growing oocytes during folliculogenesis, oogenesis, as well as oviductal cell maturation require organelle reorganization, formation of compartments responsible for mRNA synthesis and protein production and folding. The mechanisms that lead to nuclear–cytoplasmic shuttling and the formation of gap junctions within the COC are

ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-17-0367
Online version via www.reproduction-online.org

responsible for the organization of protein distribution/cytoskeleton in oocytes. Both of these mechanisms are necessary for the maintenance of proper oocyte growth and maturation as well as cumulus cells (CCs) proliferation and oophorus formation *in vivo* and *in vitro*. Previous studies showed that nuclear–cytoplasmic shuttling achieves oocyte maturational capability, proliferation of CCs and differentiation of ovarian granulosa cells (GCs) *in vitro*. Also, changes in subcellular protein distribution and protein trafficking between the cell membrane, cytoplasm and nucleus may be the main mechanism determining developmental and differentiation capability *in vitro* of both gametes and the surrounding somatic cells. Hence, changes in protein distribution during COC maturation *in vitro* may be used as a successful model for gamete maturational competence *in vitro* porcine cells.

It is well recognized that immature and mature porcine oocytes differ significantly according to morphological and molecular architecture. Therefore, recognizing genes that regulate processes behind cell morphology is crucial in determining COCs developmental competence. In this study, we analyzed the differences in the transcriptomic profile of genes involved in regulating cellular morphogenesis in porcine gametes before and after IVM. We used brilliant cresyl blue (BCB) test to analyze oocytes of increased developmental competence only (Ericsson *et al*. 1993).

Materials and methods

Experimental design

Two BCB tests were performed on the collected porcine oocytes. After the first one, only grade I BCB-positive (BCB+) immature oocytes were selected. They were divided into two groups, each containing 150 oocytes: the first group (before IVM) not subjected to *in vitro* maturation, and the second group (after IVM) where oocytes were first *in vitro*-matured, and then graded as BCB^+ after IVM, resulting in 60–70% of initial oocytes number. Both groups were exposed to subsequent molecular analyses, each performed in three biological replicates.

Animals

A total of 45 pubertal crossbred Landrace gilts with an average age of 170 days and weight of 98 kg were used in this study. The animals were bred under the same conditions and the experiments were approved by the local Ethics Committee.

Collection of porcine ovaries and cumulus-oocyte-complexes

The ovaries and reproductive tracts were recovered after slaughtering and transporting to the laboratory within 10 min at 38°C in 0.9% NaCl. To optimize *in vitro* fertilization and improve conditions for oocyte maturation, the ovaries of each animal were placed in 5% fetal bovine serum solution (FBS; Sigma–Aldrich Co.) in PBS. Thereafter, COCs were obtained via single large follicles (>5 mm), which were opened by puncturing a 5-mL syringe and 20-G needle in a sterile petridish. The COCs were washed three times in modified PBS supplemented with 36 µg/mL pyruvate, 50 µg/mL gentamycine and 0.5 mg/mL BSA (Sigma–Aldrich). COCs were selected under an inverted microscope Zeiss Axiovert 35 (Lübeck, Germany), counted and morphologically evaluated using the scale suggested by Jackowska and coworkers (Jackowska *et al.* 2009). Only COCs of grade I with homogeneous ooplasm and uniform and compact CCs were used in the experiment. A total of 300 grade I oocytes ($3 \times n = 50$ before IVM group, $3 \times n = 50$ after IVM group) were obtained.

Assessment of oocyte developmental competence by BCB test

To run the BCB staining test, oocytes were washed twice in modified Dulbecco PBS (DPBS) (SigmaAldrich) supplemented with 50 IU/mL penicillin, 50 µg/mL streptomycin (SigmaAldrich), 0.4% (w/v) BSA, 0.34 mM pyruvate, and 5.5 mM glucose (DPBSm). Thereafter, they were treated with 13 µM BCB (SigmaAldrich) diluted in DPBSm at 38.5°C, 5% CO_2 in air for 90 min. After treatment, the oocytes were transferred to DPBSm and washed twice. After washing, the oocytes were examined under an inverted microscope and classified as either stained blue (BCB^+) or colorless (BCB^-). Only the granulosa cell-free BCB^+ oocytes were used for subsequent molecular analysis (before IVM) or IVM followed by second BCB test and molecular analysis (after IVM group).

In vitro maturation of porcine COCs

After the first BCB test, the COCs that remained colorless (BCB^+) were subjected to IVM (Marques *et al*. 2007). The COCs were cultured in NunclonΔ 4-well dishes in 500 µL standard porcine IVM culture medium, TCM-199 (tissue culture medium) with Earle's salts and *L*-glutamine, (Gibco BRL Life Technologies) supplemented with 2.2 mg/mL sodium bicarbonate (Nacalai Tesque, Inc., Kyoto, Japan), 0.1 mg/mL sodium pyuryvate (SigmaAldrich), 10 mg/mL bovine serum albumin (BSA), (SigmaAldrich), 0.1 mg/mL cysteine SigmaAldrich, 10% (v/v) filtered porcine follicular fluid and gonadotropin supplements at final concentrations of 2.5 IU/mL hCG (Ayerst Laboratories, Inc., Philadelphia, PA, USA) and 2.5 IU/mL eCG (Intervet, Whitby, ON, Canada). Wells were covered with mineral oil overlay and cultured at 38°C under 5% CO_2 in air for 22 h, and then for additional 22 h in medium without hormones. After cultivation, another BCB staining test was performed and only the granulosa-cell-free BCB^+ oocytes were used for subsequent molecular analysis.

RNA extraction from porcine oocytes

Total RNA was extracted from samples using Tri Reagent (Sigma), and RNeasy MinElute cleanup Kit (Qiagen). The amount of total mRNA was determined using optical density at 260 nm, and the RNA purity was estimated using the 260/280 nm absorption ratio (higher than 1.8) (NanoDrop

spectrophotometer, Thermo Scientific). The RNA integrity and quality were checked on a Bioanalyzer 2100 (Agilent Technologies, Inc.). The resulting RNA integrity numbers (RINs) were between 8.5 and 10 with an average of 9.2 (Agilent Technologies, Inc.). The RNA in each sample was diluted to a concentration of 100 ng/μL with an OD260/OD280 ratio of 1.8/2.0. From each RNA sample, 500 ng of RNA were taken. The remaining amount of isolated RNA were used for qRT-PCR study.

Microarray expression analysis and statistics

The Affimetrix procedure was performed according to the manufacturer protocol. Briefly cDNA were obtained from total RNA (100 ng) (Ambion WT Expression Kit), biotin-labeled and fragmented by Affymetrix GeneChip WT Terminal Labeling and Hybridization (Affymetrix). Biotin-labeled fragments of cDNA (5.5 μg) were hybridized to Affymetrix Porcine Gene 1.1 ST Array Strip (48°C/20 h). Then, microarrays were washed and stained according to the technical protocol using Affymetrix GeneAtlas Fluidics Station. Subsequently, the array strips were scanned by Imaging Station of GeneAtlas System. The preliminary analysis of the scanned chips was performed using Affymetrix GeneAtlasTM Operating Software. The quality of gene expression data was checked according to quality control criteria provided by the software. Obtained CEL files were imported into downstream data analysis software. All of presented analyses and graphs were performed by Bioconductor and R programming language. Each CEL file was merged with a description file. Robust Multiarray Averaging (RMA) algorithm was used to correct background, normalize and summarize results.

Statistical significance of analyzed genes was performed by moderated *t*-statistics from the empirical Bayes method. Obtained *P* value was corrected for multiple comparisons using the Benjamini and Hochberg's false discovery rate. The selection of significantly changed gene expression was based on *P* value beneath 0.05 and expression fold higher than 2. Differentially expressed genes were subjected to the selection of genes associated with cellular component of morphogenesis. Differentially expressed gene list (separately for up and downregulated) were uploaded to the Database for Annotation, Visualization and Integrated Discovery (DAVID) software (Huang *et al.* 2009), where differentially expressed genes belonging to the cellular components morphogenesis (GO:0032989) were obtained. GO term 'cellular components morphogenesis' is defined as 'the process in which cellular structures, including whole cells or cell parts, are generated and organized'. It consists of 1166 genes annotated to the *Sus scrofa* organism. Expression data of these genes were subjected to hierarchical clusterization procedure and presented as a heatmap graph.

Additionally, whole gene expression data acquired in microarray experiment was subjected for Gene set enrichment analysis (GSEA). GSEA were performed using the GSEA 2.2.2 software by Broad Institute (Mootha *et al.* 2003, Subramanian *et al.* 2005). For the GSEA analysis, the c5.bp.v5.1.symbols.gmt (Gene ontology) database was used.

Real-time quantitative polymerase chain reaction qRT-PCR analysis

Total RNA was isolated from oocytes before and after IVM. The RNA samples were re-suspended in 20 μL of RNase-free water and stored in liquid nitrogen. RNA samples were treated with DNase I and reverse-transcribed (RT) into cDNA. qRT-PCR was conducted in a LightCycler real-time PCR detection system (Roche Diagnostics GmbH, Mannheim, Germany) using SYBR Green I as a detection dye and target cDNA was quantified using the relative quantification method. The relative abundance of *CD9*, *APP*, *ITGB1*, *SLITRK3*, *RYK*, *UBE2B*, *ROBO2*, *CTNNA2*, *SEMA5A*, *TPM1*, *TGFBR3*, *LAMB2*, *GJA1*, *WWTR1*, *EGR2*, *MAP1B*, *DAB*, *FN1*, *SOX9*, *CXCL12* and *ARHGEF2* transcripts in each sample was standardized to the internal standard glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). For amplification, 2 μL of cDNA solution was added to 18 μL of QuantiTect SYBR Green PCR (Master Mix Qiagen GmbH, Hilden, Germany) and primers (Table 1). One RNA sample of each preparation was processed without the RT-reaction to provide a negative control for subsequent PCR.

To quantify specific genes in the oocyte, expression levels of specific oocyte mRNAs were calculated relative to *PBGD* and *ACTB*. To ensure the integrity of these results, the additional housekeeping gene *18S RRNA* was used as an internal standard to demonstrate that *PBGD* and *ACTB* mRNAs were not differentially regulated in the groups of oocytes. The *18S RRNA* expression was identified as an appropriate housekeeping gene for the use in quantitative PCR studies. Expression of *PBGD*, *ACTB* and *18S RRNA* mRNAs was measured in samples from isolated oocytes.

Results

Microarray technology allowed us to investigate oocyte transcriptome changes after *in vitro* maturation (after IVM; resulting in 78% of mature oocytes based on first polar body extrusion) in relation to transcriptome profile of freshly isolated oocyte, before *in vitro* maturation (IVM). Previously, whole gene expression analysis by Affymetrix Porcine Gene 1.1 ST Array was performed and the expression profile of 12258 porcine transcripts was examined. The genes for which the fold change was higher than the cut-off value (fold>|2|) and corrected *P* value (adj *P*) <0.05, were considered as differentially expressed. The set of differentially expressed genes were used as query for subsequent detailed analysis.

The DAVID software was used for the extraction of genes belonging to cellular components morphogenesis. Up and downregulated gene sets were subjected to DAVID, searched separately and only gene sets where adj. *P* value lower than 0.05 were selected. We found that 21 genes from 'cellular components morphogenesis' Gene Ontology Biological Process (GO BP) were significantly represented in the downregulated gene set. This set of genes were subjected to hierarchical clusterization procedure and presented as heatmap (Fig. 1). The set of

Table 1 Oligonucleotide sequences used for qRT-PCR analysis.

Transcript	**Sequence** (5′–3′ direction)	**Gene accession no.**	**Product size** (bp)
CD9	CAAAGGGACGTACTCTCAAGC GACCCCGAGAAGATGACCAA	NM_214006.1	249
APP	TGGGGAAAGACACAAACCCTT CATGCACTAGTTTGATACAGCTT	NM_214372	206
ITGB1	CTGGTTCTACTTCACATACTCGG TGGCAAATTCCCTTCTGTCG	NM_213968.1	197
SLITRK3	GTTTCGTATACTGCGTCCT GAGGCTTTCTTTAACTTCACA	XM_005657095	248
RYK	ATCCACAAAGACCTGGCTGCT TGTCACTCCGAAGGCCCACA	XM_013982031	210
UBE2B	ACCGCCAACTGTTAGGTTT TTCAACAATGGCCGAAACTCT	NM_001257356.1	241
ROBO2	GGAACAGCTTCTTCTAAGGGA ATAAAGAAATTGTTCATTGCACT	XM_013982523.1	238
CTNNA2	CCTACCTTCAACGGATTGC CCTTCTGATACTTTGTTGAGGC	XM_013995995.1	204
SEMA5A	AACACCAGCATAACCAACCAC AACTGGGGAATTACAAGAAGC	XM_013984924.1	221
TPM1	GACTGGCAACAGCTTTACAGA CACCTCTTCATACTTGCGGTC	NM_001097483.2	205
TGFBR3	TGATCCACCATGAAGTGCAGT TGCCTTCCTGCGCTGTCTC	NM_214272.1	190
LAMB2	GCTGCCCAAGGATGACCACA TCCTCCTGTTCGCACTAGCTT	XM_013981664.1	130
GJA1	CAGCACTTTTCTTTCATTAGGGG CTAAGGCACTCCAGTCACC	NM_001244212.1	194
WWTR1	TGCTACAGTGTCCCCACGA CTCGCTTTTGTTCAGAGCAG	XM_003132481.4	225
EGP2	ACCATCTTTCCCAATGCCGAAC ACTTGCCCATGTAAGTGAAGGTC	NM_001097488.1	178
MAP1B	TGGACCTGTGCTATATTCCCA CCTCATCACCTCCGAGTCA	XM_003134032.4	218
DAB2	ATCAGACATCTTTGCTCCTCC CTAAAGACGCTGGGTTCCAT	XM_003133906.5	191
FN1	GAATATCTCGGTGCCATTTGCT ACTCTCGGGAATCTTCTATGTCA	XM_013984465.1	229
SOX9	AGCAGACGCACATCTCTCCCA CGCCCCTCTCGCTTCAGGTCA	NM_213843.1	190
CXCL12	GCCAGAGCCAACATCAAGCA AAACATCCCGCCGTCCTCA	NM_001009580	250
ARHGEF2	TTGCCTGTGACTATTCGCTCA CTACAGGCTCCCCGTCTCG	NM_001128463.1	220
PBGD	GAGAGTGCCCCTATGATGCT ATGATGGCACTGAACTCCT	NM_001097412.1	214
B-ACTIN	GGGAGATCGTGCGGGACAT CGTTGCCGATGGTGATGAC	DQ845171	141
18S rRNA	GTGAAACTGCGAATGGCTC CCGTCGGCATGTATTAGCT	AB117609	105

the differentially expressed genes belonging to ‘cellular components morphogenesis’ GO BP term category was also presented as dot plots where normalized log expression values (log2 signal intensity), gene symbols, fold changes and corrected *P* values were shown (Table 2). The GSEA showed that a higher percentage of gene sets are enriched in oocytes before IVM than after this procedure (Fig. 2).

Subsequently, the set of differentially expressed genes from ‘cellular components morphogenesis’ GO BP term category, were applied to the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) software for interaction predictions. STRING is a huge database that contains information of protein/gene interactions, including experimental data, computational prediction methods and public text collections. Using such prediction methods provided the molecular interaction network formed between genes of interest. Only two strong interactions were observed between *VEGFA* and *AR* and between *VEGFA* and *INSR*. (Fig. 3).

It is obvious that genes formed in one particular GO group will also belong to other GO term categories. We performed functional enrichments of GO terms based on previously uploaded gene set from ‘cellular components morphogenesis’ GO BP term. The top ten GO terms which genes belonged to ‘cellular components

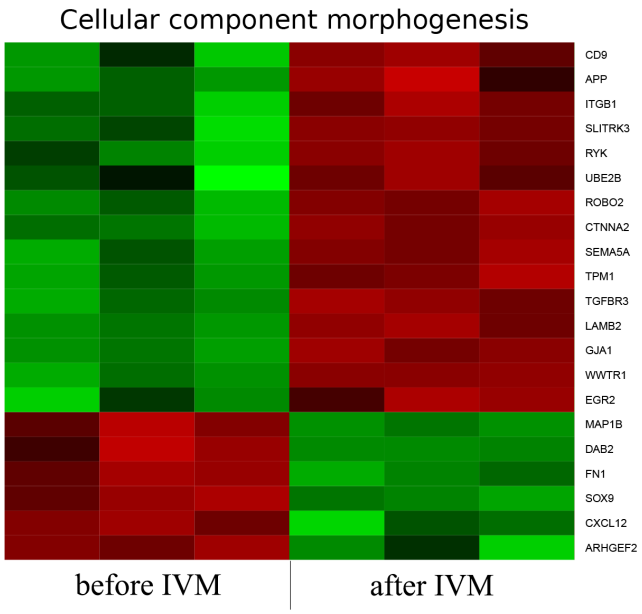

Figure 1 Heat map representation of differentially expressed genes belonging to the 'regulation of cell migration' – functional category from the DAVID GEOTERM BP database. Arbitrary signal intensity acquired from microarray analysis is represented by colors (green, higher; red, lower expression). Log2 signal intensity values for any single gene were resized to Row *Z*-Score scale (from −2, the lowest expression to +2, the highest expression for single gene).

morphogenesis' GO BP are shown in Table 3. Some of the GO BP terms were very similar to 'anatomical structure development', 'anatomical structure morphogenesis', 'cellular component organization', 'developmental process', 'cell morphogenesis', 'cell adhesion', 'biological adhesion', 'cellular developmental process', 'cellular process' and 'system development'.

qRT-PCR analysis was performed to validate microarray results, using both the same RNA samples used for PCR and microarray profiling experiments. The result from the qRT-PCR revealed increased expression of *CD9*, *APP*, *ITGB1*, *SLITRK3*, *RYK*, *UBE2B*, *ROBO2*, *CTNNA2*, *SEMA5A*, *TPM1*, *TGFBR3*, *LAMB2*, *GJA1*, *WWTR1*, *EGR2*, *MAP1B*, *DAB2*, *FN1*, *SOX9*, *CXCL12* and *ARHGEF2* in porcine oocytes before IVM as compared to analysis after IVM. The qRT-PCR assay confirmed the fold change and significance of microarray expression profiling (Fig. 4).

Table 2 Fold changes and adjusted *P* values of differentially expressed genes.

Gene	Description	Fold change	*P* value
ARHGEF2	Rho/Rac guanine nucleotide exchange factor (GEF) 2	2.6561999	0.0064318044
CXCL12	chemokine (C-X-C motif) ligand 12	3.9347058	0.0031636321
SOX9	SRY (sex determining region Y)-box 9	3.5033402	0.0006200082
FN1	fibronectin 1	2.6795807	0.0012103032
DAB2	disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)	4.0082082	0.0019126886
MAP1B	–	2.7885541	0.0010538499
EGR2	early growth response 2	0.1655038	0.0079498607
WWTR1	WW domain containing transcription regulator 1	0.3272021	0.0002540251
GJA1	gap junction protein, alpha 1.43kDa	0.2069073	0.0001076763
LAMB2	–	0.2788579	0.0001879115
TGFBR3	transforming growth factor, beta receptor III	0.1965222	0.0004059785
TPM1	tropomyosin 1 (alpha)	0.4339631	0.0016327416
SEMA5A	sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5A	0.3533917	0.0010923962
CTNNA2	–	0.2299252	0.0005121808
ROBO2	–	0.2837489	0.0011834948
UBE2B	ubiquitin-conjugating enzyme E2B	0.3827797	0.0411046593
RYK	receptor-like tyrosine kinase	0.4219030	0.0043998898
SLITRK3	SLIT and NTRK-like family, member 3	0.2391419	0.0042609506
ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	0.3662330	0.0037052151
APP	amyloid beta (A4) precursor protein	0.3241386	0.0056023228
CD9	CD9 molecule	0.3292831	0.0063323867

Discussion

In both *in vivo* oviductal and *in vitro,* COCs maturation is accompanied by substantial morphological and biochemical modifications. In immature and mature mammalian gametes the transcriptomic profile of genes upregulated and downregulated during COCs growth and development differed significantly. Genes expressed in oocytes of MII stage at high levels were determined to be involved in the cellular components of morphogenesis. Genes that regulated the process produced proteins employed in nuclear reorganization, chromatin configuration of MII stage and cellular organelle architecture. Currently, nuclear modification at the molecular level is well understood in meiosis when gametes are formed. However, nuclear modifications that contribute to the reorganization of organelles during

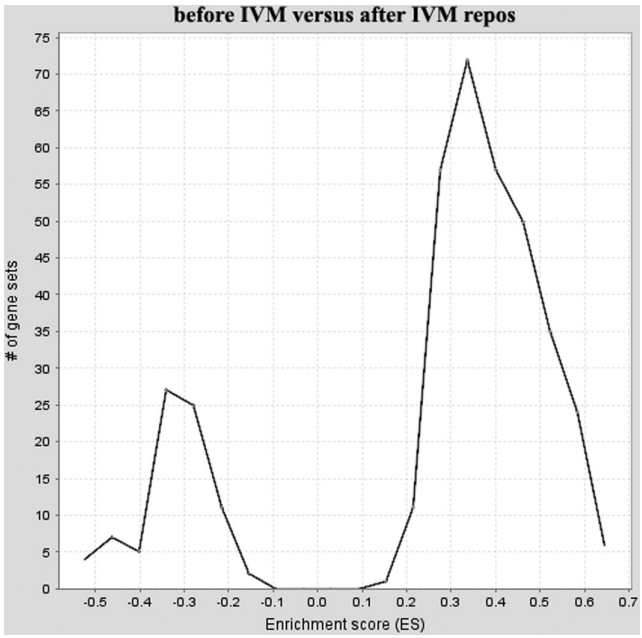

Figure 2 The global Enrichment Set histogram. Plot represents the % amount of gene sets that expressions are enriched in oocytes before in comparison to oocytes after *in vitro* maturation.

mammalian oocyte maturation both *in vivo* and *in vitro* still needs to be explored.

In this study, we analyzed the transcriptomic profile of porcine BCB+ oocytes before and after IVM

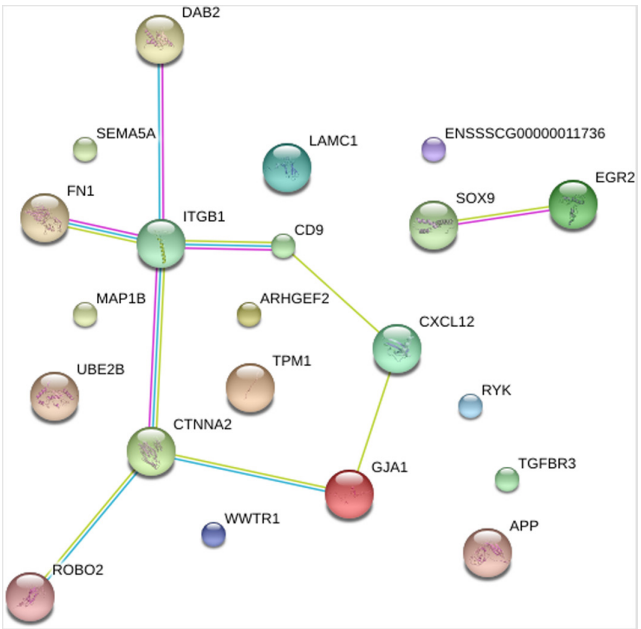

Figure 3 STRING-generated interaction network among differentially expressed genes belonging to the 'cellular component morphogenesis' ontology group. The intensity of the edges reflects the strength of interaction score. Applied prediction methods: textmining, co-expression, experimentally observed interactions.

Table 3 Top ten GO categories formed by genes differentially expressed belonging to the 'cellular component morphogenesis' ontology group.

Category	Term	Count	*P* value
GOTERM_BP_ALL	anatomical structure development	4	2.4E−3
GOTERM_BP_ALL	anatomical structure morphogenesis	3	3.4E−3
GOTERM_BP_ALL	cellular component organization	4	6.8E−3
GOTERM_BP_ALL	developmental process	4	1.2E−2
GOTERM_BP_ALL	cell morphogenesis	2	2.0E−2
GOTERM_BP_ALL	cell adhesion	3	2.6E−2
GOTERM_BP_ALL	biological adhesion	3	2.6E−2
GOTERM_BP_ALL	cellular developmental process	3	2.9E−2
GOTERM_BP_ALL	cellular process	8	2.9E−2
GOTERM_BP_ALL	system development	3	3.0E−2

GO categories were generated in STRING software. GO ID (pathway ID), GO term description (pathway description), number of the genes belonging to appropriate category (count in gene set) are shown.

to find genes responsible for pig oocyte maturation and the cellular components of morphogenesis. We implemented BCB test, since we were interested in analyzing *in vitro* system influence on transcriptomic profile of the same, good developmental competence

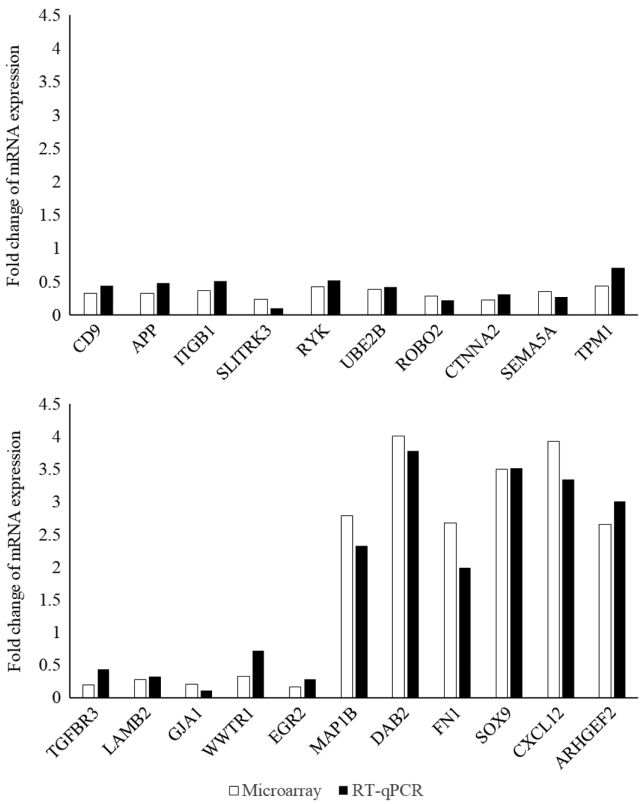

Figure 4 Comparison of gene expression fold changes in before IVM vs after IVM groups of oocytes using microarray assay and qRT-PCR. qRT-PCR analysis was normalized to the expression of three housekeeping genes (*PBGD*, β-*ACTIN*, *18S RRNA*).

oocytes. This allowed us to hypothesize that changes in genes expression were caused by IVM by its own, and not by different competency of oocytes. We found 21 differentially expressed genes potentially involved in gamete maturation and identified transcripts with possible new function. Six transcripts, such as: *MAP1B*, *ARHGEF2, DAB2*, *FN1*, *SOX9* and *CXCL12* increased their expression after IVM. We will discuss these genes first and then move on to the downregulated genes.

As the first component of cellular morphogenesis we will discuss microtubules and their role in cellular organization. Meiosis is a process where microtubules are actively involved in arranging chromosomes. Luteinizing hormone (LH) surge triggers the resumption of meiosis in oocytes, resulting in germinal vesicle breakdown (GVBD). Microtubules play a critical role in the oocyte's maturation process, since in metaphase I (MI) they form the meiotic spindle, enabling homologous chromosomes to segregate and bring about the emission of the first polar body. Then, in metaphase II, the unique organization of the meiotic spindle designates mature MII oocytes (Wang *et al.* 2008). Among our studied genes, we found two genes that may potentially be related to microtubule organization during the oocytes' maturation.

The first one, microtubule-associated protein 1B (*MAP1B*) could play a role in oocyte maturation by possibly being involved in microtubule reorganization. From previous research, we know MAP1B has a role in cellular development. It was shown to be involved in the brain development and neuronal plasticity during the neural system growth and cellular specification. The neuronal polarity was acquired, thanks to MAP1B interaction with neuronal cytoskeleton elements such as microtubules and actin filaments (Villaroel-Campos *et al.* 2014). Although, MAP1B is mainly known for its function as a neuronal protein (Gödel *et al.* 2015), a higher mRNA expression was found in human and murine podocytes in kidney, suggesting MAP1B can serve as a valuable marker protein in other cells types. Similarly, we observed an increase in *MAP1B* mRNA level in porcine oocytes after IVM compared to oocytes analyzed before IVM. To our knowledge, there is no information about the function of MAP1B protein in pig oocytes. Thus, we can only speculate that, since approximately 60% of pig oocytes after IVM reached MII stage (Marques *et al.* 2007), *MAP1B* gene may be involved in microtubule reorganization during cellular division, leading to the MII stage.

The second gene, Rho guanine nucleotide exchange factor (GEFH1), encoded by *ARHGEF2*, could be responsible for the proper formation of microtubules and used as an indicator of oocyte maturation. GEFH1 is an enzyme that cycle switches between GTP bound active state and GDP bound inactive state (Jaffe & Hall 2005). The Rho GTPases are involved in the organization of the cellular cytoskeleton and morphology, cellular migration, survival and proliferation (Aznar *et al.* 2004). ARHGEF2 is an exchange factor with specific activity for RhoA (Ren *et al.* 1998), which regulates microtubule stability and actin cytoskeleton organization during cell growth and proliferation (Krendel *et al.* 2002). Although the role of ARHGEF2 in intra- and extracellular matrix morphology was well recognized mostly in the cancer cell model, its role in regulation of Rho-signaling pathways during porcine oocyte maturation is still not entirely known. Zhong and coworkers (2005) and Zhang and coworkers (2014) indicated that RhoA plays a crucial role during mouse and porcine oocyte maturation since it promotes actin assembly, spindle formation and subsequent contractile ring formation during polar body emission. Several studies even point to RhoA contribution in early embryo division and regulation of actomyosin contractility (Bement *et al.* 2005). Thus, our observation of increased expression of *ARHGEF2* mRNA in porcine oocytes after IVM as compared to before IVM was not surprising. We can assume that higher *ARHGEF2* expression correlates with higher RhoA activity, and thus may be considered as a marker of proper formation of cellular microtubular components in a mature porcine oocyte.

Three other genes possibly responsible for changes in oocyte morphology during the maturation are the clathrin adaptor protein (*DAB2*), fibronectin (*FN1*) and SRY-Box 9 (*SOX9*). In other tissues, *DAB2*, *FN1* and *SOX9* genes are involved in the regulation of processes such as adhesion (Chao & Kunz 2009) and migration of cells in the basement membrane of connective tissues (Benito-Jardon *et al.* 2017) and transcription activation of sex-differentiation (Gonen *et al.* 2017), skeletal development and chondrogenesis (Li *et al.* 2016, Montero *et al.* 2017). Our previous experiments (unpublished data) identified *DAB2*, *FN1* and *SOX9* transcripts as differentially expressed during cell migration, proliferation and bone morphogenesis. In accordance with this trend, all three genes, in the current experiment, displayed increased expression in porcine oocytes after IVM as compared to gametes analyzed before IVM. Additionally, the current experiment seems to confirm previous observations by other authors (Gumbiner *et al.* 2005, Carroll *et al.* 2007, Rozario *et al.* 2009, 2010, Weber *et al.* 2011, Basson 2012), suggesting that processes of proliferation, migration and adhesive ability, which are associated with cell survival, share a similar genetic regulation mechanism to cellular morphogenesis in several types of somatic cells. Results from the current study indicate that these genes may influence changes in oocyte's morphology during formation of fully mature MII stage gametes.

Another possible indicator of a mature oocyte is the expression of stromal cell-derived factor (*SDF1*) gene due to its possible role in providing fertilization capability to a mature oocyte. CXCL12, also called SDF1, was identified as a chemokine (C-X-C motif)

ligand for chemokine (C-X-C motif) receptor 4 (CXCR4), which is responsible for chemotaxis of several cells types such as leukocytes (Bleul *et al.* 1996, Li *et al.* 2017), hematopoietic progenitor cells (Jin *et al.* 2006) and even primordial germ cells (PGCs). Previous research shows that the SDF1 – CXCR4 signaling axis was involved in the migration of PGCs through embryonic tissues to the gonads of the neonatal mouse, and its expression increased along with the developmental stage of the follicles (Holt *et al.* 2006). Furthermore, CXCL12 present in follicular fluid acts synergistically with vascular endothelial growth factor (VEGF), supporting angiogenesis and follicular vascularization. As a result, luteinization, follicle growth and the development of the CL *in vivo* are enhanced (Nishigaki *et al.* 2013). Finally, Zuccarello and coworkers (2011) found that human pre-ovulatory oocytes express *SDF1* mRNA and protein, and that SDF1 plays a role as a chemoattractant in spermatozoa guidance mechanism. Taking this into consideration and our data showing increased *SDF1* expression after IVM, which indirectly supports previous observations, the higher *SDF1* expression points to it playing some role in fertilization-ready mature oocytes.

The genes downregulated in the oocyte after IVM are discussed here. According to the data, the majority of differentially expressed transcripts in the oocytes were downregulated after IVM compared to before IVM. Some downregulated genes correlated with the achievement of porcine oocytes MII stage and were directly or indirectly associated with oocytes before maturation.

One of these downregulated genes, the cell-surface molecule CD9, a member of the transmembrane-4 tetraspanin superfamily, is a molecule that usually interacts with membrane proteins, such as integrins and most likely participates in cellular migration and adhesion. In humans and mice, CD9 was detected on the oocyte's membrane (Ziyyat *et al.* 2006), both in the ovary and isolated from the oviduct (Chen *et al.* 1999). Experiments with an anti-CD9 monoclonal antibody and using CD9 (−/−) knockout mice, resulted in inhibition of sperm-oocyte surface interactions, severe reduction of fertility and abnormal microvillar structure on the oocyte's surface (Runge *et al.* 2007). Therefore, it was concluded that CD9 appears to be essential for sperm-oocyte fusion, a process involving the integrin α6/β1 on the oocyte plasma membrane, acting as a receptor for sperm protein fertilin β (ADAM2). Surprisingly, in our study, porcine oocytes after IVM, which should be ready for fertilization, had decreased levels of *CD9* mRNA compared to that before IVM. This result was similar to oocytes after vitrification/refreezing where CD9 distribution pattern had changed (Zhou *et al.* 2013), although our porcine oocytes were of high quality non-vetrified cells. This discrepancy may indicate the essential impact of IVM procedures on oocytes potential for sperm binding. Another explanation can be that apart from CD9 there are other proteins, which have a complementary role in the gametes fusion process. Experiments with knockout mice showed that fertilizability of *CD9* (−/−) female can be partially abolished by expression of CD81 (Kaji *et al.* 2002). This can to some extent explain that CD9 downregulation does not necessarily imply impaired maturational and fertilization potential in porcine oocytes.

Another gene that could be involved in changes to oocyte morphology is laminin beta 2 (*LAMB2*). The protein LAMB2, a member of the laminins family, is typically involved in cell adhesion, migration and differentiation. These extracellular matrix glycoproteins are a major component of the basement membrane. Moreover, during the embryonic development this molecule is thought to mediate migration and the organization of cells into specific tissues. There is no information in the literature regarding the role of LAMB2 in oocyte maturation or oocyte-spermatozoa interaction. However, Nguyen and coworkers (2012) tested a hypothesis that suggests granulosa cells in the ovarian follicles are morphologically and functionally different, depending on their location either being adjacent to the follicular fluid lumen or aligning the basal lamina. In the study, bovine follicles had lower *LAMB2* gene expression levels in apical granulosa cells compared to that in basal cells, suggesting that cellular distribution is a crucial factor of *LAMB2* expression (Nguyen *et al.* 2012). Our study on porcine oocytes showed downregulated expression of the *LAMB2* gene after IVM. It can be supposed that the oocyte surrounded by the cumulus oophorus, which is comprised of apical granulosa cells, can have lower *LAMB2* expression due to its close proximity. Additionally, *LAMB2* expression can be diminished even further after IVM, due to oocytes being deprived of contact with the basal lamina and other surrounding cells, such as the apical granulosa cells. Although to what extent and how *LAMB2* affects oocyte morphology is not yet known and requires further research.

Another possible marker of oocyte maturation status is the expression levels of gap junction protein alpha 1 gene (*GJA1*). GJA1 protein is involved in intracellular communication via cell-cell contact, facilitating passive diffusion of small molecules, i.e.: ions, metabolites and nutrients. Bidirectional intracellular communication between the oocyte and CCs is crucial for cumulus expansion and successful oocyte maturation (Kempisty *et al.* 2014*a*,*b*). GJA1 is a member of the connexin gene family and because of its ability to rescue blocked antral follicles development (Carabatsos *et al.* 2000, Li *et al.* 2007), it was proposed as a new gene marker for oocyte maturational capability (Wang *et al.* 2009). Li and coworkers (2015) showed that CCs of mature oocytes expressed significantly lower levels of *GJA1* mRNA in comparison to immature oocytes (Li *et al.* 2015). Additionally, Shao and coworkers (2015), analyzing mouse CCs from germinal vesicle

and MII stages found that *GJA1* was downregulated after IVM (Shao *et al.* 2015). Similarly, we found downregulation of *GJA1* mRNA level in pig oocytes after IVM compared to before IVM, which is in accordance with results of previously described CCs. Since GJA1 controls the exchange of cAMP and cGMP between CCs and oocyte (Richard & Baltz 2014), downregulation of *GJA1* most likely results in their diminished diffusion and concentration in oocytes, which is necessary for resumption of meiosis to MII stage and extrusion of the first polar body (Sanchez & Smitz 2012). Thus, we may speculate that *GJA1* lowered expression in oocytes after IVM is a candidate biomarker of oocyte's maturational status. Although, COCs IVM is considerably improved nowadays, the concentration of fully grown matured cells is still unsatisfactory. Studies by Arias-Alvarez and coworkers (2016), confirmed this observation, since they found more reduced expression of *GJA1* gene in *in vivo*-matured oocytes compared to *in vitro*-matured oocytes (Arias-Alvarez *et al.* 2016).

Apart from genes which can be directly or indirectly correlated with oocyte maturation as well as fertilization feasibility, we analyzed a large set of genes with established functions outside of these processes. All of them presented diminished mRNA expression level after IVM. The first group consisted of genes encoding proteins whose activity and function were found in the nervous system and its related processes such as neurite growth, plasticity development and axonal navigation. Aamyloid beta precursor protein (APP) is a protein mainly related to the formation of amyloid plaques in the brain of patients with Alzheimer's disease (Borel *et al.* 2017), however, contribution to oocytes maturation cannot be excluded, especially since Khan and coworkers (2016), presented the correlation between different schemes of FSH superstimulation and APP expression levels in oocytes of cows, concluding that together with other genes APP could be a potential biomarker of follicle differentiation and predictor of oocyte competence (Khan *et al.* 2016).

Similar to *APP*, *SLITRK3* (SLIT And NTRK-Like Family Member 3) (Wang *et al.* 2015), *ROBO2* (Roundabout Guidance Receptor 2) (Kidd *et al.* 1998), *SEMA5A* (Semaphorin A) (Purohit *et al.* 2014), *RYK* (receptor-like tyrosine kinase) (Onishi *et al.* 2014), *CTNNA2* (Catenin Alpha 2) (Vite *et al.* 2015) and *EGR2* (Early Growth Response 2) (Sevilla *et al.* 2015) were predominantly associated with nervous system processes. But, their role in oocyte maturation and morphogenesis is unknown and needs to be studied further.

The secondary group of genes could not be assigned to any specific system or mechanism of function. All exert broad spectrum of actions but have never been associated with an oocyte and folliculogenesis like: UBE2B (Ubiquitin-Conjugating Enzyme E2 B), that targets damaged or abnormal proteins for destruction by modification with ubiquitin (Polge *et al.* 2016). TPM1 (Tropomyosin 1 Alpha) that provides cell stability and contractility of smooth and striated muscle (Sewanan *et al.* 2016). TGFBR3 (Transforming Growth Factor Beta Receptor 3) a membrane-bound receptor that acts as a co-receptor with other receptors for TGF-β cytokine (Villarreal *et al.* 2016). And finally, WWTR1 (WW Domain Containing Transcription Regulator 1), which is related to embryonic stem cells self-renewal, organ size control and tumor suppression (Zhou & Lei 2016).

All mentioned and analyzed genes need to be investigated further to determine their potential as biomarkers for oocyte maturation and fertilization capability. This includes genes with protein products involved in body functions other than oocyte maturation and fertilization capability, but whose qualities show potential in their involvement. Moreover, these genes may be characterized as molecules of new function in regulatory processes of porcine morphogenesis and organogenesis with special attention to development and their regulatory pathways. They may be recognized not only as new markers of oogenesis but as markers of regulatory processes such as embryogenesis, fetal growth and their compound developmental systems.

Declaration of interest

The authors declare that they have no conflict of interest.

Funding

Publication of this article was made possible by grant number 2014/13/D/NZ9/04798 'SONATA' and UMO-2011/03/N/NZ4/00305 'PRELUDIUM' from the Polish National Science Centre.

References

Arias-Alvarez M, Garcia-Garcia RM, Lopez-Tello J, Rebollar PG, Gutierrez-Adan A & Lorenzo PL 2016 In vivo and in vitro maturation of rabbit oocytes differently affects the gene expression profile, mitochondrial distribution, apoptosis and early embryo development. *Reproduction, Fertility, and Development*. Epub (doi:10.1071/RD15553)

Aznar S, Fernandez-Valeron P, Espina C & Lacal JC 2004 Rho GTPases: potential candidates for anticancer therapy. *Cancer Letters* **206** 181–91. (doi:10.1016/j.canlet.2003.08.035)

Basson MA 2012 Signaling in cell differentiation and morphogenesis. *Cold Spring Harbor Perspectives in Biology* **4** a008151. (doi:10.1101/cshperpect.a008151)

Bement WM, Benink HA & von Dassow G 2005 A microtubule-dependent zone of active RhoA during cleavage plane specification. *Journal of Cell Biology* **170** 91–101. (doi:10.1083/jcb.200501131)

Benito-Jardon M, Klapproth S, Gimeno-LLuch I, Petzold T, Bharadwaj M, Muller DJ, Zuchtriegel G, Reichel CA & Costell M 2017 The fibronectin synergy site re-enforces cell adhesion and mediates a crosstalk between integrin classes. *eLife* **6** e22264. (doi:10.7554/eLife.22264)

Bleul CC, Fuhlbrigge RC, Casasnovas JM, Aiuti A & Springer TA 1996 A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). *Journal of Experimental Medicine* **184** 1101–1109. (doi:10.1084/jem.184.3.1101)

Borel F, Marzocca F, Delcros JG, Rama N, Mehlen P & Ferrel JL 2017 Molecular characterization of Netrin-1 and APP receprot binding: New

leads to block the progression of senile plaques in Alzheimer's disease. *Biochemical and Biophysical Research Communications* **488** 466–470. (doi:10.1016/j.bbrc.2017.05.056)

Carabatsos MJ, Sellitto C, Goodenough DA & Albertini DF 2000 Oocyte-granulosa cell heterologous gap junctions are required for the coordination of nuclear and cytoplasmic meiotic competence. *Developmental Biology* **226** 167–179. (doi:10.1006/dbio.2000.9863)

Carroll DK, Brugge JS & Attardi LD 2007 p63, cell adhesion and survival. *Cell Cycle* **6** 255–261. (doi:10.4161/cc.6.3.3799)

Chao WT & Kunz J 2009 Focal adhesion disassembly requires clathrin-dependent endocytosis of integrins. *FEBS Letters* **583** 1337–1343. (doi:10.1016/j.febslet.2009.03.037)

Chen MS, Tung KS, Coonrod SA, Takahashi Y, Bigler D, Chang A, Yamashita Y, Kincade PW, Herr JC & White JM 1999 Role of the integrin-associated protein CD9 in binding between sperm ADAM 2 and the egg integrin alpha6beta1: implications for murine fertilization. *PNAS* **96** 11830–11835. (doi:10.1073/pnas.96.21.11830)

Dunlop CE & Anderson RA 2014 The regulation and assessment of follicular growth. *Scandinavian Journal of Clinical and Laboratory Investigation Supplementum* **244** 13–17. (doi:10.3109/00365513.2014.936674)

Eppig JJ, Wigglesworth K & Pendola FL 2002 The mammalian oocyte orchestrates the rate of ovarian follicular development. *PNAS* **99** 2890–2894. (doi:10.1073/pnas.052658699)

Ericsson SA, Boice ML, Funahashi H & Day BN 1993 Assessment of porcine oocytes using brilliant cresyl blue. *Theriogenology* **39** 214. (doi:10.1016/0093-691X(93)90069-H)

Galeati G, Giaretta E, Zannoni A, Bucci D, Tamanini C, Forni M & Spinaci M 2016 Embelin supplementation of in vitro maturation medium does not influence nuclear and cytoplasmic maturation of pig oocytes. *Journal of Physiology and Pharmacology* **67** 513–519.

Gonen N, Quinn A, O'Neill HC, Koopman P & Lovell-Badge R 2017 Normal levels of Sox9 expression in the developing mouse testis depend on the TES/TESCO enancer, but this does not act alone. *PLoS Genetics* **13** e1006520. (doi:10.1371/journal.pgen.1006520)

Gödel M, Temerinac D, Grahammer F, Hartleben B, Kretz O, Riederer BM, Propst F, Kohl S & Huber TB 2015 Microtubule associated protein 1b (MAP1B) is a marker of the microtubular cytoskeleton in podocytes but is not essential for the function of the kidney filtration berrier in mice. *PLoS ONE* **10** e0140116. (doi:10.1371/journal.pone.0140116)

Gumbiner BM 2005 Regulation of cadherin-mediated adhesion in morphogenesis. Nature reviews. *Molecular Cell Biology* **6** 622–634. (doi:10.1038/nrm1699)

Holt JE, Jackson A, Roman SD, Aitken RJ, Koopman P & McLaughlin EA 2006 CXCR4/SDF1 interaction inhibits the primordial to primary follicle transition in the neonatal mouse ovary. *Developmental Biology* **293** 449–460. (doi:10.1016/j.ydbio.2006.02.012)

Huang da W, Sherman BT & Lempicki RA 2009 Systemic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols* **4** 44–57. (doi:10.1038/nprot.2008.211)

Hulinska P, Martecikova S, Jeseta M & Machatkova M 2011 Efficiency of in vitro fertilization is influenced by the meiotic competence of porcine oocytes and time of their maturation. *Animal Reproduction Science* **124** 112–117. (doi:10.1016/j.anireprosci.2011.02.004)

Jackowska M, Kempisty B, Antosik P, Bukowska D, Budna J, Lianeri M, Rosinska E, Wozna M, Jagodzinski PP & Jaskowski JM 2009 The morphology of porcine oocytes is associated with zona pellucida glycoprotein transcript contents. *Reproductive Biology* **9** 79–85. (doi:10.1016/S1642-431X(12)60097-7)

Jaffe AB & Hall A 2005 Rho GTPases: biochemistry and biology. *Annual Review of Cell and Developmental Biology* **21** 247–269. (doi:10.1146/annurev.cellbio.21.020604.150721)

Jin DK, Shido K, Kopp HG, Petit I, Shmelkov SV, Young LM, Hooper AT, Amano H, Avecilla ST & Heissig B *et al.* 2006 Cytokine-mediated deployment of SDF-1 induces revascularization through recruitment of CXCR4+ hemangiocytes. *Nature Medicine* **12** 557–567. (doi:10.1038/nm1400)

Jin JX, Lee S, Khoirinaya C, Oh A, Kim GA & Lee BC 2016 Supplementation with spermine during in vitro maturation of porcine oocytes improves early embryonic development after parthenogenetic activation and somatic cell nuclear transfer. *Journal of Animal Science* **94** 963–970. (doi:10.2527/jas.2015-9761)

Kaji K, Oda S, Miyazaki S & Kudo A 2002 Infertility of CD9-deficient mouse eggs is reversed by mouse CD9, human CD9, or mouse CD81; polyadenylated mRNA injection developed for molecular analysis of sperm-egg fusion. *Developmental Biology* **247** 327–334. (doi:10.1006/dbio.2002.0694)

Kempisty B, Ziolkowska A, Ciesiolka S, Piotrowska H, Antosik P, Bukowska D, Nowicki M, Brussow KP & Zabel M 2014*a* Study on connexin gene and protein expression and cellular distribution in relation to real-time proliferation of porcine granulosa cells. *Journal of Biological Regulators and Homeostatic Agents* **28** 625–635.

Kempisty B, Ziolkowska A, Piotrowska H, Antosik P, Bukowska D, Zawierucha P, Jaskowski JM, Brussow KP, Nowicki M & Zabel M 2014*b* Expression and cellular distribution of cyclin-dependent kinase 4 (Cdk4) and connexin 43 (Cx43) in porcine oocytes before and after in vitro maturation. *Acta Veterinaria Hungarica* **62** 84–95. (doi:10.1556/AVet.2013.039)

Khan DR, Landry DA, Fournier E, Vigneault C, Blondin P & Sirard MA 2016 Transcriptome meta-analysis of three follicular compartments and its correlation with ovarian follicle maturity and oocyte developmental competence in cows. *Physiological Genomics* **48** 633–643. (doi:10.1152/physiolgenomics.00050.2016)

Kidd T, Brose K, Mitchell KJ, Fetter RD, Tessier-Lavigne M, Goodman CS & Tear G 1998 Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors. *Cell* **92** 205–215. (doi:10.1016/S0092-8674(00)80915-0)

Krendel M, Zenke FT & Bokoch GM 2002 Nucleotide exchange factor GEF-H1 mediates cross-talk between microtubules and the actin cytoskeleton. *Nature Cell Biology* **4** 294–301. (doi:10.1038/ncb773)

Li J & Dong S 2016 The signaling pathways involved in chondrocyte differentiation and hypertrophic differentiation. *Stem Cells International* **2016** Epub. (doi:10.1155/2016/2470351)

Li TY, Colley D, Barr KJ, Yee SP & Kidder GM 2007 Rescue of oogenesis in Cx37-null mutant mice by oocyte-specific replacement with Cx43. *Journal of Cell Science* **120** 4117–4125. (doi:10.1242/jcs.03488)

Li SH, Lin MH, Hwu YM, Lu CH, Yeh LY, Chen YJ & Lee RK 2015 Correlation of cumulus gene expression of GJA1, PRSS35, PTX3, and SERPINE2 with oocyte maturation, fertilization, and embryo development. *Reproductive Biology and Endocrinology* **13** 93. (doi:10.1186/s12958-015-0091-3)

Li J, Yin G, Chen M, Yang S, Wu A, Liang J & Yuan Z 2017 Expression of CXCL12 and its receptor CXCR4 in patients with adenomyosis. *Oncology Letters* **13** 2731–2736. (doi:10.389/ol.2017.5762)

Marques MG, Nicacio AC, de Oliveira VP, Nascimento AB, Caetano HV, Mendes CM, Mello MRB Milazzotto MP, Assumpcao ME & Visintin JA 2007 In vitro maturation of pig oocytes with different media, hormone and meiosis inhibitors. *Animal Reproduction Science* **97** 375–381. (doi:10.1016/j.anireprosci.2006.02.013)

Montero JA, Lorda-Diez CI, Francisco-Morcillo J, Chimal-Monroy J, Garcia-Porrero JA & Hurle JM 2017 Sox9 expression in amniocytes: species-specific differences in the formation of digits. *Frontiers in Cell and Developmental Biology* **5** 23. (doi:10.3389/fcell.2017.00023)

Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstrale M & Laurila E *et al.* 2003 PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nature Genetics* **34** 267–273. (doi:10.1038/ng1180)

Nguyen T, Lee S, Hatzirodos N, Hummitzsch K, Sullivan TR, Rodgers RJ & Irving-Rodgers HF 2012 Spatial differences within the membrana granulosa in the expression of focimatrix and steroidogenic capacity. *Molecular and Cellular Endocrinology* **363** 62–73. (doi:10.1016/j.mce.2012.07.009)

Nishigaki A, Okada H, Okamoto R, Shimoi K, Miyashiro H, Yasuda K & Kanzaki H 2013 The concentration of human follicular fluid stromal cell-derived factor-1 is correlated with luteinization in follicles. *Gynecological Endocrinology* **29** 230–234. (doi:10.3109/09513590.2012.736551)

Onishi K, Hollis E & Zou Y 2014 Axon guidance and injury-lessons from Wnts and Wnt signaling. *Current Opinion in Neurobiology* **27** 232–240. (doi:10.1016/j.conb.2014.05.005)

Polge C, Leulmi R, Jarzaquet M, Claustre A, Combaret L, Bechet D, Heng AE, Attaix D & Taillandier D 2016 UBE2B is implicated in myofibrillar protein loss in catabolic C2C12 myotubes. *Journal of Cachexia, Sarcopenia and Muscle* **7** 377–387. (doi:10.1002/jcsm.12060)

Purohit A, Sadanandam A, Myneni P & Singh RK 2014 Semaphorin 5A mediated cellular navigation: connecting nervous system and cancer. *Biochimica et Biophysica Acta* **1846** 485–493. (doi:10.1016/j.bbcan.2014.09.006)

Ren Y, Li R, Zheng Y & Busch H 1998 Cloning and characterization of GEF-H1, a microtubule-associated guanine nucleotide exchange factor for Rac and Rho GTPases. *Journal of Biological Chemistry* **273** 34954–34960. (doi:10.1074/jbc.273.52.34954)

Richard S & Baltz JM 2014 Prophase I arrest of mouse oocytes mediated by natriuretic peptide precursor C requires GJA1 (connexin-43) and GJA4 (connexin-37) gap junctions in the antral follicle and cumulus-oocyte complex. *Biology of Reproduction* **90** 137. (doi:10.1095/biolreprod.114.118505)

Rozario T & DeSimone DW 2010 The extracellular matrix in development and morphogenesis: a dynamic view. *Developmental Biology* **341** 126–140. (doi:10.1016/j.ydbio.2009.10.026)

Rozario T, Dzamba B, Weber GF, Davidson LA & DeSimone DW 2009 The physical state of fibronectin matrix differentially regulates morphogenetic movements in vivo. *Developmental Biology* **327** 386–398. (doi:10.1016/j.ydbio.2008.12.025)

Runge KE, Evans JE, He ZY, Gupta S, McDonald KL, Stahlberg H, Primakoff P & Myles DG 2007 Oocyte CD9 is enriched on the microvillar membrane and required for normal microvillar shape and distribution. *Developmental Biology* **304** 317–325. (doi:10.1016/j.ydbio.2006.12.041)

Sanchez F & Smitz J 2012 Molecular control of oogenesis. *Biochimica et Biophysica Acta* **1822** 1896–1912. (doi:10.1016/j.bbadis.2012.05.013)

Sevilla T, Sivera R, Martinez-Rubio D, Lupo V, Chumillas MJ, Calpena E, Dopazo J, Vilchez JJ, Palau F & Espinos C 2015 The EGR2 gene is involved in axonal Charcot-Marie-Tooth disease. *European Journal of Neurology* **22** 1548–1555. (doi:10.1111/ene.12782)

Sewanan LR, Moore, JR, Lehman W & Campbell SG 2016 Predicting effects of tropomyosin mutations on cardiac muscle contraction through myofilament modeling. *Frontiers in Physiology* **7** 473. (doi:10.3389/fphys.2016.00473)

Shao L, Chian RC, Xu Y, Yan Z, Zhang Y, Gao C, Gao L, Liu J & Cui Y 2015 Genomic expression profiles in cumulus cells derived from germinal vesicle and MII mouse oocytes. *Reproduction, Fertility, and Development*. Epub (doi:10.1071/RD15077)

Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR & Lander ES *et al.* 2005 Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *PNAS* **102** 15545–15550. (doi:10.1073/pnas.0506580102)

Villarreal MM, Kim SK, Barron L, Kodali R, Baardsnes J, Hinck CS, Krzysiak TC, Henen MA, Pakhomova O & Mendoza V *et al.* 2016 Binding properties of the transforming growth factor-β coreceptor betaglycan: proposed mechanism for potentiation of receptor complex assembly and signaling. *Biochemistry* **55** 6880–6896. (doi:10.1021/acs.biochem.6b00566)

Villarroel-Campos D & Gonzalez-Billault C 2014 The MAP1B case: an old MAP that is new again. *Developmental Neurobiology* **74** 953–971. (doi:10.1002/dneu.22178)

Vite A, Li J & Radice GL 2015 New functions for alpha-catenins in health and disease: from cancer to heart regeneration. *Cell and Tissue Research* **360** 773–783. (doi:10.1007/s00441-015-2123-x)

Wang L, Wang ZB, Zhang X, FitzHarris G, Baltz JM, Sun QY & Liu XJ 2008 Brefeldin A disrupts asymmetric spindle positioning in mouse oocytes. *Developmental Biology* **313** 155–66. (doi:10.1016/j.ydbio.2007.10.009)

Wang HX, Tong D, El-Gehani F, Tekpetey FR & Kidder GM 2009 Connexin expression and gap junctional coupling in human cumulus cells: contribution to embryo quality. *Journal of Cellular and Molecular Medicine* **13** 972–984. (doi:10.1111/j.1582-4934.2008.00373.x)

Wang CJ, Zhang ZZ, Xu J, Wang M, Zhao WY, Tu L, Zhuang C, Liu Q, Shen YY & Cao H *et al.* 2015 SLITRK3 expression correlation to gastrointestinal stromal tumor risk rating and prognosis. *World Journal of Gastroenterology* **21** 8398–8407. (doi:10.3748/wjg.v21.i27.8398)

Weber GF, Bjerke MA & DeSimone DW 2011 Integrins and cadherins join forces to form adhesive networks. *Journal of Cell Science* **124** 1183–1193. (doi:10.1242/jcs.064618)

Zhang Y, Duan X, Cao R, Liu HL, Cui XS, Kim NH, Rui R & Sun SC 2014 Small GTPase RhoA regulates cytoskeleton dynamics during porcine oocyte maturation and early embryo development. *Cell Cycle* **13** 3390–3403. (doi:10.4161/15384101.2014.952967)

Zhong ZS, Huo LJ, Liang CG, Chen DY & Sun QY 2005 Small GTPase RhoA is required for ooplasmic segregation and spindle rotation, but not for spindle organization and chromosome separation during mouse oocyte maturation, fertilization, and early cleavage. *Molecular Reproduction and Development* **71** 256–261. (doi:10.1002/mrd.20253)

Zhou X & Lei QY 2016 Regulation of TAZ in cancer. *Protein and Cell* **7** 548–561. (doi:10.1007/s13238-016-0288-z)

Zhou GB, Zeng Y, Meng QG, Liu Y, Dai YP, Zhu SE, Bunch TD & Hou YP 2013 Decreased expression of CD9 in bovine oocytes after cryopreservation and the relationship to fertilization capacity. *Molecular Reproduction and Development* **80** 451–459. (doi:10.1002/mrd.22181)

Ziyyat A, Rubinstein E, Monier-Gavelle F, Barraud V, Kulski O, Prenant M, Boucheix C, Bomsel M & Wolf JP 2006 CD9 controls the formation of clusters that contain tetraspanins and the integrin alpha 6 beta 1, which are involved in human and mouse gamete fusion. *Journal of Cell Science* **119** 416–424. (doi:10.1242/jcs.02730)

Zuccarello D, Ferlin A, Garolla A, Menegazzo M, Perilli L, Ambrosini G & Foresta C 2011 How the human spermatozoa sense the oocyte: a new role of SDF1-CXCR4 signaling. *International Journal of Andrology* **5** 554–565. (doi:10.1111/j.1365-2605.2011.01158.x)

Received 8 March 2017
First decision 4 May 2017
Revised manuscript received 4 July 2017
Accepted 21 July 2017