Expression of cyclin-dependent kinase inhibitors (*CDKN1, CDKN5*) in developmentally competent and incompetent porcine oocytes

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ABSTRACT: Although several reports have been published regarding the cyclin-dependent kinases (Cdks) in developmentally competent mammalian oocytes, still little is known about their expression pattern in developmentally incompetent female gametes. Since Cdks are the main cell cycle division regulators, also described as "checkpoints" in the MI to MII transition, the aim of this study was to investigate the differential mRNA expression of genes encoding *Cdkn1* and *Cdkn5* in developmentally competent and incompetent porcine oocytes. Porcine cumulus-oocyte complexes (COCs) were collected from crossbred Landrace gilts after slaughter and partly subjected to brilliant cresyl blue (BCB) staining and *in vitro* maturation (IVM). Three groups of COCs were analysed: (i) oocytes soon after collection without BCB staining, (ii) oocytes which remained colourless after staining (BCB^-) and (iii) COCs which stained blue (BCB^+). BCB^+ COCs were additionally cultured in standard porcine medium (TCM199) for 44 h. All oocytes were analysed using QT-PCR targeting *CDKN1* and *CDKN5* mRNA expression. The highest *CDKN1* mRNA expression was found in oocytes without BCB staining and IVM (Group I) compared to BCB^- and BCB^+ oocytes, ($P < 0.001$, $P < 0.01$, respectively). An increased *CDKN5* mRNA level was observed in BCB^+ oocytes (Group III) compared to oocytes of Group I and Group II ($P < 0.01$), respectively. The mRNA expression of both genes was always higher in BCB^+ compared to BCB^- oocytes. Based on this study it can be supposed that *CDKN1* and *CDKN5* are differentially expressed in developmentally distinct porcine oocytes and in a maturation stage-dependent manner. Moreover, *CDKN1* may play a role as a molecule which regulates cell cycle arrest and the specific "block of maturation" from MI to MII.

Keywords: *Cdkn1*; *Cdkn5*; oocyte; developmental competence

The maturation of mammalian COCs is an important step in reaching full developmental competence (Grupen and Armstrong 2010; Jaskowski et al. 2010). However, maturation *in vivo* differs significantly from *in vitro* maturation, even if both of these processes are determined by extrinsic and intrinsic factors. The extrinsic factors consist of the follicular/ovarian environment, which includes concentration and presence of several proteins, hormones, and metabolites important for proper

Supported by the Polish Ministry of Scientific Research and Higher Education (Grant No. 0233/IP1/2011/71 "IuventusPlus").

growth and differentiation of COCs during folliculogenesis as well as normal development (Petro et al. 2012). The conditions of maturation *in vitro* can, however, only mimic the *in vivo* environment. Therefore, the concentration and composition of compounds, which supplement the culture medium, play a significant role in the achievement of full developmental competence by oocytes (Herrick et al. 2004; Kim et al. 2005; Song et al. 2010). The important intrinsic factors include the accumulation of a large amount of mRNAs and proteins during folliculo- and oogenesis, and a proper communication between the cumulus cells and the oocyte (Norris et al. 2010; Antosik et al. 2011). The "molecular corridor" of communication between cumulus cells and the oocyte is formed mainly by gap junction structures. Their protein structure allows an active transport of small substances and metabolites between oocytes and the surrounding somatic cumulus cells. It has been shown that this specific crosstalk is based on paracrine interactions between these two cell types. Nitta et al. (2010) constructed an oocyte-specific cDNA library to identify which connexin genes are expressed in oocytes and follicle cells. They found the diversity in the connexin genes expressed in porcine oocytes as well as in a gap junction connecting oocytes and follicular cells. Moreover, Areekijseree and Chuen-Im (2012) investigated the effects of pFSH, LH, and oestradiol supplementation in culture medium on ultrastructures of porcine cumulus oocyte complexes (pCOCs). They found that changes in cumulus cell morphology were a sign of the beginning of oocyte maturation.

Fujii et al. (2011) investigated the involvement of CAK (CDK-activating kinase) in the meiotic progression of immature porcine oocytes. CAT phosphorylates threonine 161 (T161) of CDC2, a catalytic subunit of maturation/M-phase promoting factor (MPF) which is crucial for MPF activation during mitosis. Moreover, CAT consists of a catalytic subunit of CDK7. They found that the overexpression of CDK7 accelerated meiotic events, such as meiotic resumption, T161 phosphorylation, and MPF activation. It is suggested that CDK7 activates CDC2 by T161 phosphorylation and that CAK is required for proper meiotic progression during porcine oocyte maturation.

It is presumed that several other proteins, such as cyclin-dependent kinases (Cdks) also play an important role during oocyte maturation and cell-to-cell communication. Moreover, the expression of Cdks and CXs (connexins) is presumably regulated by similar molecular mechanisms (Antosik et al. 2011). Generally, Cdks play an important role during G2-to-M transition and cell cycle regulation. The cyclin-dependent kinase inhibitors 1 and 5 (*Cdkn1* and *Cdkn5*) are responsible for cell arrest in a p53 signalling pathway-dependent manner, which is the first signal manifesting DNA damage in mammalian cells (Kazmierczak et al. 2010; Zhang et al. 2011). The effect of inhibition of Cdks by several compounds was investigated using oocytes of a few mammalian species. In bovine oocytes, the inhibition of Cdks did not significantly influence the MPF- and MAPK-dependent maturation (Quetglas et al. 2010). Since only scant data are available which would indicate a role of Cdks in the regulation of mammalian oocyte maturation, this study was aimed at investigating the expression profiles of *CDKN1* and *CDKN5* after IVM in developmentally competent and incompetent porcine oocytes.

MATERIAL AND METHODS

Animals and collection of porcine ovaries and cumulus-oocyte complex (COC) classification. A total of 26 crossbred Landrace gilts with a median age of 160 days (range 140–180 days) and median weight of 100 kg (95–120 kg) were used in this study. The experiments were approved by the Local Ethics Committee.

The ovaries and reproductive tracts were recovered at slaughter and transported to the laboratory within 30 min at 38.5 °C in 0.9% NaCl.

In the laboratory, the ovaries were placed in 5% foetal bovine serum solution (FBS) (Sigma-Aldrich, St. Louis, MO, USA) in phosphate buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA). Thereafter, individual follicles at sizes of 3–5 mm were opened by puncturing 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 visualised under a stereoscopic microscope, counted and carefully morphologically evaluated using the four grade scale suggested by Jackowska et al. (2009). Only COCs of grade I, which had a homogeneous cytoplasm and a complete and compact cumulus oophorus were used in further steps of the experiment.

Assessment of oocyte developmental competence using the brilliant cresyl blue (BCB) test. A portion of COCs was not subjected to BCB staining (Group I). The other oocytes were washed two times in a modified Dulbecco phosphate buffered saline (PBS-DPBS), 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 remaining colourless (BCB^-, Group II) or having stained blue (BCB^+, Group III). The BCB^+ and BCB^- oocytes were transferred into cultivation.

***In vitro* maturation of porcine COCs**. After staining, the COCs were cultured in Nunclon™Δ 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 final concentrations of 2.5 IU/ml hCG (Ayerst Laboratories, Inc. Philadelphia, PA, USA) and 2.5 IU/ml eCG (Intervet, Whitby, ON, Canada). Wells were covered with a mineral oil overlay and cultured for 44 h at 38 °C under 5% CO_2 in air.

Real-time quantitative PCR (QT-PCR) analysis of *CDKN1* and *CDKN5* mRNA expression. Total RNA was isolated as previously described (Kempisty et al. 2008, 2009) from grade I oocytes without BCB staining (Group I, n = 40), BCB- (Group II, n = 40) and BCB^+ oocytes (Group III, n = 40) using an RNeasy mini column (Qiagen GmbH, 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 into cDNA (RT). QT-PCR was conducted in a LightCycler real-time PCR detection system (Roche Diagnostics GmbH, Mannheim, Germany) using SYBR® Green I as a detection dye, and target cDNA was quantified using the relative quantification method. For amplification, 2 µl of total (20 µl) cDNA solution was added to 18 µl of QuantiTect® SYBR® Green PCR (Master Mix Qiagen GmbH, Hilden, Germany) and primers (Table 1). One RNA sample of each preparation was processed without the RT-reaction to provide a negative control in subsequent PCR. To ensure that granulosa cells did not contaminate the oocytes, we demonstrated the absence of the cytochrome P450 aromatase transcript by RT and QT-PCR.

The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and *ACTB* genes were amplified as housekeeping 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 those of *GAPDH* and *ACTB*. To ensure the integrity of these results, an additional housekeeping gene, *18S rRNA*, was used as an internal standard to ensure that *GAPDH* and *ACTB* mRNAs were not regulated in

Table 1. Oligonucleotide sequences used for RQ-PCR analysis

Transcript	Sequence (5'-3' direction)	Gene accession No.	Product size (bp)
CDKN1	ACAGGCGGAGTACCCCAA GCCCTTTTCCACCTCCTGC	NM214316.1	190
CDKN5	GGGAAGGCACCTATGGAAC AGGGCAGAACTTGGCACTC	NM001044621.2	115
CYP 19	GTCCTTTTTGGCAGCATTG CAGAAAATAGCCAGGACCT	U92246	102
GAPDH	CTGCACCACCAACTGCTT TTCTGGGTGGCAGTGATG	AF069649	105
ACTB	GGGAGATCGTGCGGGACAT CGTTGCCGATGGTGATGAC	DQ845171	141
18S rRNA	GTGAAACTGCGAATGGCTC CCGTCGGCATGTATTAGCT	AB117609	105

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 *ACTB* did not vary when normalised against *18S rRNA* (results not shown).

Statistical analysis. ANOVA followed by the Tukey post-test was used to compare the results of real-time QT-PCR quantification. The experiments were carried out in at least three replicates. The quantification of the relative abundance (RA) of investigated mRNAs was expressed as the mean of the transcript: *GAPDH*/*ACTB*/*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, USA) was used for the statistical calculations.

RESULTS

QT-PCR analysis revealed expression patterns of *CDKN1* and *CDKN5* in oocytes of all groups, i.e., Group I (without staining), Group II (BCB^{-}) as well as Group III (BCB^{+}). We could detect higher (10-fold) expression of *CDKN1* in all groups of porcine oocytes, compared to the expression of *CDKN5*. We observed the highest *CDKN1* mRNA levels in oocytes analysed before staining compared to BCB^{-} and BCB^{+} oocytes ($P < 0.001$, $P < 0.01$, respectively) (Figure 1). A higher expression was also noted in BCB^{+} compared to BCB^{-} oocytes ($P < 0.05$). *CDKN5* mRNA levels were highest in BCB^{+} oocytes in comparison to those of Groups I and II, respectively ($P < 0.001$). The expression of *CDKN1* and *CDKN5* genes was always lower in BCB^{-} oocytes. A similar expression pattern was observed regardless of which of the three housekeeping genes (*GAPDH*, *ACTB* and *18S rRNA*), was used for normalisation.

DISCUSSION

The developmental competence of mammalian oocytes highly influences their ability to mature *in vivo* and/or *in vitro*, to be successfully fertilised, to form a zygote and finally to develop into an embryo suitable for implantation. Oocytes characterised by a decreased developmental competence have a low ability to reach the embryo stage and their development is disturbed (Nemcova et al. 2006; Petro et al. 2012). There are numerous published data regarding the assessment of developmental competence of oocytes of several mammalian species, including cows, pigs and dogs. Developmental competence is mainly evaluated using the brilliant cresyl blue (BCB) test. In this assay, the activity of glucose-6-phosphate dehydrogenase (G6PDH) is measured: oocytes with blue colour are regarded to be fully developmentally competent, while colourless gametes are regarded as incompetent (Roca et al. 1998; Kempisty et al. 2011). There are numerous studies using both BCB positive (BCB^{+}) and BCB negative (BCB^{-}) oocytes (Wongsrikeao et al. 2006; Catala et al. 2012). Therefore, this study was aimed at investigating the key regulators of cell cycle division-cyclin-dependent kinase inhibitors (Cdkns) in developmentally competent and incompetent porcine oocytes.

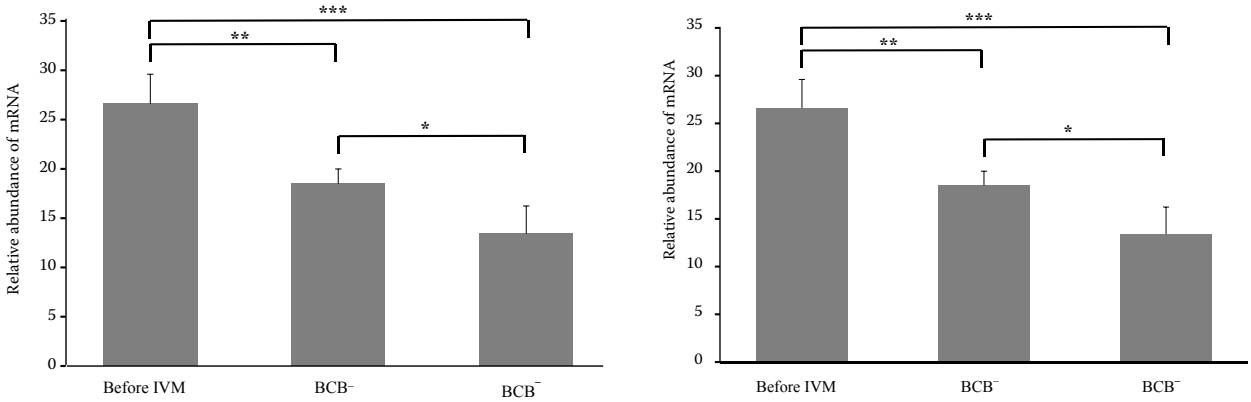

Figure 1. Relative abundance of *CDKN1* and *CDKN5* transcripts in competent and incompetent porcine oocytes. The porcine oocytes, before IVM, BCB^{+} and BCB^{-}, isolated from of pubertal gilts were immediately used to isolate RNA, which was reverse-transcribed into cDNA. The presence of *CDKN1* (A) and *CDKN5* (B) transcripts was evaluated by QT-PCR analysis. Results are presented as mean ± SEM with the level of significance, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

The cyclin-dependent kinases are important cell cycle regulators, described also as "checkpoints" during the cell cycle division. Activation of these kinases requires cyclins, the phosphorylation of which is catalysed by cyclin-dependent kinase-activating kinase (CAK). It has been shown that formation of a complex between cdc2 (p34) and cyclin B leads to G2-to-M transition in cell division (Ren et al. 2011). Moreover, activation of the cyclin-dependent kinase machinery is required for the activation of cell division and cell proliferation as well as normal meiotic progression during porcine oocyte maturation (Fujii et al. 2011; Bertoli et al. 2013). Therefore, abnormal activity and/or expression of *Cdkn* are determined as the most important players in the induction of improper cell proliferation and carcinogenesis (Graf et al. 2010). However, it was also found that these proteins may play an important role during oocyte maturation, embryo development and foetal growth, where cell division regulation is crucial for normal growth (Gotoh et al. 2011). On the other hand, cyclin-dependent kinase (Cdkn) inhibitors also play a significant role in cell division control, which they exert via the p53 signalling pathway.

In fact, the activity and/or expression of *Cdkn* are important for the balance in the division cycle during folliculo- and oogenesis (Tomic et al. 2004; Wang et al. 2013). However, still little is known about the differential expression of these mRNAs and/or proteins in relation to the developmental competence of mammalian oocytes. In our study, oocytes were analysed after follicle aspiration, after BCB staining (BCB^- oocytes; developmentally incompetent) and after BCB^+ staining and IVM (developmentally competent oocytes) regarding their *CDKN1* and *CDKN5* mRNAs expression. In oocytes immediately after aspiration, an increased expression of *CDKN1* and a decreased mRNA level of *CDKN5* were found. It can be assumed that differential mRNA expression or different activity of *CDKN1* and *CDKN5* may be associated with the stage of porcine oocyte maturation. Moreover, it may be suggested that the level or activity of these kinase inhibitors is associated with the expression of several kinase-activation regulatory proteins involved in, e.g., phosphorylation. Different activity of *Cdkn* may also be associated with different activities of cyclin-dependent kinases, but this remains to be investigated. Adhikari et al. (2012), after using knockout mice with oocyte-specific deletions of *CDK1* and *CDK2*, found that *CDK1* is the sole Cdk essential and sufficient to drive resumption of meiosis in mouse oocytes. They also observed that *CDK1* maintains the phosphorylation status of protein phosphatase 1 and lamin A/C in oocytes. On the other hand, it is also suggested that the deletion of several kinases such as *CDK3*, *CDK4* or *CDK6* in mice results in normal oocyte maturation, indicating that these *Cdk* are not essential for the meiotic maturation of oocytes. Higher expression of *CDKN1* in (possibly immature) porcine oocytes may be associated with regulation by effecting a protein-specific "block" in cell cycle division and/or low mRNA synthesis. Presumably, this inhibitor down-regulates the expression of *CDKS* and, therefore, keeps the oocytes in a pre-activation stage of oocyte growth and development. On the other hand, *CDKN5* revealed a differential but typical mRNA expression pattern, in which the highest transcript level was noted in BCB^+ oocytes before IVM. It may be presumed that the *Cdkn* protein is not involved in the process of "growth silence" of porcine oocytes. Furthermore, lower expressions of both *CDKN1* and *CDKN5* mRNAs in BCB-oocytes indicate (before IVM) that, as previously shown in several studies, that a double BCB staining test may down-regulate the expression of several genes important for the maturation and fertilisation ability of mammalian oocytes (Wongsrikeao et al. 2006; Kempisty et al. 2011).

Although data are available on the role of *Cdkn* and their regulation during oogenesis, oocyte maturation and embryogenesis, only a small number of studies report on the function of *Cdkn* in mammalian oocytes. Liu et al. (2007), found that Cdkn and Foxo3a (a component of the phosphatidylinositol 3-kinase (PI3K) signalling pathway) inhibit oocyte growth during folliculogenesis. Furthermore, they observed several aberrations in follicular development which may lead to premature ovarian failure. Our results partially confirm the results obtained by Gutierrez et al. (2006). They found that in Xenopus oocytes RINGO/Speedy proteins, which are also described as the main inducers of Cdk1 and Cdk2, accumulated in meiosis I entry and, then are down-regulated. Additionally, they showed that RINGO protein expression may contribute to the maintenance of G2 arrest. However, it is suggested that the degradation of RINGO proteins may be required for the meiosis I to meiosis II transition in Xenopus oocytes. Our observations seem to indicate a similar mechanism of oocyte arrest before maturation, in which *Cdkn* plays the main role. The importance of RINGO/Speedy proteins in cell cycle arrest, in cell cycle division and MI into MII transition was shown by Cheng et al. (2005). They found that for Cdk binding to RINGO/

Speedy, a conserved 140-aa domain called also as the RINGO/Speedy box is required and that a point mutation in this region abolished this binding of Cdk. Moreover, Kume et al. (2007) examined the effect of SPDY on the meiotic maturation of porcine oocytes. They observed that *xRINGO* mRNA accumulated abnormally in porcine oocytes and arrested them in the first meiotic metaphase (M1), whereas *pigSPDYA2* accelerated the meiotic progression, and *pigSPDYA2* mRNA-injected oocytes completed meiotic maturation within 30 h. They concluded that *pigSPDYA2* plays important roles in the meiotic maturation of porcine oocytes and that the degradation of SPDY was necessary for the proper maturation of female gametes.

In conclusion, it can be assumed that Cdks and Cdkns play a significant role during oocyte-specific cell cycle progression and cell cycle arrest, and are linked with high activity of Cdkns or aberrant expression of Cdks and/or RINGO/Speedy associated proteins.

Acknowledgement

The authors wish to acknowledge MSc. Marta Jackowska (University of Life Science, Poznan, Poland) for technical support.

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Received: 2012–02–14
Accepted after corrections: 2013–09–20

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