NEW MOLECULAR MARKERS INVOLVED IN IMMUNE SYSTEM HOMEOSTASIS AND HEMOPOIETIC ORGAN DEVELOPMENT ARE DIFFERENTIALLY REGULATED DURING OOCYTES IN VITRO MATURATION

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Abstract

The growth and maturation of the oocyte is a dynamic process which requires a variable supply of hormones, growth factors and energy. These needs are met partially by the surrounding somatic cells and the cumulus-oocyte complex, which communicate bi-directionally via gap junctions. Identifying and analyzing protein expression in the oocyte can provide insight in its development and growth. Further, like bone marrow stem cells, if relevant marker genes are found in oocytes, there is a potential for the oocyte to be manipulated into becoming hemopoietic stem cells. In this study, porcine oocytes were isolated and subjected to microarray analysis to compare the oocyte gene expression in vivo and in vitro maturation (IVM). Genes identified belonged to both 'hemopoietic or lymphoid organ development' (GO:0048534) and 'immune system development' (GO:0002520), and the markers can be used to identify several activities such as cell migration, neurogenesis and proliferation. The following are the identified genes and all were downregulated after IVM to varying degrees: ID2, VEGFA, TGFB3, INHBA, CDK6, BCL11A, MYO1E, ITGB1, EGR1, NOTCH2, SPTA1, KIT and TPD52. Our results should provide new markers to further investigate oocyte development and growth regulation.

Running title: Markers of hemopoietic organ development

Keywords: pig, oocyte, microarray, IVM

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Introduction

The long process of creating a viable female gamete for reproduction involves the growth and maturation of the oocyte. It is critical that the needs of the oocyte is met, such as receiving hormones, growth factors or energy, so that folliculogenesis and oogenesis can successfully occur [1]. Due to the dynamic requirements of the oocyte during its development, gene expression will change between the transition of *in vivo* and *in vitro* maturation (IVM) which could give information on how the gamete cells interacts with its surrounding somatic cells [2].

The spheroid structure of the ovarian follicle consists of an oocyte, layers of two cell population of granulosa cells called cumulus cells (CCs) and mural granulosa cells (GCs), and also theca cells. Granulosa cells and theca cells release hormones that stimulate essential morphological and metabolic changes in the oocyte [3]. Increasing evidence shows that the quality and capability of gamete cells, to achieve successful development, ovulation and fertilization, are dependent on how it interacts with the somatic cells and their extracellular relationship [4]. CCs which directly surround the oocyte are connected via gap junctions (made up of connexons), and have bi-directional communication which exchanges proteins [5]. This specific relationship is called the cumulus-oocyte complex (COC).

Bone marrow stem cells have been successfully influenced to differentiate into human embryonic progenitor cells which can then further develop into specialized hematopoietic cells such as erythrocytes or leucocytes [6]. This was accomplished through identifying a h-ESC marker (CD34+) and applying an appropriate culture medium to induce hemopoiesis. In a similar approach, identifying marker genes for hematopoietic or lymphoid organ development in oocytes could make them a new source of hematopoietic stem cells [7]. Furthermore, more could be learnt about oocyte regulation, proliferation and development by identifying biomarkers related to immune system development [8].

To study protein synthesis and identify biomarkers for hemopoiesis or immune system development in a porcine oocyte model, microarray techniques can be used [9].

Oocytes from pubertal crossbred Landrace gilts were isolated. Then the protein expression of genes belonging to GO BP ontology terms 'hemopoietic or lymphoid organ development' or 'immune system development' was selected and analyzed for comparison of *in vivo* and *in vitro* maturation expressions. These results provide insight into the development, growth and differentiation capability of the oocyte.

Material and methods

Experimental design

Oocytes were collected and subjected to two Brilliant Cresyl Blue (BCB) tests and divided into two

groups. The first group ("before IVM") included oocytes graded as BCB-positive (BCB⁺) and directly exposed to microarray assay. The second group ("after IVM") included BCB⁺ oocytes which were then *in vitro* matured, and if classified as BCB⁺ in second BCB test passed to molecular analyses.

Animals

A total of 45 pubertal crossbred Landrace gilts bred on a commercial local farm were used in this study. They had a mean age of 155 days (range 140 – 170 days) and a mean weight of 100 kg (95–120 kg). All animals were bred under the same conditions and fed the same forage (depending on age and reproductive status). All experiments were approved by the Local Ethic Committee (approval no. 32/2012).

Collection of porcine ovaries and cumulus-oocyte-complexes (COCs)

The ovaries and reproductive tracts were recovered at slaughter and transported to the laboratory within 40 min. at 38°C in 0.9% NaCl. To provide optimal conditions for subsequent oocyte maturation and fertilization *in vitro*, the ovaries of each animal were placed in a 5% fetal bovine serum solution (FBS; Sigma-Aldrich Co., St. Louis, MO, USA) in PBS. Single large follicles (>5mm) were opened by puncturing with a 5ml syringe and 20-G needle in a sterile Petri dish, and COCs were recovered. The COCs were washed three times in modified PBS supplemented with 36 µg/ml pyruvate, 50 µg/ml gentamycin, and 0.5 mg/ml BSA (Sigma-Aldrich, St. Louis, MO, USA). The COCs were selected under an inverted microscope Zeiss, Axiovert 35 (Lübeck, Germany), counted, and morphologically evaluated. Only COCs of grade I possessing homogeneous ooplasm and uniform, compact cumulus cells were considered for further use, resulting in a total of 300 grade I oocytes (3 x n=50 "before IVM" group, 3 x n=50 "after IVM" group).

Assessment of oocyte developmental competence by BCB test

Brilliant Cresyl Blue (BCB) test was used for assessment of porcine oocytes' quality and maturity. The glucose-6-phosphate (G6PDH) enzyme converts BCB stain from blue to colorless. In oocytes that completed the growth activity of the enzyme decreases and the stain cannot be reduced, resulting in blue oocytes (BCB⁺). To perform the BCB staining test, oocytes were washed twice in modified Dulbecco's Phosphate Buffered Saline (DPBS) commercially supplemented with 0.9 mM calcium, 0.49 mM magnesium, 0.33 mM pyruvate, and 5.5 mM glucose (Sigma-Aldrich, St. Louis, MO, USA), and additionally with 50 IU/ml penicillin, 50 µg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and 0.4% Bovine Serum Albumin (BSA) [w/v] (Sigma-Aldrich, St. Louis, MO, USA). They were then treated with 13 µM

BCB (Sigma-Aldrich, St. Louis, MO) diluted in DPBS at 38.5°C, 5% CO₂ for 90 min. After treatment, the oocytes were transferred to DPBS and washed twice. During washing, the oocytes were examined under an inverted microscope and classified as stained blue (BCB⁺) or colorless (BCB⁻). Only the granulosa cell-free BCB⁺ oocytes were used for subsequent molecular analyses (“before IVM” group) or IVM followed by second BCB test and molecular analyses (“after IVM” group).

In vitro maturation of porcine cumulus-oocyte-complexes (COCs)

After the first BCB test, the BCB⁺ COCs were subjected to IVM. The COCs were cultured in Nunc[™]Δ 4-well dishes (Thermo Fisher Scientific, Waltham, MA, USA) in 500 µl standard porcine IVM culture medium: TCM-199 (tissue culture medium) with Earle’s salts and L-glutamine (Gibco BRL Life Technologies, Grand Island, NY, USA), supplemented with 2.2 mg/ml sodium bicarbonate (Nacalai Tesque, Inc., Kyoto, Japan), 0.1 mg/ml sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA), 10 mg/ml BSA (Bovine Serum Albumin) (Sigma-Aldrich, St. Louis, MO, USA), 0.1 mg/ml cysteine (Sigma-Aldrich, St. Louis, MO, USA), 10% (v/v) filtered porcine follicular fluid, and gonadotropin supplements at final concentrations of 2.5 IU/ml hCG (human Chorionic Gonadotropin) (Ayerst Laboratories, Inc., Philadelphia, PA, USA) and 2.5 IU/ml eCG (equine Chorionic Gonadotropin) (Intervet, Whitby, ON, Canada). Wells were covered with mineral oil overlay and cultured at 38°C under 5% CO₂ in air for 22h, and then for additional 22h in medium without hor-

mones. After cultivation, the second BCB staining test was performed, and BCB⁺ oocytes were used for further molecular analyses.

Microarray expression analysis and statistics

Experiments were performed in three replicates. Total RNA (100 ng) from each pooled sample was subjected to two round sense cDNA amplification (Ambion® WT Expression Kit). The obtained cDNA was used for biotin labeling and fragmentation 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. The array strips were scanned employing Imaging Station of GeneAtlas System. The preliminary analysis of the scanned chips was performed using Affymetrix GeneAtlas™ Operating Software. 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 analyzes were performed using BioConductor software, based on the statistical R programming language. For background correction, normalization and summation of raw data, the Robust Multiarray Averaging (RMA) algorithm implemented in “affy” package of BioConductor was applied. Biological annotation was taken from BioConductor “oligo” package where annotated data frame object was merged with normalized data set, leading to a complete gene data table. Statistical significance of

FIGURE 1 Heat map representations of differentially expressed genes belonging to the “hemopoietic or lymphoid organ development” and “immune system development” GO BP terms. Arbitrary signal intensity acquired from microarray analysis is represented by colours (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)

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|.

Functional annotation clustering of differentially expressed genes was performed using DAVID (Database for Annotation, Visualization and Integrated Discovery). Gene symbols for up- or down-regulated genes from each of the compared groups were loaded to DAVID by “RDAVIDWebService” BioConductor package. For further analysis we have chosen the enriched GO terms which has at least 5 genes and p value (Benjamini) lower than 0.05. The enriched GO terms were subjected to hierarchical clusterization algorithm and presented as heat maps.

Subsequently we analyzed the relation between the genes belonging to chosen GO terms with GOplot package. The GOplot package had calculated the z-score: the number of up- regulated genes minus the number of down- regulated genes divided by the square root of the count. This information allowed estimating the change course of each gene-ontology term.

Interactions between chosen differentially expressed genes/proteins belonging to ontology group were investigated by STRING10 software (Search Tool for the Retrieval of Interacting Genes). List of gene names were used as query for interaction prediction. Searching criteria based on co-occurrences of genes/proteins in scientific texts (text mining), co-expression and experimentally observed interactions. The results of such analysis generated gene/protein interaction network where the intensity of the edges reflects the strength of interaction

score. Besides interaction prediction, STRING also allowed us to perform functional enrichments of GO terms based on previously uploaded gene sets.

Ethical approval

The research related to animal use has been complied with all the relevant national regulations and instructional policies for the care and use of animals. Bioethical Committee approval no. 32/2012 from 30.06.2012.

Results

Whole transcriptome profiling by Affymetrix microarray allowed us to analyze the gene expression changes in freshly isolated oocytes, before in vitro procedure (“before IVM”), in relation to after in vitro maturation (“after IVM”). By Affymetrix® Porcine Gene 1.1 ST Array we have examined expression of 12258 porcine transcripts. Genes with fold change higher than |2| and with corrected p value lower than 0.05 were considered as differentially expressed. This set of genes consisted of 419 different transcripts. Subsequently, the genes were used for identification of significantly enriched GO BP terms.

DAVID (Database for Annotation, Visualization and Integrated Discovery) software was used for extraction of the genes belonging to regulation of “hemopoietic or lymphoid organ development” and “immune system development” gene ontology Biological Process term (GO BP). We found that 13 genes from these GO BP term were significantly represented in down-regulated gene sets. This set of genes was subjected to hierarchical clusterization procedure and presented as heat maps (Fig. 1).

Set of the differentially expressed genes belonging to “hemopoietic or lymphoid organ development” and “immune system development” GO BP terms

FIGURE 2 The circle plot showing the differentially expressed genes and z-score “hemopoietic or lymphoid organ development” and “immune system development” GO BP Terms. The outer circle shows a scatter plot for each term of the fold change of the assigned genes. Red circles display down- regulation and green ones up- regulation. The inner circle shows the z-score of each GO BP term. The width of each bar corresponds to the number of genes within GO BP term and the color corresponds to the z-score

FIGURE 3 The representation of the mutual relationship between differently expressed genes that belongs to “hemopoietic or lymphoid organ development” and “immune system development” GO BP Terms. The ribbons indicate which gene belongs to which categories. The middle circle represents logarithm from fold change (LogFC) between before IVM and after IVM respectively. The genes were sorted by logFC from most to least changed gene

with their official gene symbols, ratio, Entrez Gene IDs and corrected p values were shown in table 1.

The enrichment of each GO BP term as well KEGG pathway were calculated as z-score and shown on the circle diagram (Fig. 2)

Moreover, in Gene Ontology database genes that formed one particular GO group can also belong to other different GO term categories. By this reason we explore the gene intersections between selected GO BP terms. The relation between those GO BP terms was presented as circle plot (Fig. 3) as well as heatmap.

STRING-generated interaction network was created with differentially expressed genes belonging to the “fatty acid metabolic process” ontology group. The intensity of the edges reflects the strength of interaction score (Fig. 4).

Discussion

In our study, a group of 13 genes expressed in our porcine oocyte model were selected and analyzed: *ID2*, *VEGFA*, *TGFBR3*, *INHBA*, *CDK6*, *BCL11A*, *MYO1E*, *ITGB1*, *EGR1*, *NOTCH2*, *SPTA1*, *KIT* and *TPD52*. All genes belonged to both ontology groups which are ‘hemopoietic or lymphoid organ development’ and ‘immune system development’. Furthermore, they were all downregulated after IVM compared to before. This provides an indication of the cell type’s potential to differentiate into hemopoietic or lymphoid cell lines [10,11].

The most downregulated gene after IVM was found to be *ID2*. The differentiation of CD4⁺T cells, and their regulatory T (T_{reg}) population which are responsible for immune homeostasis, is regulated

FIGURE 4 STRING-generated interaction network between genes that belongs to the “hemopoietic or lymphoid organ development” and “immune system development” GO BP Terms. The intensity of the edges reflects the strength of interaction score

partly by Id2 (inhibitor of DNA binding 2) and its family member Id3 [12,13]. The Id proteins (1-4) in general control lymphocyte development and homeostasis through their inhibitory effect on helix-loop-helix (HLH) DNA transcription factors [14]. Our results for *ID2* downregulated expression in the oocyte after IVM is supported by Chermuła et al.’s own study [15], which may indicate a role in the development and maturation of the oocyte.

Vascular endothelial growth factor-A (*VEGFA*) was the second most downregulated gene in our two ontology groups. This is a well-studied gene for its role in neuron differentiation for vascularization and neurogenesis [16], and from our study is known to bind and show an increase in activity with *ITGB1* and *KIT* (refer to **Fig. 4**). Inflammation results in increased expression of *VEGFA*, as the immune system needs blood vessels to form in order to fulfill, nutrient and energy requirements for the inflamed area [17]. In the ovarian follicle, the important process of angiogenesis, which provides energy and promotes cell migration, relies on *VEGFA* [18]. The observation of its downregulation after the occurrence of IVM is also shown by Chermuła et al. [19].

Following *VEGFA* in the downregulated genes, is transforming growth factor-beta receptor 3 (*TGFBR3*). Its protein TGFβ (also known as betaglycan), catalyzes endocrine hormones inhibin A and B to

TGFβ whilst also inhibiting the activity of activin and bone morphogenetic proteins (BMPs) [20]. Activin promotes follicle stimulating hormone (FSH) secretion, whilst inhibin does the reverse, and therefore the expression of *TGFBR3* must have a clear influence on the growth of the ovarian follicle [21]. Various studies are connecting unusual expression of *TFGβ* to the development of cancer and inflammatory disease, as cell migration concerning the immune system and proliferation of cells become unregulated [22,23].

The fourth most downregulated gene was Inhibin-β A (*INHBA*) is known to be expressed in cumulus cells to which it affects oocyte competence and its differential expression is a potential marker for small and dominant granulosa cell follicles [24,25]. Various studies have shown that high expression of *INHBA* is associated with adverse effects for cancer treatment due to poor immune system response [26,27]. This highlights the protective role of *INHBA*’s regulation even in oocyte development. Overexpression of cyclin-dependent kinase 6 (*CDK6*) is also linked to unregulated cell proliferation for the G1/S cell cycle transition, and also by inhibiting miRNA-320d, leading to cancer progression [28,29]. Other CDKs appear to compensate in *CDK6* is absent in oocyte development, and the resulting ovary will still be healthy [30].

BCL11A is the sixth most downregulated gene. The transcription factor is known to be involved in hemopoietic development, specifically in monitoring the expression of fetal hemoglobin (HbF) [31]. Several components on beta globin genes cluster is occupied by *BCL11A*; therefore, it is recognized a key therapeutic target for sickle-cell disease [32]. While we have identified a change in expression from before and after IVM, more research should be done to investigate the genes possible effects on oocyte development and growth such as providing increased blood cells and energy to the oocyte.

MYO1E is the seventh most downregulated gene. The adhesion and B cell migration for high endothelial venules incorporate the involvement of Myo1e, and is found to be necessary for the FAK/PI3K/RAC-1 signaling pathway [33]. Being specialized capillary blood vessels, high-endothelial venules are responsible for the trafficking of white blood cells [34]. Therefore, *MYO1E* must have a crucial role in preserving oocyte competence and protecting the cell population. In a study done by Borys et al., *MYO1E* was also found to be downregulated after IVM in oocytes [35].

The eight most downregulated gene is Integrin B-1 (*ITGB1*). Xu et al. have shown that *ITGB1* is a potential therapeutic target because of its effects the cell migration and metastasis of esophageal squamous cell carcinoma (ESCC) [36]. Although more information is needed to know the gene's role in folliculogenesis and oogenesis, *ITGB1* is involved in implantation of the ovary and trophoblastic differentiation [37].

Early growth response 1 (*EGR1*), which encodes a nuclear transcription factor, was the fifth least downregulated gene. Various signaling molecules such as hormones, neurotransmitters, growth and differentiation factors will increase the activity of the gene [38]. Possibly through its interaction with NF- κ or inflammatory cytokines, *EGR1* has been shown to induce apoptosis for various cell types including granulosa cells during follicular atresia [39]. FSH is also induced by *EGR1*, an essential gonadotropin for the ovarian follicle suggesting the gene promotes the growth of the oocyte [40].

Another gene we selected and analyzed in our ontology groups is *NOTCH2*. The expression of *NOTCH2* is essential for healthy development of the primordial follicle, according to knockout mice studies of the gene for polycystic ovary syndrome (PCOS) [41]. Our results that *NOTCH2* is downregulated after IVM compared to before in oocytes is supported by the results Stefańska et al. [42], suggesting it has a regulatory role in the transition.

The second least downregulated gene is *KIT*, a cytokine receptor and produces protein members of the tyrosine-kinase family. Cell proliferation of the oocyte and anti-apoptosis of the pre-antral follicle is aided by the phosphorylation from *KIT* ligands [43]. Furthermore, the protein encoded by *KIT* can be

used as a marker for hematopoietic stem cells [44]. Finally, tumor protein D52 (*TPD52*) was the least downregulated gene from both ontology groups. Overexpression of *TPD52* is linked to increased cell migration and tumor growth [45,46]. Not much is known about this gene's effect on the oocyte, and further research should be done.

Through our study, various genes which are present in the oocyte and effect the immune system and hemopoiesis have been analyzed. The expression of these genes suggests the oocyte could have stem cell like qualities in long term in vitro cultures; however, after IVM these are downregulated. This downregulation could be for regulatory purposes during the development and maturation of the oocyte, as these genes influence important cellular activities such as cell migration, neurogenesis and proliferation. Furthermore, the COC will be affected by the change of protein expression in the oocyte and this effect should be studied. New IVM oocyte biomarkers have found from the differential expression of these 13 genes from our microarray approach.

Conclusions

To summarize, the gene expression of a porcine oocyte model was compared to before and after IVM. Thirteen genes were found to belong to ontological groups 'hemopoietic or lymphoid organ development' (GO:0048534) and 'immune system development' (GO:0002520). Possibly due to their regulatory roles for the developing and mature oocyte, these genes were all downregulated after IVM and could be used as biomarkers. The regulation of genes roles related to 'immune system development' has also been emphasized great importance in maintaining homeostasis, preventing cancer aggression and improving treatment resistance. From the effects of cell migration, neurogenesis or proliferation, the proteins encoded have an impact on cell function and competence of the oocyte. Furthermore, the oocyte and its surrounding granulosa cells are indicated to have potential stem cell characteristics for the hematopoietic and lymphoid cell lines which could be further investigated for future studies.

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Conflict of interest statement

The authors declare they have no conflict of interest.

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