Assessment of transcript and protein levels contributing to cell cycle control and gap junction connections in morphologically variable groups of porcine cumulus-oocyte complexes

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ABSTRACT: Oocytes and somatic cumulus cells are connected by an extensive network of gap junctions. These connections contribute in a major way to oocyte maturation and developmental competence. Cumulus-oocyte complexes (COCs) were cultured in standard porcine IVM culture medium (TCM 199) for 44 h. The morphological classification of COCs is based on the number of cumulus cell layers and the degree of their compaction, as well as on cytoplasm composition (homogenous, heterogeneous). The obtained COCs were divided into four grades according to this classification system. By assessing the activity of glucose-6-phosphate dehydrogenase (G6PDH) using the brilliant cresyl blue (BCB) test, real-time quantitative PCR (RQ-PCR) reaction methods and confocal microscopic observations, we determined the transcript levels of connexins 43 and 45, cyclin dependent kinases (cdk5 and cdk5r), and cdk inhibitors 1 and 3 (p27kip1 and cdkn3) as well as cdk4 protein in morphologically different groups of porcine oocytes isolated from puberal gilts. To assess their nuclear status the completely denuded oocytes were subjected to DAPI staining. We found statistically increased cdkn3, cdk5 and connexin 45 mRNA levels in oocytes graded as I as compared to II, III, and IV. The cdkn1, cdk5r and connexin 43 transcript contents were higher only when comparing between oocytes graded as I, III and IV. The cdk4 protein in oocytes graded I and II is localized mainly in the *zona pellucida*, although in grade III COCs the expression of this protein is decreased and observed only in the cytoplasm. Grade IV COCs do not demonstrate a significant presence of cdk4 protein. With regards to nuclear maturation, the percentage of MII stage oocytes was significantly ($P < 0.05$) higher in grade I and II oocytes as compared to grade III and IV oocytes. Our results demonstrate for the first time that cdk4 protein localization and all of the investigated transcript levels are associated with COC morphology and may be related to further maturation ability as well as developmental competence of oocytes.

Keywords: pig; oocyte; connexins; cyclin-dependent kinases

In all mammalian species, individual oocytes differ significantly in their ability to mature, undergo successful fertilization, and give rise to proper embryo growth (Miyano and Manabe, 2007; Obata et al., 2007; Assidi et al., 2008; Mermillod et al., 2008; Smith et al., 2009). Oocyte quality is crucial for embryo viability and subsequent ability of the embryo to grow and develop (Kelly et al., 2007; Patrizio et al., 2007; Singh and Sinclair, 2007; Ciray et al., 2008). Since the developmental competence of

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the female gamete is defined as the potential of the oocyte to undergo maturation steps and develop to the blastocyst stage or live offspring, proper characterization and selection of good quality oocytes is the most important step before *in vitro* fertilization (IVF) and *in vitro* production (IVP) programs in pigs.

One of the most important criteria, now in use for some time, is primarily based on the morphology of the oocyte. Morphological criteria are mostly used to select good quality cumulus-oocyte-complexes (COCs) for IVF and IVP of embryos in several species of mammals (Combelles and Racowsky, 2005; Balaban and Urman, 2006; Sato et al., 2007). Generally, oocytes with compact cumulus and homogeneous ooplasm are considered viable and healthy (Pujol et al., 2004). However, the definitive characteristics of developmentally competent COCs are still unknown.

Oocytes are coupled to surrounding somatic cells by highly specific cell membrane components called gap junctions. These membrane connections contain specific channels to mediate the transfer of small metabolites, signal proteins and regulatory molecules from one cell to another. The connection between oocyte and granulosa cells *via* gap junctions is essential for proper oocyte maturation and embryo growth (Carabatsos et al., 2000).

Gap junctions are collections of intracellular membrane channels and are composed of connexins (Cx's). The Cx's are members of a large family of more than 20 proteins, which participate in communication between oocytes and the complex of cumulus cells. Therefore, Cx's play a significant role in the process of oocyte maturation.

The cyclin-dependent kinases (CDKs) are involved in cell cycle progression. These kinases are associated with activating molecules called cyclins. The activation of CDKs and the subsequent phosphorylation of their substrates regulate the cell cycle. It was recently demonstrated that all of the presently investigated genes may contribute to the regulation of maturation mechanisms in oocytes (Font de Mora et al., 1997; Akamatsu et al., 1998; Motlik et al. 1998; Vozzi et al., 2001; Calder et al., 2003; Sato and Yokoo, 2005; Feuerstein et al., 2007; Whitten et al., 2007).

Therefore, the aim of the present study was to determine the possible association between the morphology of COCs and specific mRNA and protein contents of molecules contributing to the maturation ability of porcine female gametes.

MATERIAL AND METHODS

Animals

A total of thirty crossbred Landrace puberal gilts with median age of 160 days (range 140–180 days) and median weight 100 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 cumulus-oocyte complex (COCs) classification

The ovaries were collected from thirty crossbreed Landrace puberal gilts. After slaughter the ovaries and reproductive tract were recovered and transported to the laboratory within 30 min at 38.5 °C in 0.9% NaCl.

The ovaries from these animals were placed in 5% fetal bovine serum solution (FBS) (Sigma-Aldrich Co. St. Louis, MO) in phosphate buffered saline (PBS). The follicles were opened by puncturing individual follicles in a sterile petri dish, and the COCs were recovered. These 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). The COCs were then visualized under a stereoscopic microscope, counted, and morphologically evaluated carefully, using the four grade scale suggested by Jackowska et al. (2009).

To summarize the classification, COCs graded I have a homogeneous cytoplasm and a complete and compact cumulus oophorus; grade II COCs have a homogeneous cytoplasm and an incomplete but compact cumulus oophorus; 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 and dark cytoplasm and completely absent cumulus oophorus.

In vitro maturation of porcine COCs

After measurements, the collected COCs were cultured in Nunclon$^{TM}\Delta$ 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 (Sigma-Aldrich), 10 mg/ml BSA, (Sigma-Aldrich, St. Louis, MO, USA), 0.1 mg/ml cysteine (Sigma-Aldrich), 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.

Assessment of nuclear maturation

After culture of COCs, the cumulus cells were removed by vortexing. The oocytes were then fixed for 15 min in 2.5% (w/v) glutaraldehyde (Merck, Darmstadt, Germany) in PBS, following by washing twice with PBS, staining with 0.1 µg/ml 4,6-diamino-2-phenylindole (DAPI; Sigma, St. Louis, MO, USA) in PBS, and mounting on slides. The evaluation of nuclear status was performed by epifluorescence microscopy (BH2-RFCA; Olympus, Tokyo, Japan). The oocytes were classified as either MII – oocytes with either a polar body or two shiny chromatin spots – or aberrations – oocytes with an abnormal chromosomal organization.

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

After cultivation, oocytes (graded as I, II, III, and IV) were washed two times in modified Dulbecco phosphate buffered saline (PBS-DPBS) (Sigma-Aldrich Co. St. Louis, MO) supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin (Sigma-Aldrich), 0.4% [w/v] BSA, 0.34mM pyruvate, and 5.5mM glucose (DPBSm). Oocytes were treated with 26µM BCB (Sigma-Aldrich) 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 two times. During the washing procedure, the oocytes were examined under an inverted microscope (Zeiss, Axiovert 35, Lübeck, Germany) and classified as either having stained blue (BCB^+) or remained colorless (BCB^-). Only BCB^+ oocytes, which had completed their growth phase and may have reached higher developmental competence, were used in the experiment. The BCB staining test, usually applied before IVM, has been clearly demonstrated to be a useful tool in assessing the developmental competence of COCs and for the pre-selection of female gametes for IVF and embryo production (Alm et al., 2005; Bhojwani et al., 2007; Egerszegi et al., 2010). However, several factors such as oocyte diameter, animal sexual maturity and gonadotropin stimulation during IVM have also been shown to possibly impact on the developmental competence of BCB^+ oocytes (Wu et al., 2007). We assessed the developmental competence of porcine oocytes after IVM to acquire a higher number of BCB^+ COCs as well as to define the efficiency of *in vitro* culture.

Confocal microscopic observation

Oocytes (graded as I, II, III, and IV) were incubated with 300 µg/ml bovine testicular hyaluronidase (Sigma-Aldrich) for 5 min at 38 °C to remove cumulus cells. Oocytes were fixed with 2.5% paraformaldehyde in PBS and 0.2% Triton-X 100 for 30 min at room temperature (RT) and washed three times in PBS/PVP (polyvinyl pyrrolidine), (0.2%). To block nonspecific binding, samples were incubated with 3% BSA in PBS with 0.1% Tween 20 for 30 min at RT. Oocytes were incubated for 12 h at 4 °C with rabbit polyclonal anti-Cdk4 antibodies (Ab) (H-22), (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 with rabbit polyclonal anti-Cdk4 Ab were incubated for 1 h at RT with fluorescein isothiocyanate (FITC) – conjugated goat anti-rabbit IgG Ab diluted 1 : 200 in PBS/0.1% Tween 20.

Real-time quantitative PCR (RQ-PCR) analysis of transcript levels in morphologically different groups of porcine oocytes

Total RNA was isolated from 40 oocytes (from each puberal gilt) described as grade I (n = 10), grade II (n = 10), grade III (n = 10), and grade IV (n = 10), using an RNeasy mini column (Qiagen GmbH, Hilden, Germany), (Kempisty et al., 2008, 2009; Jackowska et al., 2009). The RNA samples were resuspended in RNase-free water and stored in liquid nitrogen. RNA samples were treated with DNase I and reverse-transcribed into cDNA. RQ-PCR

was conducted in a LightCycler real-time PCR detection system (Roche Diagnostics GmbH, Mannheim, Germany) using SYBR® Green I as detection dye, and target cDNA was quantified using the relative quantification method. For amplification, cDNA solution was added to 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 in subsequent PCR.

The housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (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 ß-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 ß-actin mRNA was not regulated in the four morphologically different groups of oocytes. This gene has been identified as an appropriate housekeeping gene for use in quantitative PCR studies (Thellin et al., 1999). The expression of GAPDH and ß-actin did not vary when normalized against 18S rRNA (results not shown).

Statistical analysis

One-way ANOVA followed by the Tukey post-test was used to compare the results of real-time RT-PCR quantification. The experiments were carried out in at least three replicates. The results quantifying relative abundance (RA) of investigated mRNAs are expressed as the mean of the transcript: GAPDH/ß-actin/18S rRNA ratio. 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 (GraphPad Software, San Diego, CA) was used for the statistical calculations.

RESULTS

It was recently demonstrated that oocytes with homogenic ooplasm and compact cumulus cells, and thus grade I in the scale suggested by Jackowska et al. (2009), are considered to be healthy and have an increased developmental potential. Therefore, in our study we compared oocytes of grade I to the other oocyte grades: II, III, and IV.

We found an increased CDKN1 transcript level in oocytes graded as I only as compared to III and IV, ($P < 0.05$) (Figure 1A). When comparing the CDKN3

Table 1. Oligonucleotide sequences used for RQ-PCR analysis

Transcript	Sequence (5'-3' direction)	Gene accession No.	Product size (bp)
CDKN 1	ACAGGCGGAGTACCCCAA GCCCTTTTCCACCTCCTGC	NM214316	190
CDKN 3	AGACTGCCAGCGATGAAGC TCGTGACAGGGGTAGCCAT	NM214320	120
CDK 5	GGGAAGGCACCTATGGAAC AGGGCAGAACTTGGCACTC	NM001044621	115
CDK 5R	TGTTTTGTCACTCTCGAAG CTGCCAGTTTAATTGTTCC	NM001123097	87
Cx 43	AGGGAAGGTGTGGCTGTCA ACGTGAGAGATGGGGAAGG	AY382593	162
Cx 45	GCAAACCAGTTCCGTCACCAT CGCAAAGGCATCATAGCAGAC	NM001097519	226
GAPDH	CTGCACCACCAACTGCTT TTCTGGGTGGCAGTGATG	AF069649	105
β-actin	GGGAGATCGTGCGGGACAT CGTTGCCGATGGTGATGAC	DQ845171	141
18S rRNA	GTGAAACTGCGAATGGCTC CCGTCGGCATGTATTAGCT	AB117609	105

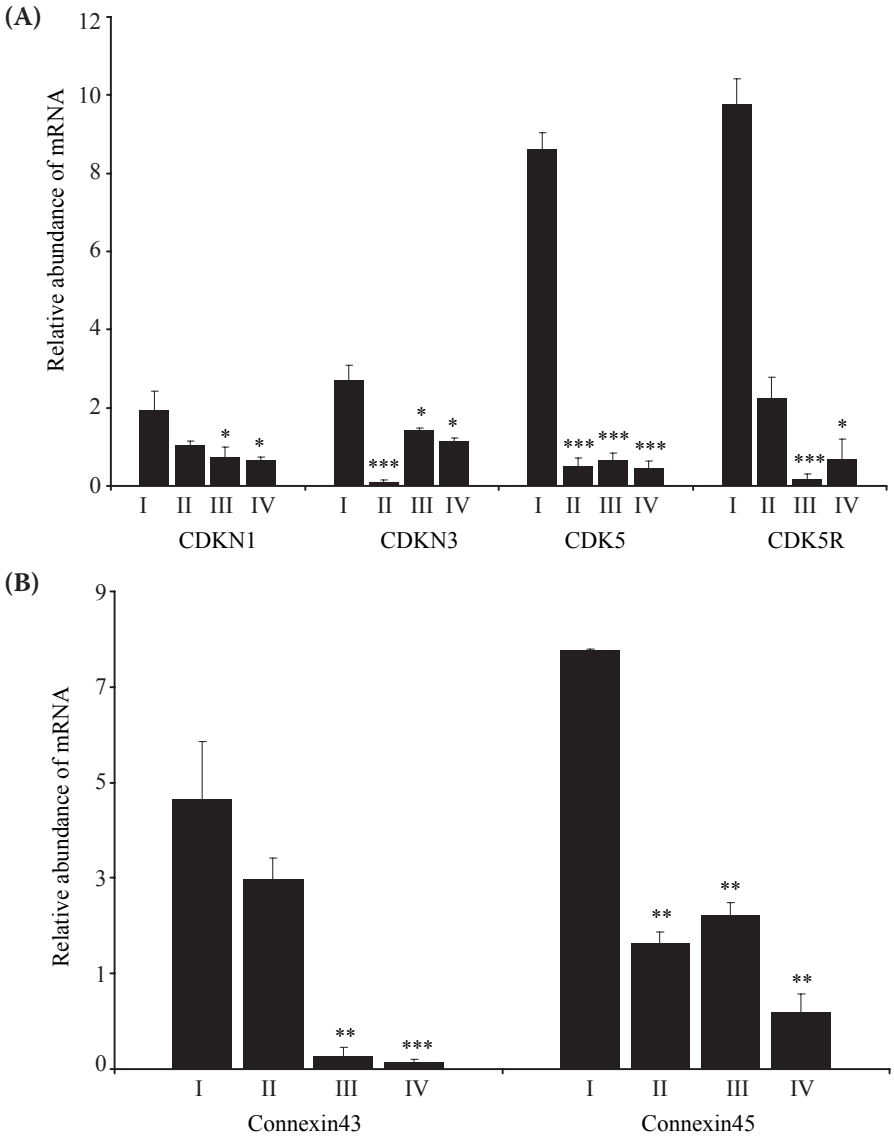

Figure 1. Relative abundance of CDKN1, CDKN3, CDK5, CDK5R, Cx43 and Cx45 mRNAs in morphologically variable groups of porcine oocytes. RNA from porcine oocytes was isolated immediately after recovery of these oocytes (n = 40), (grade I oocytes, n = 10; grade II oocytes, n = 10; grade III oocytes, n = 10; grade IV oocytes, n = 10). The RNA was reverse-transcribed into cDNA. RQ-PCR was used to evaluate the presence and quantity of CDKN1, CDKN3, CDK5, CDK5R (**A**), Cx43, Cx45 (**B**) transcripts. Each sample was determined in triplicate. Results are presented as mean ±S EM with the level of significance: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

and CDK5 mRNA abundance we observed higher transcript levels between oocytes graded as I and II, ($P < 0.001$), I and III ($P < 0.05$, $P = 0.001$), and I and IV ($P < 0.05$, $P < 0.001$), for CDKN3 and CDK5 respectively (Figure 1A). The CDK5R and Cx43 mRNA content was higher in oocytes graded as I when comparing to grades III ($P < 0.001$, $P < 0.01$) and IV, ($P < 0.05$, $P < 0.001$), respectively (Figure 1). The Cx45 mRNA level was increased in oocytes graded as I as compared to others; ($P < 0.01$) (Figure 1B).

Using confocal microscopy, we observed a higher level of cdk4 protein in grade I oocytes as compared to the other oocyte grades (II, III, IV) (Figure 2). In 91% of the studied grade I oocytes, the cdk4 protein was expressed in the periphery of the oocyte cytoplasm under the oolema (Figure 2A). In grade II oocytes the cdk4 protein was localized both in the periphery of the oocyte cytoplasm under the oolema and in the cytoplasm, although cytoplasmatic localization was observed in 66% of

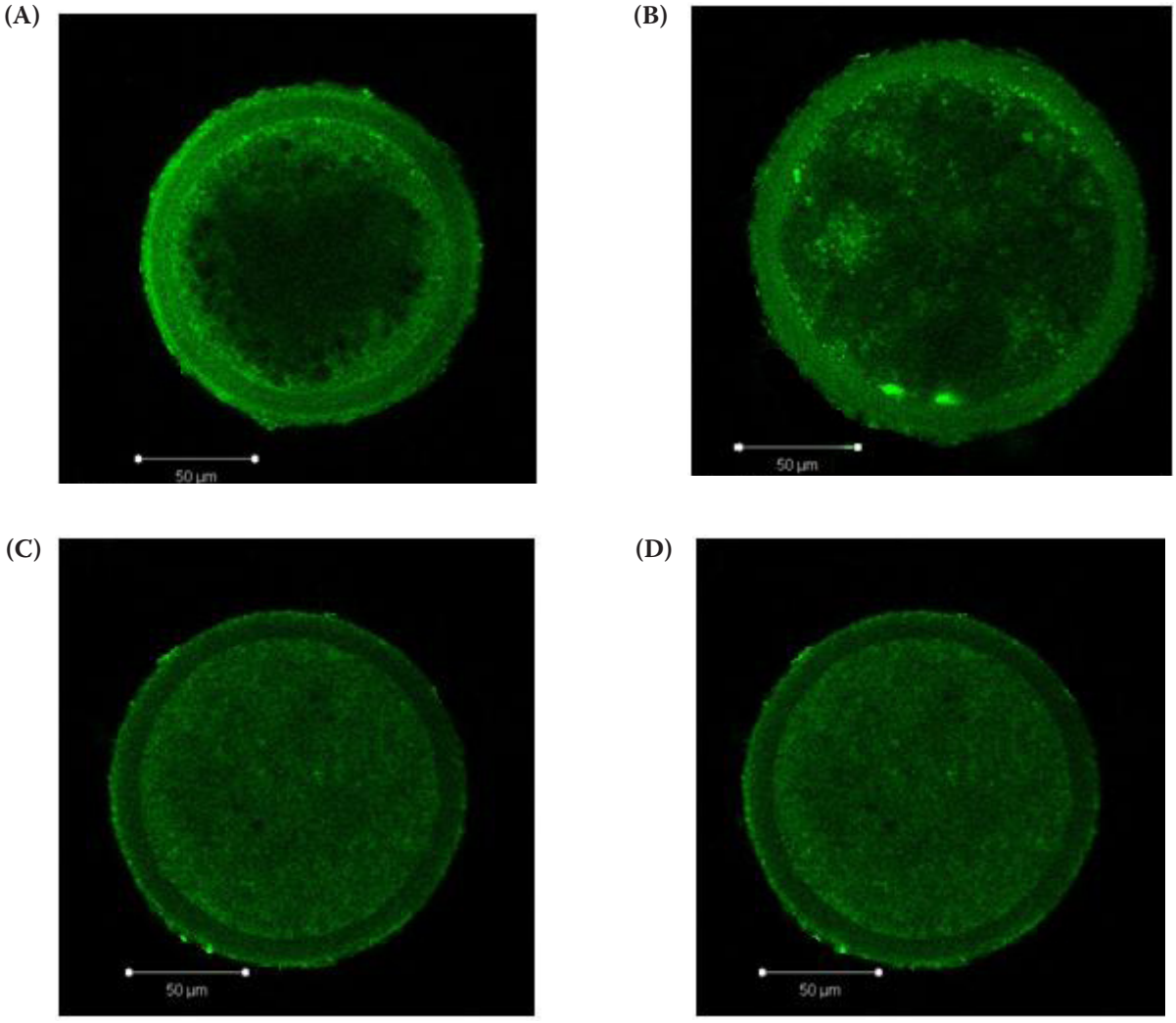

Figure 2. Confocal microscopic observation of Cdk4 protein in four morphologically different oocytes. Porcine oocytes graded as I (**A**), II (**B**), III (**C**), and IV (**D**) were stained with rabbit polyclonal anti- Cdk4 Ab, (H-22). The treated oocytes were labeled for 40 min with fluorescein isothiocyanate (FITC) -conjugated goat anti-rabbit IgG antibody in a 1 : 200 dilution of PBS/0.1% Tween 20. Bars are 50 µm

these oocytes (Figure 2B). Eighty four percent of the investigated grade III oocytes were characterized by cytoplasmatic localization of cdk4 protein (Figure 2C). We did not detect any significant signals from cdk4 protein expression in grade IV oocytes (Figure 2D).

When assessing the progression of meiosis, we found a higher number of MII oocytes graded as I (n = 54) (28.0 ± 3.5) and II (n = 51) (31.5 ± 3.7) as compared to gametes graded as III (n = 21) (13.5 ± 2.2) and IV (n = 17) (10.5 ± 1.6), ($P < 0.05$), (Table 2). When analyzing the developmental competence of porcine COCs using the BCB staining test, we found an increased number of BCB^+ oocytes as compared to BCB^- in the COCs graded as I ($P < 0.05$). In the group of COCs graded as II we did not observe a statistical difference between the number of BCB^+ and BCB^- oocytes. An increased number of BCB^- as compared to BCB^+ COCs was found in the grade III and IV COCs ($P < 0.01$), (Table 2).

DISCUSSION

Oocyte quality is determined by several factors, mainly growth in the specific follicular environment and gamete morphology. The morphology of oocytes is under the influence of many elements, which results in the ability of the female gamete to achieve maturation, successful fertilization

Table 2. Total and (mean ± SEM) number of four morphologically different types of oocytes recovered and identified as BCB^-, BCB^+, and MII assessed after IVM

Morphological grades of oocytes	Number of oocytes recovered	Number of BCB^- oocytes	Number of BCB^+ oocytes	Significant difference between BCB^- and BCB^+ oocytes	Number of MII oocytes
Grade I	112 (51.5 ± 4.8)	48 (21.5 ± 3.3)	64 (35.5 ± 4.1)	0.05	54 (28.0 ± 3.5)
Grade II	127 (56.5 ± 6.5)	61 (38.5 ± 4.0)	66 (39.4 ± 4.2)	0.12	51 (31.5 ± 3.7)
Grade III	198 (74.5 ± 7.5)	131 (68.5 ± 6.8)	67 (46.5 ± 4.2)	0.01	21 (13.5 ± 2.2)
Grade IV	224 (101.5 ± 9.4)	160 (79.5 ± 7.6)	64 (48.0 ± 5.9)	0.01	17 (10.5 ± .6)

Total number of oocytes is that which was 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. Identification of MII oocytes was made using DAPI staining after IVM

and proper further embryo growth and development. The morphological evaluation of oocytes includes: perivitelline space, perivitelline debris, oocyte shape, morphology of the *zona pellucida*, first polar body structure, cytoplasmic granularity, cytoplasmic vacuoles and cytoplasmic coloration. The most recent findings indicate that cytoplasmic coloration and cumulus cell layers (compact/expanded) are the most important predictors of oocyte quality, which may determine the further maturation and fertilization ability of the oocyte, as well as further embryo development (Patrizio et al., 2007; Wang and Sun, 2007).

In this study we evaluated the abundance of mRNA molecules encoding connexins and cyclin-dependent kinases in morphologically different oocytes. Increased levels of all the investigated transcripts and cdk4 protein in oocytes graded as I as compared to other grades may suggest an association between the gametes' morphology and their maturation ability. There are only a few studies indicating the role of connexins and cdk proteins in the regulation of important processes in porcine and bovine reproduction (Shimada et al., 2001; Calder et al., 2003; Marchal et al., 2003; Luciano et al., 2004). Calder et al. (2003) used Cx43 as a marker of the developmental competence of bovine COCs maturing *in vitro*. Calder also also found a significant association between a relatively high level of Cx43 mRNA and an increased quality of bovine oocytes. Most recent findings based on the porcine model indicate that lipid raft clustering of Cx43 plays a role in the breakdown of gap junction connections during IVM of porcine COCs (Sasseville et al. 2009). Based on the porcine model, there is no data demonstrating an association between the morphology of COCs and the expression of genes that are important for oocyte maturation. This study is the first to present such observations.

However, much data on connexin expression have been reported from mice knock out models (Ackert et al., 2001; Gittens and Kidder, 2005; Li et al., 2007). Li et al. (2007) demonstrated that connexin37 is the main protein responsible for communication between oocytes and granulosa cells. Mice lacking this protein were sterile, as their gametes did not complete development. Using the Cx37 (–/–) null mutant mouse line, Li et al. (2007) proved that Cx43 is a physiological equivalent to Cx37 and therefore may take over the function in communication between the gamete and somatic cells. Similar results were obtained by Gittens et al. (2005), who demonstrated that both follicle development and oocyte maturation were impaired in ovaries containing wild-type gametes and Cx43 (–/–) deficient granulosa-cells. However, much more interesting results were reported by Ackert et al. (2001), who reported that mice lacking Cx43 had several morphological dysmorphisms, including: poorly developed zona pellucida, vacuolated cytoplasm of both granulosa cells and oocytes, and absence of cortical granules in the oocyte. These results confirm our hypothesis that expression of Cx43 may correlate with the role of connexins in the morphogenesis of both gametes and somatic cells, which may have consequences with respect to oocyte quality and further early embryo development. Our results also confirmed previous results

showing that Cx43 is an important marker in the assessment of oocyte maturation potential and developmental competence (Calder et al., 2003).

There are several reports indicating that CDKs regulate folliculogenesis and oogenesis, resulting in cell cycle progression and oocyte maturation (Font de Mora et al., 1997; Motlik et al., 1998). Li et al. (2007) demonstrated that CDK-5 (–/–) null mutant mice present an increased number of atretic immature oocytes within the ovaries and a higher number of degenerating oocytes. They also demonstrated a decreased quality of oocytes as a result of increased elimination of these cells via apoptosis. These results may support our findings that decreased levels of CDK mRNA and protein in oocytes with decreased quality may be a result of the directing of these cells to the apoptotic pathway. Moreover, we found differences in the localization of cdk4 protein in oocytes of different morphological quality. In oocytes graded I and II, this protein was localized in the periphery of the oocyte cytoplasm under the oolema and, in the case of grade II oocytes, in the cytoplasm. In grade III and IV oocytes, which are of decreased quality, cdk4 protein was localized mostly in the cytoplasm, if it was demonstrable at all. A similar study was conducted by Kohoutek et al. (2004), who demonstrated that the investigated cdk4 protein is involved in important regulation processes of oocyte development. However, they also found that the cdk4 protein was localized in the nucleus in growing oocytes and in fully grown GV-stage oocytes. Moreover, the other important cell control proteins such as cyclin D3 and p27 or cdk6 were localized also in the cytoplasm of growing and fully grown oocytes. When they assessed the localization of these regulators in relation to the meiotic maturation of oocytes, they showed homogenous distribution of all investigated proteins, including cdk4, throughout the cytoplasm. This study may be the first to demonstrate a variable localization of cdk4 within oocytes, which may be associated with the quality of these oocytes as well as their developmental competence or may be related to the function of this protein. Rajareddy et al. (2007) demonstrated that the ovaries of mice lacking CDKN1 (p27kip1, –/–) were largely depleted, leading to premature ovarian failure. Thus, they showed that CDKN1 is an important factor in the process of proper ovarian development. Disturbances in folliculogenesis as a cause of dysmorphisms in ovarian morphology may lead to infertility.

CONCLUSIONS

In this study we investigated the abundance of the mRNAs and proteins responsible for gap junction communication and oocyte maturation. We demonstrated that the specific transcript and protein levels may be related to oocyte morphology. Moreover, we showed that the expression of gap junction genes has specific localization from the zona pellucida to the cytoplasm according to the morphological quality of oocytes, which may have an impact on proper oocyte maturation, acquisition of cytoplasmic competence and proper embryo growth. Therefore, the levels in oocytes of gap junction proteins may be an important indicator of oocyte developmental ability.

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