Journal of Reproduction and Development, Vol. 55, No. 6, 2009

—Full Paper—

Follicular Size is Associated with the Levels of Transcripts and Proteins of Selected Molecules Responsible for the Fertilization Ability of Oocytes of Puberal Gilts

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Abstract. The maturation and developmental competence of the oocyte is acquired during folliculogenesis. It is still unclear whether follicle size is associated with the levels of transcript and protein encoding molecules contributing to the fertilization ability of the porcine oocyte. Follicles were dissected from porcine ovaries after slaughter and classified as small (< 3 mm), medium (3–5 mm) or large (>5 mm), aspirated cumulus-oocyte complexes were cultured in standard porcine IVM culture medium (TCM 199) for 44 h. In developmentally competent oocytes, assessed by determining the activity of glucose-6-phosphate dehydrogenase (G6PDH) using a brilliant cresyl blue (BCB) test, real-time quantitative PCR reaction methods, western-blot and confocal microscopy analysis were applied to determine the transcript levels of porcine *zona pellucida* glycoproteins pZP1, pZP2, pZP3, pZP3 alpha and integrins beta 1 and beta 2, as well as the levels of pZP3 and integrin beta 2 proteins. We observed significantly higher levels of pZP1, pZP3 and integrin beta1 and beta2 transcripts in oocytes collected from medium follicles as compared with small follicles (P<0.001). Moreover, we found an increased content of all investigated mRNAs in oocytes isolated from large follicles as compared with small follicles (P<0.001). Western-blot analysis demonstrated a higher level of pZP3 protein in oocytes isolated from large and medium follicles as compared with small follicles (P<0.001). Our results suggest that the levels of transcripts and proteins for selected molecules contributing to the fertilization ability of oocytes are associated with follicular size in puberal gilts.

Key words: Follicular size, Integrins, Oocyte, Pig, ZPs glycoproteins

(**J. Reprod. Dev. 55:** 588–593, 2009)

Oocyte quality has a significant influence on early embryonic development and survival, establishment and maintenance of pregnancy, fetal development and even the health of offspring in postnatal life [1–5]. The developmental competence of an oocyte may be affected by follicle size [6–8]. The growth of the oocyte within the follicle is accompanied by expression of a series of genes [9–11]. Some of these genes are involved in cell cycle control and meiotic progression, resulting in full meiotic competence of the oocyte [12, 13]. Several other proteins are involved in cellular progress as characterized by complete developmental competence of the gamete prior to fertilization [14, 15]. During the growth phase, several messenger RNA (mRNA) and their respective proteins are accumulated and stored long-term to be used after fertilization and subsequent proper embryo development [1, 16]. The abilities of fertilization and embryo development are acquired by an oocyte only after a long period of growth and development. Specific follicular growth and somatic follicular cell-oocyte interactions lead to the acquisition of high developmental potential for the oocyte.

The porcine *zona pellucida* is composed of four major glycoproteins: ZP1, ZP2, ZP3 and ZP3α, described also as ZP4. These *ZPs* genes are described in pigs as the porcine zona glycoproteins *pZP1*, *pZP2*, *pZP3* and *pZP3α*. They are responsible for the major sperm-receptor activity of the zona and play a significant role during fertilization [17–20].

Integrins are major receptor proteins within the extracellular matrix (ECM) that mediate several functions connected with cell life and metabolism, such as cell adhesion, migration, cytoskeletal organization, proliferation, survival and differentiation. Several lines of experiments have identified the role of integrin *β1* as a candidate molecule involved in sperm-oocyte interactions [21–25].

It has recently been demonstrated that oocytes isolated from small follicles have not completed cytoplasmic maturation, which may affect normal embryo growth. Low developmental competence of these oocytes may be associated with decreased levels of stored mRNA and proteins [6, 7, 26]. However, it is not clear whether oocytes isolated from different sized follicles reflect different fusibility and fertilization abilities. Therefore, the aim of the present study was to determine the mRNA pattern of porcine sperm-oocyte interaction genes *pZP1*, *pZP2*, *pZP3*, *pZP3α*, *integrin β1* and *integrin β2* and the protein levels of pZP3 and integrin *β2* in oocytes isolated from follicles of various sizes.

Accepted for publication: July 10, 2009
Published online in J-STAGE: August 11, 2009
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Table 1. Oligonucleotide sequences used for RQ-PCR analysis

Transcript	Sequence (5'-3' direction)	Gene accession no.	Product size (bp)
pZP1	AGAGGAGACAGTGGGAGAC AAGAGGGTCCACCACAGAG	S74651	219 bp
pZP2	CCAGGTATTGTCACTTGCC CGCACTCTTTTGGTACAGG	NM213848	185 bp
pZP3	GCTGGAGGTTCTTCGTCTG TACGGTGGGTGGCTTTGAG	NM213893	113 bp
pZP3 alpha	TGGCTCTGCTTTCCGCTGT GAGTTGCTGTGTCCTGGCT	NM214045	136 bp
Integrin β1	ATGCCTACTTCTGCTCGATGT TCCCTTTGCTACGGTTGGTTA	NM213968	133 bp
Integrin β2	GGCTACCCCATCGACCTGT TTGACGAAGGGAAGCACCG	NM213908	173 bp
CYP 19	GTCCTTTTTGGCAGCATTG CAGAAAATAGCCAGGACCT	U92246	102 bp
GAPDH	CTGCACCACCAACTGCTT TTCTGGGTGGCAGTGATG	AF069649	105 bp
β-actin	GGGAGATCGTGCGGGACAT CGTTGCCGATGGTGATGAC	DQ845171	141 bp
18S rRNA	GTGAAACTGCGAATGGCTC CCGTCGGCATGTATTAGCT	AB117609	105 bp

Material and Methods

Animals

A total of thirty crossbred puberal Landrace gilts with a median age of 170 days (range 160–180 days) and median weight of 98 kg (95–120 kg) were used in this study. The animals were bred under the same conditions. The experiments were approved by the local Ethics Committee.

Collection of porcine ovaries and COCs

Ovaries and reproductive tracts were recovered from gilts immediately after slaughter and transported to the laboratory within 20 min at 38 C in 0.9% NaCl. Thereafter, the ovaries were placed in 5% fetal bovine serum solution (FBS; Sigma-Aldrich St. Louis, MO, USA) in phosphate buffered saline (PBS). Follicles were classified into three size categories: small (<3 mm), medium (3–5 mm) and large (>5 mm).

The follicles were opened by individual puncturing with a 5-ml syringe and 20-G needle in a sterile petri dish, and the cumulus-oocyte complexes (COCs) were recovered. COCs were washed three times in modified PBS supplemented with 36 μg/ml pyruvate, 50 μg/ml gentamycin and 0.5 mg/ml bovine serum albumin (BSA; Sigma-Aldrich). They were selected under an inverted Zeiss microscope (Axiovert 35, Lübeck, Germany), counted and morphologically evaluated with special care using the scale suggested by De Loos *et al*. (1992) [27]. Only COCs of grade 1 with homogenic ooplasm and having uniform and compact cumulus cells were considered for use in the subsequent steps of the experiment.

In vitro maturation of porcine COCs

The selected grade 1 COCs were cultured in Nunclon$^{TM}\Delta$ 4-well dishes (Nunc, GmbH, Co. KG, Germany) in 500 μl standard porcine *in vitro* maturation (IVM) medium (TCM-199 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, Kyoto, Japan), 0.1 mg/ml sodium pyruvate (Sigma-Aldrich), 10 mg/ml BSA (Sigma-Aldrich), 0.1 mg/ml cysteine (Sigma-Aldrich), 10% (v/v) filtered porcine follicular fluid and gonadotropin supplements at final concentrations of 2.5 IU/ml human chorionic gonadotropin (hCG; Ayerst Laboratories, Philadelphia, PA, USA) and 2.5 IU/ml equine chorionic gonadotropin (eCG; Intervet, Whitby, ON, Canada). Wells were covered with a mineral oil overlay and cultured for 44 h at 38 C under 5% CO_2 in air.

Assessment of oocyte developmental competence by brilliant cresyl blue (BCB) test

After cultivation, oocytes were washed two times in modified Dulbecco PBS (DPBSm, Sigma-Aldrich) supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin (Sigma-Aldrich), 0.4% [w/v] BSA, 0.34 mM pyruvate and 5.5 mM glucose (DPBSm). Oocytes were treated with 26 μM brilliant cresyl blue (BCB, Sigma-Aldrich) diluted in DPBSm at 38.5 C under 5% CO_2 in air for 90 min. After treatment, the oocytes were transferred to DPBSm and washed two times. During the washing procedure, the oocytes were examined under an inverted microscope and classified as either having stained blue (BCB^+) or remained colorless (BCB^-). Only BCB^+ oocytes that had completed their growth phase and could possibly have reached developmental competence were used in the experiment. The BCB^+ COCs were incubated with bovine testicular hyaluronidase (Sigma-Aldrich) for 2 min at 38 C to separate the cumulus cells. These cells were removed by vortexing the oocytes in 1% sodium citrate buffer and by mechanical displacement using a small-diameter glass micropipette. The

cumulus-cell-free oocytes were used for further analysis.

Confocal microscopic observation

Oocytes were isolated from large, medium and small follicles (each n=10 per slide) and incubated with 300 μg/ml benzothiadiazole (BTH) for 5 min in 38 C to remove cumulus cells. They were then fixed with 2.5% paraformaldehyde in PBS and 0.2% Triton- × 100 (Sigma-Aldrich) for 30 min at room temperature and washed three times in PBS/polyvinylpyrrollidone (PBS/PVP, 0.2%). To block nonspecific binding, samples were incubated with 3% BSA in PBS with 0.1% Tween 20 (Sigma-Aldrich) for 30 min at room temperature. The oocytes were incubated for 12 h at 4 C with goat polyclonal anti-pZP3 antibody (Ab) (N-20) or goat polyclonal anti-integrin β2 Ab (C-20), both from Santa Cruz Biotechnology (Santa Cruz, CA, USA), diluted 1:500 in PBS/1.5% BSA/0.1% Tween 20. After several washes with PBS / 0.1% Tween 20, the samples were incubated for 1 h at room temperature with fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG Ab produced in rabbits, diluted 1:200 in PBS / 0.1% Tween 20. Following washing in PBS/ 0.1% Tween 20, the oocytes were mounted on glass slides in an antifade drop and observed under an LSN 510 confocal system on a Carl ZEISS Axiovert 200M microscope. FITC was excited with a wavelength of 488 nm from an argon laser, and emissions were imaged through a 505–530 nm filter.

Real-time quantitative polymerase chain reaction (RQ-PCR) analysis

Total RNA was isolated from oocytes collected from small (n= 10), medium (n=10) and large (n=10) follicles using Qiagen GmbH RNeasy mini column (Hilden, Germany). The RNA samples were resuspended 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. RQ–PCR was conducted in a Light-Cycler real-time PCR detection system (Roche Diagnostics GmbH, Mannheim, Germany) using SYBR® Green I as the detection dye, and target cDNA was quantified using a relative quantification method. The relative abundance of pZPs (pZP1, pZP2, pZP3 and pZP3α) and integrin β1 and β2 transcripts in each sample was standardized using an internal standard of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). For amplification, 2 μl of total (20 μl) cDNA solution were added to 18 μl of QuantiTect® SYBR® Green and primers for PCR (Table 1). One RNA sample of each preparation was processed without the RT reaction to provide a negative control in the subsequent PCR. To ensure that granulosa cells did not contaminate the oocytes, we demonstrated the absence of cytochrome P450 aromatase transcript by RT and RQ-PCR.

The housekeeping genes *GAPDH* and *β-actin* were amplified as references for mRNA quantification.

To quantify specific gene expression in oocytes, the levels of expression of specific oocyte mRNAs in each sample were calculated relative to *GAPDH* and *β-actin*. To ensure the integrity of these results, an additional housekeeping gene, *18S rRNA*, was used as an internal standard to ensure that GAPDH and β-actin mRNA were not regulated in the different groups of oocytes. The gene for *18S rRNA* expression has been identified as an appropriate housekeeping gene for use in quantitative PCR studies [28]. Expression of GAPDH, β-actin and 18S rRNA mRNA was measured in cDNA samples from freshly isolated COCs. The expression of *GAPDH* and *β-actin* did not vary when normalized against *18S rRNA* (data not shown).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting analysis

Oocytes isolated from an additional, similarly selected pool of small (n=20), medium (n=20) and large (n=20) follicles were treated by RIPA lysis buffer. Next, the proteins were resuspended in sample buffer and separated on 15% Tris-glycine gel using SDS-PAGE. Gel proteins were transferred to nitrocellulose, which was blocked with 5% milk in Tris-buffered saline/Tween. Immunodetection was performed with goat polyclonal anti-pZP3 Ab (N-20) and goat polyclonal anti-integrin β2 Ab (C-20), followed by incubation with donkey anti-goat HRP-conjugated Abs. The membranes were also incubated with anti-actin HRP-conjugated Ab (clone I-19; Santa Cruz Biotechnology, Santa Cruz, CA, USA) to ensure equal protein loading of the lanes.

Bands were revealed using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Rockford, IL, USA).

Statistical analysis

Results were estimated using Kruskal-Wallis test and one-way analysis of variance (ANOVA) with Dunn's *post-hoc* test. $P<0.05$, $P<0.01$ and $P<0.001$ were determined as the levels of significance.

Results

After slaughter, we recovered a relatively small number ($P<0.01$) of grade I and II oocytes as compared with grade III and IV oocytes, respectively. We also found an increased number of BCB^+ grade I oocytes, as compared with BCB^- grade I oocytes ($P<0.01$), after cultivation. The numbers of grade II BCB^+ and BCB^- oocytes were similar ($P=0.09$). Moreover, in the grade III and IV oocytes, the mean number of BCB^- oocytes was higher than that of the BCB^+ oocytes ($P<0.01$, $P<0.001$, respectively; Table 2).

Using RQ-PCR, we determined the levels of pZP1, pZP2, pZP3, pZP3α, integrin β1 and integrin β2 transcripts in porcine oocytes isolated from small, medium and large follicles of puberal gilts. We found increased transcript levels ($P\leq0.001$) of pZP1, pZP3, integrin β1 and integrin β2 in oocytes isolated from medium follicles as compared with small follicles. When comparing medium and large follicles, we observed increased transcript levels in oocytes isolated from large follicles only for pZP2, and pZP3α ($P\leq0.01$). We determined that the mRNA levels of pZP1, pZP2, pZP3, pZP3α, integrin β1 and integrin β2 were higher in large follicles as compared with small follicles ($P\leq0.01$, Fig. 1, Table 1). No differences were observed between medium and large follicles in terms of the pZP1 and integrin β1 transcript contents. We found an increased level of pZP3 mRNA in medium follicles as compared with small and large follicles ($P\leq0.001$).

Western blot analysis demonstrated an increased level of pZP3 protein in oocytes collected from large and medium follicles as compared with small follicles ($P\leq0.01$, Fig. 2). The concentration of integrin β2 was at a low stable level. However, using confocal

Table 2. Total and mean numbers (± SEM) of four morphologically different types of oocyte recovered and identified as BCB^- and BCB^+

Morphological grades of oocytes	Number of oocytes recovered	Number of BCB^- oocytes	Number of BCB^+ oocytes	Significant difference between BCB^- and BCB^+ oocytes
Grade I	83 (32.3 ± 4.2)	22 (15.4 ± 2.9)	61 (41.3 ± 6.1)	0.01
Grade II	98 (36.6 ± 5.4)	43 (27.3 ± 3.7)	55 (32 ± 4.5)	0.09
Grade III	106 (41.4 ± 7.4)	76 (48.2 ± 5.7)	30 (19.4 ± 3.6)	0.01
Grade IV	135 (51.6 ± 8.3)	97 (62.2 ± 6.3)	38 (24.6 ± 5.1)	0.001

Total number of oocytes recovered from all gilts; BCB^- and BCB^+ oocytes recovered from all gilts. The numbers of BCB^- and BCB^+ oocytes were determined after IVM. The experiments were carried out in at least three replicates.

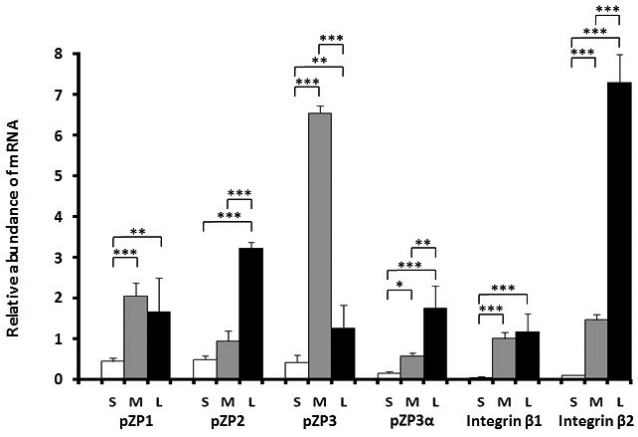

Fig. 1. Relative abundance of pZP1, pZP2, pZP3, pZP3α, integrin β1 and integrin β2 transcripts in porcine oocytes isolated from small, medium and large follicles. The porcine oocytes (n=30) isolated from small (n=10, white bars), medium (n=10, grey bars) and large (n=10, black bars) follicles of puberal gilts were immediately used to isolate RNA, which was reverse-transcribed into cDNA. The presence of transcripts of pZPs (pZP1, pZP2, pZP3 and pZP3α), integrin β1 and integrin β2 was evaluated by RQ-PCR analysis. Each sample was determined in triplicate from three independent cell collections from thirty separate animals. Results are presented as means ± SEM with the level of significance (* P<0.05, ** P<0.01, *** P<0.001).

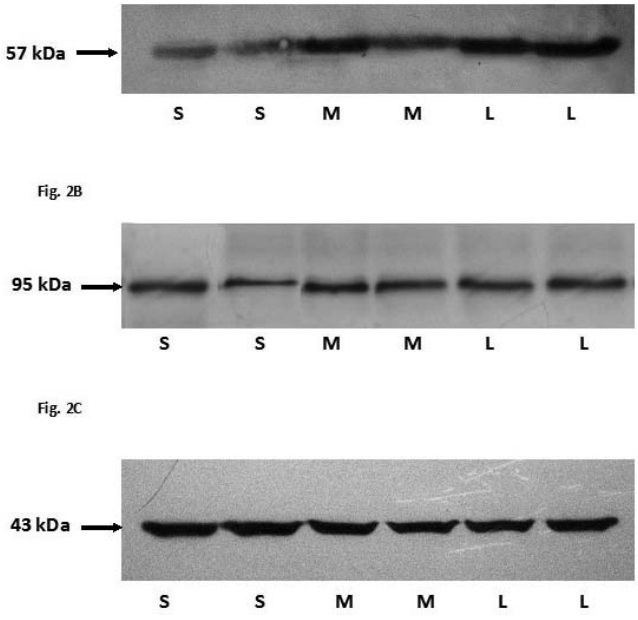

Fig. 2. Western-blot analysis. For western blot analysis, proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane that was then immunoblotted with goat polyclonal anti-pZP3 Ab (N-20; Fig. 2A) and goat polyclonal anti-integrin β2 Ab (C-20; Fig 2B), followed by incubation with donkey anti-goat HRP-conjugated Ab. To equalize protein loading, the membrane was reblotted with anti-actin HRP-conjugated Ab (Fig. 2C). Optical density (OD) was evaluated by using a Gel Logic 200 Imaging System (Kodak, Rochester, NY, USA). S, small follicles; M, medium follicles; L, large follicles.

microscopic observations, we also determined a higher concentration of both proteins in oocytes isolated from large and medium follicles as compared with gametes collected from small follicles (Fig. 3).

Discussion

During folliculogenesis, oocytes grow and mature in the follicular environment. It has been demonstrated that cellular and molecular associations between the gamete and somatic follicular cells are crucial for proper oocyte maturation and acquisition of developmental potential [29]. As the follicle grows, follicular somatic cells undergo several biochemical and structural changes. During each stage of follicle development, the somatic cells secrete several growth factors that have various effects on the maturation and development of the oocyte. The heterogeneity of follicular size has been implicated as the most important factor determining the developmental competence of oocytes in different stages of life [30–32].

The developmental and meiotic competence of oocytes isolated from different sized follicles has been intensively investigated in several species [8, 33–36]. It has been suggested that oocytes originating from large and medium follicles display an increased developmental potential compared with oocytes isolated from small follicles [8, 37]. However, little is known about the fusibility and fertilization ability of oocytes originating from follicles of various sizes.

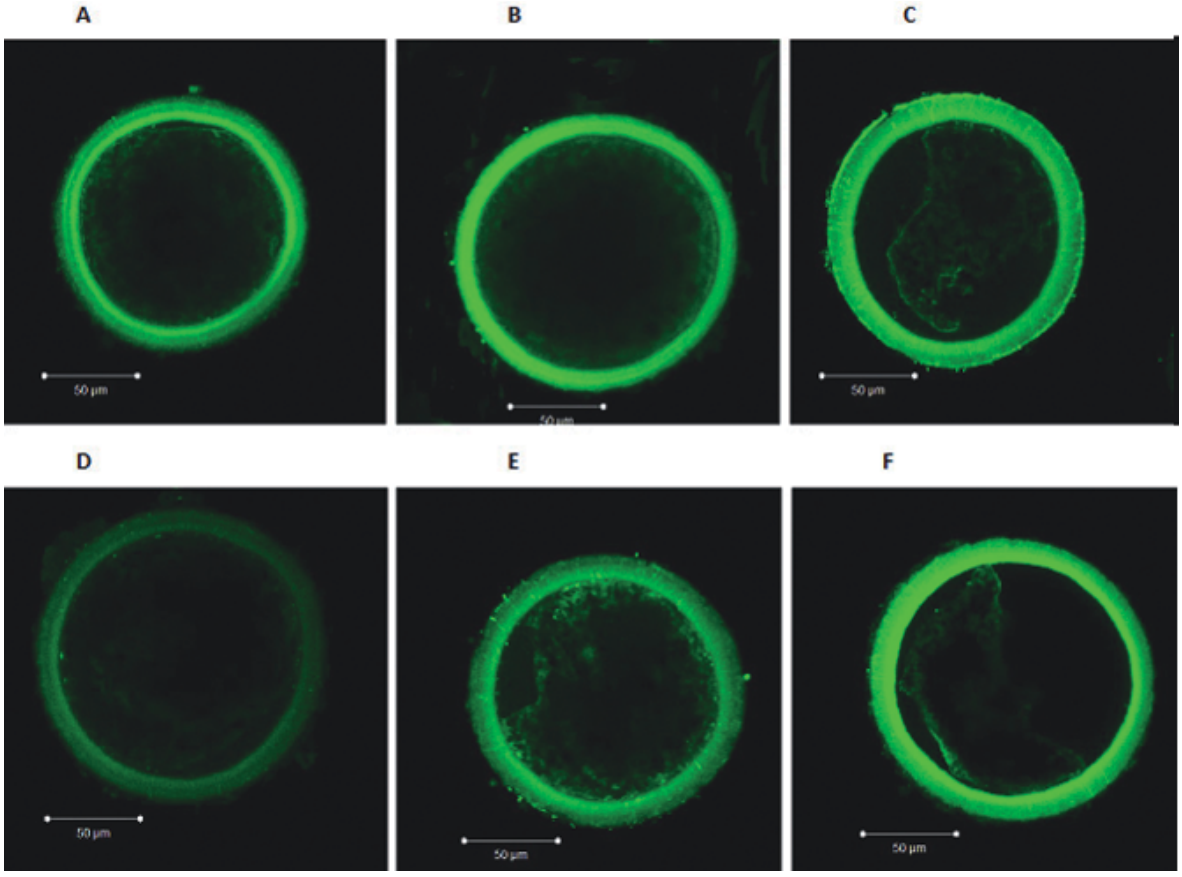

Fig. 3. Confocal microscopic observation of ovarian oocytes isolated from small, medium and large follicles. The porcine oocytes were isolated from small (A and D), medium (B and E) and large (C and F) follicles and then stained with porcine pZP3 (A–C; goat polyclonal anti-pZP3 Ab, N-20) and integrin β2 (D–F; goat polyclonal anti-integrin β2 Ab, C-20). The treated oocytes were labeled for 40 min with FITC-conjugated anti-goat IgG Ab at a 1:200 dilution in PBS. Bars are 50 μm.

In the present study, we determined the transcript levels for pZPs, integrin β1 and integrin β2 in oocytes isolated from small, medium and large porcine follicles. We observed increased mRNA levels for all of these genes in oocytes originating from medium and large follicles as compared with those isolated from small follicles. Our results confirm the hypothesis that follicles of a diameter less than 3 mm are not fully developmentally competent and are cytoplasmatically and molecularly deficient in development [8]. Similarly, Iwata *et al.* (2004) demonstrated that bovine oocytes isolated from large follicles resume nuclear maturation and progress to the MII stage significantly faster compared with oocytes collected from small follicles [34]. Marchal *et al.* (2002) postulated that more oocytes from medium and large porcine follicles are penetrated by spermatozoa during *in vitro* fertilization than oocytes from small follicles [8]. Moreover, they suggested that more oocytes from medium and large follicles developed to the blastocyst stage.

It has been demonstrated that the follicular population is morphologically and biochemically heterogeneous [38]. Moreover, oocytes from medium and large follicles show an increased concentration of steroids in follicular fluid, as well as a high number of granulosa cells and LH receptors [36]. All of these factors may have marked influence on follicular growth, oocyte maturation and oocyte development. It has also been demonstrated that larger and more mature follicles contain better quality oocytes as determined by sperm penetration and male pronucleus formation [39, 40]. Based on our results, it is possible that oocytes isolated from medium and large follicles have increased sperm penetrability because of higher expression of sperm-oocyte interaction genes compared with oocytes collected from small and developmentally incompetent follicles.

Comparing the morphological quality of COCs, we found that higher quality COCs, indicated by homogeneous cytoplasm and a complete cumulus oophorus, were collected from medium and large follicles (data not shown). Expansion of the cumulus layer precedes nuclear maturation and is an important indicator of oocyte reproductive potential [41–43]. Therefore, COCs collected from medium and large follicles may display increased developmental, meiotic and fertilization competence.

The follicular fluid and medium supplements have an important influence on mRNA abundance [44]. Furthermore, our results suggest that the origin of the oocyte and its state of maturation play a

significant role, as the expression of almost all of the genes investigated in our study varied according to follicular size.

In the present study, we demonstrate an increased level of transcripts of sperm-oocyte interaction genes in oocytes collected from follicles of different sizes. The altered transcript levels in the follicles up to 3 mm in size, collected from puberal gilts, indicate that these follicles contain oocytes with insufficient fertilization competence. The results reflect the different intrafollicular environments of maturing oocytes. These observations may be helpful in qualifying follicles and oocytes for use in *in vitro* fertilization and production programs in pigs.

Acknowledgements

This study was supported by grant No. 1682/B/PO1/2007/33 from the Polish Ministry of Scientific Research and Information Technology. B Kempisty acknowledges support from a Fellowship of the Polish Science Foundation.

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