The morphology of porcine oocytes is associated with *zona pellucida* glycoprotein 3 and integrin beta 2 protein levels

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ABSTRACT: The morphology and quality of oocytes is set during oo- and folliculo-genesis, and is completed during final maturation. Early embryonic development is associated with the morphology of the cumulus-oocyte-complex (COC). However, current knowledge of the possible relationships between oocyte morphology and the level of proteins within the oocyte, which may reflect fertilization ability, is insufficient. Using western-blot analysis and confocal microscopic observation, we determined the levels of integrin beta-2 (integrin β2) and glycoprotein 3 (pZP3) protein levels in the porcine *zona pellucida* of four morphologically different types of oocytes, graded according to their cytoplasmic composition and cumulus structure. The level of integrin β2 protein was increased in grade I and II oocytes as compared to other grades ($P < 0.05$). Moreover, the level of pZP3 protein was 3–4 fold higher in grade I oocytes ($P < 0.01$). We suggest that COC morphology may be associated with oocyte fertilization ability with respect to its sperm-oocyte interaction gene expression, which is the result of increased accumulation of specific proteins prior to fertilization in higher quality oocytes. Higher quality oocytes may also reflect better fertilization ability.

Keywords: pig; oocyte; morphology; glycoproteins; integrins

During the process of oo- and folliculo-genesis, oocytes acquire normal morphology (high quality of oocytes as defined in Materials and Methods), the ability to resume meiosis, and, after ovulation, the ability to be successfully fertilized by a spermatozoon (Coticchio et al., 2004; Russell et al., 2006; Sirard et al., 2006). Moreover, the mature female gamete must undergo several cellular and molecular changes. The molecular modifications include processes which result in the accumulation of specific mRNAs, which are the template of the embryonic genome (Sirard, 2001; Gosden, 2002; Watson, 2007; Wrenzycki et al., 2007; Mtango et al. 2008). Although the use of confocal microscopy in establishing reliable criteria for predicting developmental potential is controversial, recent findings indicate that one of the most important factors in the evaluation of the oocyte's ability to mature and to be fertilized is the morphology of the gamete (Coticchio et al., 2004; Sirard et al., 2006; Miyano and Manabe, 2007; Kempisty et al., 2008; Jackowska et al., 2009). In a previous study we demonstrated an association between oocyte morphology and the presence of specific mRNAs of genes encoding porcine *zona pellucida* glycoproteins (pZPs; Jackowska et al., 2009). These results have clearly shown that the oocytes with the best morphology and quality may have increased ferti-

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lization ability. The experiments presented herein should further buttress these results. The analysis of protein levels is necessary in determining if the morphology of porcine oocytes is strictly correlated with the oocyte contents of sperm-oocyte interaction proteins.

The *zona pellucida* is an extracellular matrix that surrounds the growing and developing oocyte, as well as ovulated eggs and preimplantation embryos (Hasegawa and Koyama, 2007). The porcine *zona pellucida* is composed of four glycoproteins: pZP1, pZP2, pZP3 and pZP3α (also called pZP4) (Rath et al., 2006). The morphology of the *zona pellucida* is permanently modified during oocyte growth and early embryonic development (Ducibella et al., 1995; Tatemoto and Terada, 1999; Rath et al., 2005; Michelmann et al., 2007). Several factors, e.g., maturation *in vivo* or *in vitro*, specific supplements to *in vitro* maturation (IVM) media, as well as the stage of embryonic development from zygote to preimplantation stage may influence *zona pellucida* morphology (Rath et al., 2005). Furthermore, the oocyte and the *zona pellucida* undergo several morphological and biochemical modifications following fertilization (Rath et al., 2006; Kempisty et al., 2009). Therefore, we suggest that there may be an association between oocyte morphology and the concentration of specific proteins which may be responsible for the fertilization ability of the female gametes.

Integrins are cell adhesion proteins responsible for several cellular processes, including cell proliferation and differentiation in mammals, including humans, mice, and cows (Ziyyat et al., 2006; Barraud-Lange et al., 2007; Feugang et al., 2009; Roberts et al., 2009). These proteins are also major receptors by which cells attach to their extracellular matrices, and are involved in signal transduction pathways. The most recent findings indicate a possible role for these molecules in gamete-specific interaction and fusion during fertilization in several species (Rubinstein et al., 2006; Vjugina and Evans, 2008; Vjugina et al., 2009). However, there is only one study to date that has indicated a role for integrins in the process of fertilization in pigs (Linfor and Berger, 2000).

It has previously been reported that the brilliant cresyl blue test (BCB) is one of the most important indicators for the selection of competent oocytes (Roca et al., 1998; Wongsrikeao et al., 2006; Torner et al., 2008). This test is based on the determination of glucose-6-phosphate dehydrogenase (G6PDH) activity, which is synthesized in growing oocytes but is inactive in oocytes that have finished their growth phase. Decreased expression or complete loss of expression of G6PD is highly associated with oocytes that have become competent, and therefore mature. These oocytes fail to enzymatically break down BCB and thus stain positively blue (BCB^+; Roca et al., 1998). Oocytes that have not matured are colorless (BCB^-).

The role of ZP glycoproteins and integrins during the processes of recognition, adhesion, and interaction of gametes has been well described. However, there are no indications on the association between oocyte morphology and the expression of the above genes. Therefore, the aim of this study was to analyze the expression pattern of ZP glycoproteins and integrins in morphologically distinct oocytes.

MATERIAL AND METHODS

Animals

A total of 20 puberal crossbred Landrace gilts with a mean age of 155 days (range 140–170 days) and mean weight of 100 kg (95–120 kg) were used in this study. The experiment was performed in gilts during their natural oestrus. All animals were checked for oestrus once daily for 15 min using fence-line contact with a mature boar. After commencement of oestrus, detection continued over the following days, and on Day 17 of the subsequent oestrous cycle gilts were slaughtered.

The experiments were approved by the local Ethics Committee.

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

Ovaries were collected from gilts at slaughter on Day 17 of the oestrous cycle. The ovaries and reproductive tract were recovered and transported to the laboratory within 10 min at 38°C in 0.9% NaCl. The oocytes were isolated from follicles of 3 to 5 mm in size, as well as from follicles greater than 5 mm in diameter if such were found, according to the procedures published by Antosik et al. (2009). The recovered oocytes were randomly divided into two groups; a group of oocytes used in the present study and a second group of oocytes that were used

in the recent paper by Jackowska et al. (2009), described in that study.

The ovaries were then placed in 5% fetal bovine serum solution (FBS, Sigma-Aldrich Co., St. Louis, MO) in PBS. The follicles (large, medium and small) were opened by puncturing of individual follicles in a sterile petri dish, and the 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 bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA). This was followed by COC selection under a stereoscopic microscope based on morphological evaluation using the four grade scale suggested by Jackowska et al. (2009). Grade I COCs display a homogeneous cytoplasm and a complete cumulus oophorus; grade II have a homogeneous cytoplasm and an incomplete but compact cumulus oophorus with more than two layers; COCs of grade III are characterized by a heterogeneous cytoplasm and a greater-than-one-cell-layer cumulus oophorus; and grade IV oocytes have a strongly heterogenous cytoplasm and either a partial or completely absent cumulus oophorus, respectively (Jackowska et al., 2009).

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

Before cultivation, COCs were assessed by washing two times in modified Dulbecco PBS (DPBS, Sigma-Aldrich Co., St. Louis, MO) supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin (Sigma-Aldrich Co., St. Louis, MO), 0.4% [w/v] BSA, 0.34mM pyruvate, and 5.5mM glucose (DPBSm).COCs were then treated with 26 μM BCB (Sigma-Aldrich Co., St. Louis, MO) 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. During the washing procedure, the oocytes were examined under an inverted microscope (Zeiss, Axiovert 35, Lubeck, Germany) and classified as either having stained blue (BCB^+) or having remained colorless (BCB^-). Only BCB^+ COCs, which had reached developmental competence, were used in the experiment.

In vitro maturation of porcine COCs

Developmentally competent COCs were cultured in Nunclon™ 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, Grand Island, NY, USA) supplemented with 2.2 mg/ml sodium bicarbonate (Nacalai Tesque, Inc., Kyoto, Japan), 0.1 mg/ml sodium pyruvate, 10 mg/ml BSA, , 0.1 mg/ml cysteine (all supplements from Sigma-Aldrich, St. Louis, MO, USA), 10% (v/v) filtered porcine follicular fluid and gonadotropin supplements at a final concentration 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 a mineral oil overlay and cultured for 44 h at 38°C under 5% CO_2 in air.

Thereafter, COCs were incubated with hyaluronidase (Sigma-Aldrich Co., St. Louis, MO, USA) for 2 min at 38°C to separate cumulus cells and granulosa cells. The cells were removed by vortexing the oocytes in 1% sodium citrate buffer and by mechanical displacement using a small-diameter glass micropipette. To determine the effect of oocyte morphology on nuclear maturation, COCs were fixed after maturation and the nuclear stage was assessed following 4,6-diamino-2-phenylindole (DAPI) staining according to the method described previously by Beker et al. (2002). Only mature oocytes, stage MII, were used in further analyses.

Preparation of zona-free oocytes

After oocyte maturation, the *zona pellucida* was dissolved in acid Tyrode's solution (pH 2.1) for 1 to 2 min, and zona-free oocytes were washed three times in TYH medium (119.37mM NaCl, 4.78mM KCl, 1.71mM $CaCl_2 \cdot 2H_2O$, 1.19 mM $MgSO_4 \cdot 7H_20$, 1.19mM KH_2PO_4, 25.07mM $NaHCO_3$, 5.56mM glucose, 0.5mM sodium pyruvate, 75 μg/ml potassium penicillin G, 50 μg/ml streptomycin sulfate, 2 μg/ml of phenol red) (Toyoda et al., 1971) supplemented with PVA (1 mg/ml, cold water soluble; Sigma) or BSA (4 mg/ml, FractionV, Amersham Biosciences, Cleveland, Ohio).

Confocal microscopic observation

Oocytes were fixed with 2.5% paraformaldehyde in PBS and 0.2% Triton-X 100 (Sigma-Aldrich, St. Louis, MO, USA) 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. Oocytes were then 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, 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 confocal system LSN 510 on Carl ZEISS microscope Axiovert 200M. FITC was excited with a wavelength of 488 nm from an argon laser, and emissions were imaged through a 505–530 nm filter. Negative controls were also performed, in which the primary antibody was not introduced, for both integrin β2 and pZP3. The homology of the antigenic peptide used to generate the anti-pZP3 Ab with the Sus scrofa sequence (Swiss-Prot P42098) is 90%, while the homology of the antigenic peptide used to generate the anti-integrin β2 Ab with the Sus scrofa sequence (Swiss-Prot P53714) is 100%.

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

Oocytes isolated from an additional, similarly selected pool of grade I–IV COCs (n = 15 per group) were treated by RIPA lysis buffer. Next, the proteins were resuspended in sample buffer and separated on a 15% Tris-glycine gel by 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 horseradish peroxidase (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 Inc., Rockford, IL, USA).

Statistical analysis

One-way ANOVA followed by the Tukey post-test was used to compare the results of densitometric analyses of protein levels. The differences were considered to be significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$. The software program GraphPad Prism version 4.0 (GraphPadSoftware, San Diego, CA) was used for the statistical calculations (Prochazka et al., 2004).

RESULTS

A relatively small number ($P < 0.01$) of grade I and II oocytes were recovered for this study compared to oocytes graded III and IV, respectively. We also found an increased number of BCB^+ grade I oocytes as compared to BCB^- grade I oocytes ($P < 0.05$). The number of grade II BCB^+ and BCB^- oocytes was similar ($P = 0.09$). Moreover, the mean number of BCB^- oocytes was higher than that of BCB^+ oocytes ($P < 0.05$, $P < 0.01$, respectively) in

Table 1. Total and mean number (± SEM) of four morphologically different types of oocytes 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	131 (43.6 ± 3.1)	53 (17.6 ± 3.05)	78 (33.6 ± 2.6)	0.05
Grade II	136 (45.3 ± 5.3)	73 (30.6 ± 3.1)	63 (30.3 ± 3.4)	0.09
Grade III	185 (61.6 ± 5.2)	101 (47 ± 5.1)	84 (21 ± 2.6)	0.05
Grade IV	283 (94.3 ± 6.2)	184 (61.3 ± 5.3)	99 (33 ± 6.9)	0.01

Total number of oocytes is that recovered from all gilts in all replicates; mean number of oocytes is derived from the total number of recovered oocytes divided by the number of replicates; BCB^- and BCB^+ oocytes recovered from all gilts. The experiments were carried out in three replicates

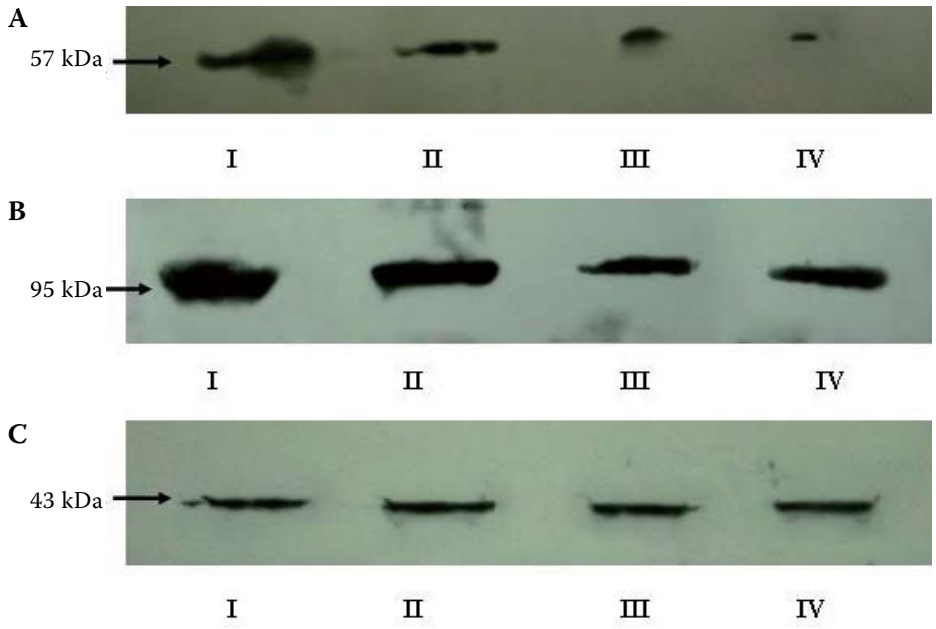

Figure 1. Western blot analysis. For western blot analysis, proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane that was then immunoblotted with goat polyclonal anti-pZP3 Ab (N-20) (**A**), and goat polyclonal anti-integrin β2 Ab (C-20) (**B**), followed by incubation with donkey anti-goat HRP-conjugated Ab. To equalize protein loading, the membrane was reblotted with anti-actin HRP-conjugated Ab (**C**). Optical density (OD) was evaluated by using Gel Logic 200 Imaging System (Kodak, Rochester, NY, USA). Oocytes were graded on a four-grade morphological scale as follows; I, II, III, IV, as suggested by Jackowska et al. (2009)

those graded III and IV (Table 1). The percentage of MII oocytes, determined by DAPI staining, for COCs graded I, II, III, and IV was 40.5, 26, 11, and 8.5, respectively.

After using western blot analysis, we determined the pZP3 and integrin β2 protein levels in four morphologically different types of oocytes (Figure 1A–B). We found an increased level ($P < 0.05$, $P < 0.01$, $P < 0.001$) of pZP3 protein in grade-I oocytes as compared to other grades (Figure 1A, 2A). The integrin β2 level was increased in oocytes graded I and II as compared to those graded III and IV ($P < 0.05$), (Figure 1B, 2B). The differences between the other grades of gametes (III and IV) were less visible and not statistically significant for both pZP3 and integrin β2 (Figure 1A–B, 2A– B).

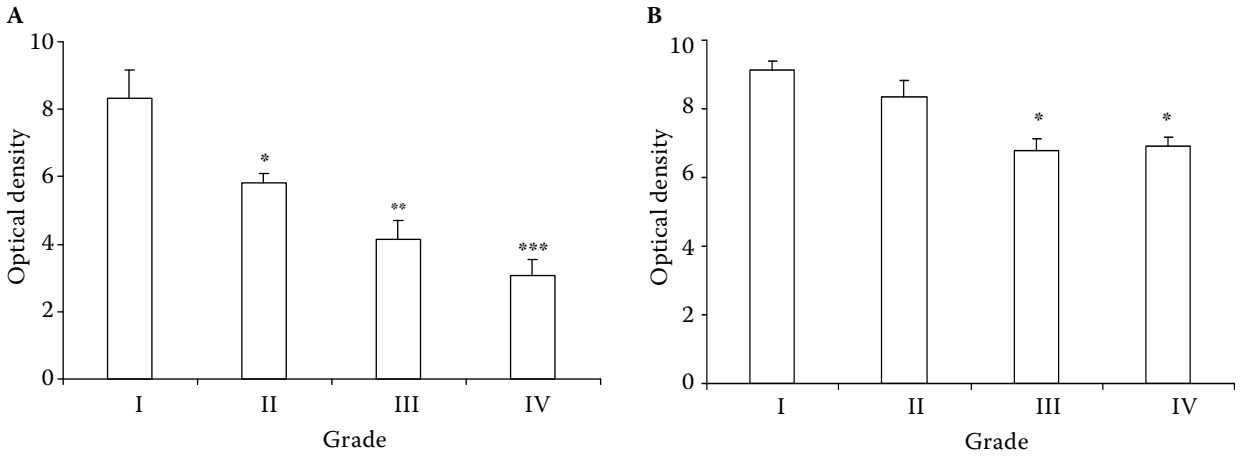

Figure 2. Optical density (OD) analysis. OD was evaluated using Gel Logic 200 Imaging System (Kodak, Rochester, NY, USA). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ were determined as the levels of significance. OD for pZP3 (**A**) and integrin β2 (**B**) was analyzed. In this experiment, four morphologically different types of oocytes were classified as previously described by Jackowska et al. (2009)

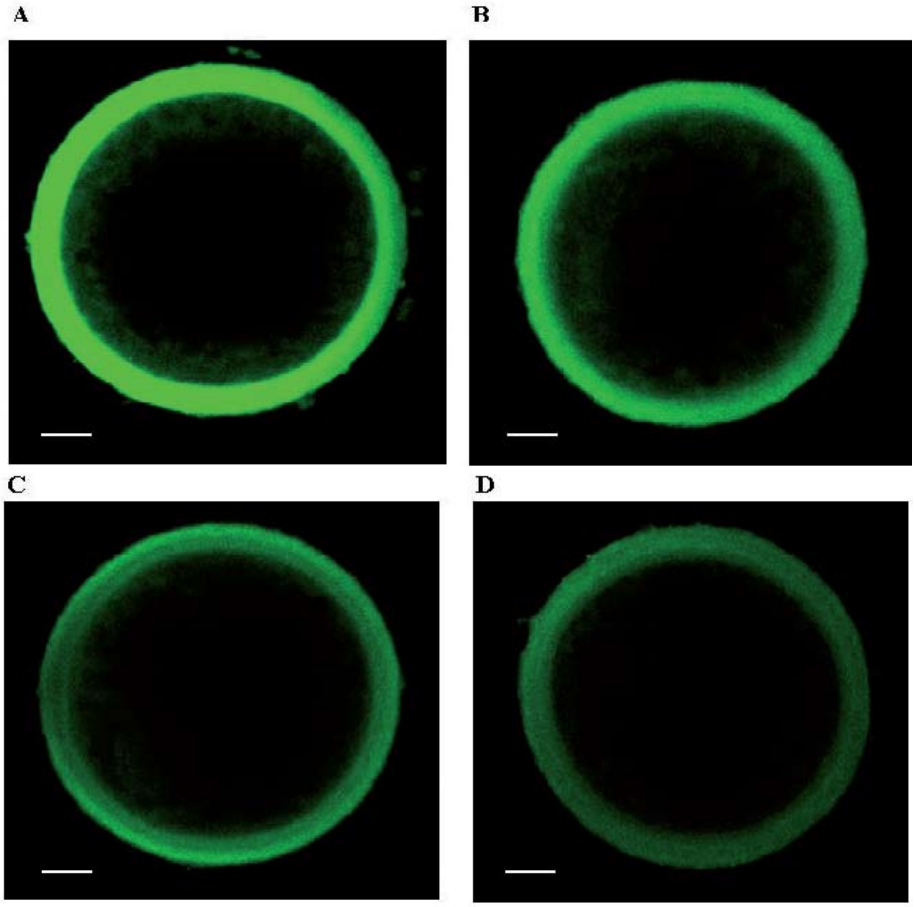

Figure 3. Confocal microscopic observation of four morphologically different groups of oocytes. Porcine oocytes graded I, II, III, and IV were stained with porcine pZP3 Ab (**A–D**) (goat polyclonal anti-pZP3 Ab, N-20) and integrin β2 Ab (goat polyclonal anti-integrin β2 Ab, C-20) (**E–H**) for zona pellucida-specific localization. The treated oocytes were labeled for 40 min with FITC-conjugated anti-goat IgG Ab at a 1 : 200 dilution in PBS. Following washing in PBS/0.1% Tween 20, the oocytes were mounted on glass slides in an antifade drop and observed under confocal system; bars are 20 μm

Regarding the oocyte *zona pellucida* distribution of pZP3 and integrin β2, we observed a large pZP3-containing *zona pellucida* in approximately 90% of all grade I oocytes. A decreased expression of this protein was visualized in the *zona pellucida* of other grades of oocytes (Figure 3 A–D). Staining with an anti-integrin β2 antibody showed a morphologically identical structure. Similarly, we observed an increased expression of integrin β2 in approximately 90% of the entire investigated grade I oocytes, and a decreased level of this protein in the other grades (Figure 3 E–H).

DISCUSSION

In our previous work we reported 3–4 fold higher mRNA levels of the porcine *zona pellucida* glycoproteins – pZP1, pZP2, pZP3 and pZP3α (pZP4) – in mature oocytes graded as I as compared to the other grades of gametes (Jackowska et al. 2009). Analysis of differences between each of the grades of oocytes other than grade I did not indicate statistical significance.

In the present study we described the association between oocyte morphology, oocyte developmental competence and the level of proteins responsible for the oocyte quality of porcine oocytes after IVM. Our results indicate that COCs with the best morphology and quality, graded as I in the scale suggested by Jackowska et al. (2009), may reflect increased fertilization ability. As the quality of oocytes and their proper morphology is forming during the processes of oogenesis and folliculogenesis, the fertilization ability of oocytes should also be established at these stages. When oocytes

grow and develop, they undergo intense processes of mRNA and protein synthesis, and accumulate those components that comprise the embryonic genome. Thus, in oocytes of higher quality, these processes must be much more intensive than in the other, lower quality oocytes. This accumulated material is one of the most important factors determining early embryonic development during the preimplantation stage. The increased levels of some mRNAs and proteins in "high quality" oocytes may suggest that these gametes are also characterized by increased "molecular quality" (accumulation of specific mRNA and proteins), which is the key to successful embryo growth and development. However, to improve the "molecular quality" of an oocyte, further analysis from a total transcriptome and proteome must be performed. In our previous study (Jackowska et al., 2009), we suggested an association between follicular size and the expression of pZP3 protein in porcine oocytes. In the present study we found an increased level of this protein in oocytes isolated from large and medium follicles as compared to small follicles, using both western blot and confocal microscopic analysis. In the present paper the oocytes were selected according to their morphological characteristics, dissected from various sized follicles, and pooled in one group from which the oocytes were morphologically evaluated. Direct correlations between follicular size and quality of the resulting oocytes have previously been described by several authors (Humblot et al., 2005; Mourot et al., 2006; Feng et al., 2007). However, Stankiewicz et al. (2009) found several differences in the viability and size of oocytes isolated from the same polyovular follicles, indicating that even the same follicle can contain a highly heterogeneous group of oocytes. On the other hand, Antosik et al. (2009) demonstrated that porcine follicular size is highly associated with the expression of genes encoding proteins responsible for the fertilization ability of oocytes. They concluded that the follicular size may be one of the most important factors determining the process of successful fertilization.

The biochemical and morphological ultrastructure of the *zona pellucida* is permanently modified, especially during oocyte maturation prior to fertilization and early embryo development. It has been shown that the structure of the ZP is different between oocytes matured *in vivo* or *in vitro* (Rath et al., 2005). Moreover, specific culture conditions and media supplements may significantly affect the *zona pellucida* ultrastructure, varying from a porous, net-like structure to a nearly smooth and compact surface (Michelmann et al., 2007). Thus, they suggested that the specific protein concentrations may be also affected by IVM conditions. Our results require further investigation with analyses of protein contents in oocytes collected from ovaries and without the IVM phase.

In this study, a higher number of BCB^+ oocytes were found in grade I gametes. Our experiment indicates that an oocyte's quality may be associated with its developmental competence. However, it was found that the BCB staining test may have a toxic effect on oocytes (Wongsrikeao et al., 2006). Future investigations will need to be performed with analysis of the role of the BCB staining test on the expression of sperm-egg interaction genes, too.

Roca et al. (1998) have shown that BCB^+ oocytes reflect an increased fertilization ability and penetrability over BCB^- gametes. It has also been demonstrated that the BCB staining test may serve as a preliminary test to select for oocyte viability (Torner et al., 2008). Since the BCB test results depend on the fertilization ability of gametes, it is also suggested that the molecular pathway related to this indicator may be associated with increased or decreased expression of ZP glycoproteins and integrins. Rath et al. (2005) suggested that the ZP must also undergo maturation during oocyte growth and development, and following fertilization. Their results indicate that the ZP undergoes several biochemical changes during the final maturation phase of the oocyte prior to fertilization. We suggest that the changes in oocyte morphology and in the morphology of the ZP may be correlated with the expression of ZP glycoproteins.

There are some reports on the role that integrins play in fertilization in many mammalian species (Burke et al., 2004; Kaji and Kudo, 2004; Sengoku et al., 2004; Kempisty et al., 2009). However, there has been no indication of an association between oocyte morphology and integrin expression until now. Here we have described that an increase in integrin protein levels is associated with higher oocyte quality. Similar to ZP glycoproteins, high levels of integrin proteins can be the result of increased accumulation of transcripts and proteins in oocytes characterized by the best morphology prior to fertilization.

In conclusion, it was demonstrated that the expression of sperm-oocyte fusion proteins is associated with cumulus-oocyte-complex morphology.

We suggest that the "morphological quality" of an oocyte may be associated with its "molecular quality", with a special relationship to the porcine oocyte's fertilization ability. Future studies may examine, using IVF and *in vitro* production (IVP) in pigs, the use of primary microscopic selection of COCs to increase embryonic yields.

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