

EXPRESSION AND CELLULAR DISTRIBUTION OF CYCLIN-DEPENDENT KINASE 4 (CDK4) AND CONNEXIN 43 (CX43) IN PORCINE OOCYTES BEFORE AND AFTER *IN VITRO* MATURATION

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It is recognised that connexin 43 (Cx43) and cyclin-dependent kinase 4 (Cdk4) are involved in the cumulus cell-oocyte communication via gap junctions and the control of cell cycle progress. However, little is known about their mRNA expression pattern and encoded proteins distribution in porcine oocytes during *in vitro* maturation (IVM). Cumulus-oocyte complexes (COCs) were collected from 31 puberal crossbred Landrace gilts and analysed for their Cdk4 and Cx43 mRNA expression using RQ-PCR and for the respective protein expression by confocal microscopic observations. An increased Cdk4 and Cx43 mRNA expression was found in oocytes after IVM ($P < 0.001$ and $P < 0.05$, respectively). Confocal microscopic observations revealed a significant increase of Cdk4 protein expression in the cytoplasm of oocytes during the maturation process. The localisation of Cx43 changed from zona pellucida before to cytoplasm of oocytes after IVM. It is supposed that the increased expression of Cdk4 and Cx43 mRNA in oocytes after IVM is linked with the accumulation of a large amount of templates during the process of oocyte maturation. The translocation especially of Cx43 from the zona pellucida into the cytoplasm may be associated with a decrease in gap junction activity in fully grown porcine oocytes. Both Cdk4 and Cx43 can be used as ‘checkpoints’ of oocyte maturation.

Key words: Cyclin-dependent kinase, connexin, expression, porcine oocyte, *in vitro* maturation

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The maturation of mammalian cumulus-oocyte-complexes (COCs) both *in vivo* and *in vitro* is a complex process which includes several fine-tuned molecular, morphological and cellular changes. Up to the MII stage, the maturation process is characterised by the expression of cell-cycle-specific genes and proteins (Chiba, 2011; Schatten and Sun, 2011). Cyclin-dependent kinases (Cdks) are well-described markers of cell cycle control, known also as ‘the cell cycle checkpoints’. The cyclin-dependent kinase 4 (Cdk4) is a serine kinase and its activation leads to the phosphorylation of target proteins and thereby promotes cell cycle progression (Berthet and Kaldis, 2006). The complex between Cdk4 and cyclin-D is responsible for the regulation of cell proliferation during the G1 phase. Moreover, Cdk4 is down-regulated by p16, described also as a cyclin-dependent kinase inhibitor 2 (Cdkn2a) (Feng et al., 2012). The cell cycle regulatory mechanism of Cdk4 was studied mainly in uncontrolled cell proliferation during carcinogenesis (Roberts et al., 2012). However, it is supposed that the cell-cycle-promoting ability of Cdk4 is also involved in the control of cell growth during folliculo- and oogenesis. Cdk4 was found to be involved in G1/S progression during oocyte maturation in mice (Kohoutek et al., 2004).

Another important factor that determines the maturation ability of COCs is the interaction between the surrounding somatic cumulus cells and the oocyte. Both cell types communicate by cell-to-cell transfer of ions, small molecules and second messengers via intercellular channels of gap junctions, which are formed by proteins called connexins (Cxs). The most important proteins crucial for this structure include connexin 37 (Cx37), connexin 43 (Cx43) and connexin 45 (Cx45) (Söhl and Willecke, 2004). Our previous study demonstrated that Cx43 was differentially localised within oocytes which were recovered from follicles of different size (Antosik et al., 2009; Antosik et al., 2011). Although several data are available on the role of Cdk4 and Cx43 in cell cycle control and cell-to-cell communication in mammalian oocytes, especially in oocytes of mice, there is no information on the expression of these genes and the cellular distribution of the respective proteins in porcine oocytes during *in vitro* maturation. Therefore, the aim of the present study was to compare the Cdk4 and Cx43 mRNA expressions as well as their respective protein distribution in porcine oocytes before and after *in vitro* maturation (IVM).

Materials and methods

Animals

A total of 31 crossbred Landrace gilts (mean age: 160 days; mean body weight: 100 kg) were used in this study. The experiments were approved by the local Ethics Committee.

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

The ovaries were collected after slaughter and transported to the laboratory within 30 min at 38.5 °C in 0.9% NaCl. Thereafter, the ovaries were placed in 5% fetal bovine serum solution (FBS) (Sigma-Aldrich Co., St. Louis, MO, USA) in phosphate buffered saline (PBS), and COCs were recovered in a sterile Petri dish by puncturing individual follicles 3–5 mm in size. COCs were washed three times in modified PBS, supplemented with 36 µg/ml pyruvate, 50 µg/ml gentamicin and 0.5 mg/ml bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) and then morphologically evaluated under a stereoscopic microscope, using the four-grade scale suggested by Jackowska et al. (2009). Only grade-I COCs (n=100), which have a homogeneous cytoplasm and a complete and compact cumulus oophorus, were used in further steps of the experiment.

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

Before *in vitro* cultivation, oocytes (n = 100) were washed twice in modified Dulbecco's 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, 0.34 mM pyruvate and 5.5 mM glucose (DPBSm) and then treated with 26 µM BCB (Sigma-Aldrich) diluted in DPBSm at 38.5 °C, 5% CO₂ in air for 90 min according to Wongsrikeao et al. (2006) and Kempisty et al. (2011). 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, Lübeck, Germany) and classified as either having stained blue (BCB⁺) or remaining colourless (BCB⁻). Only BCB⁺ in a number of approximately 44%, i.e. developmentally competent oocytes, were used for *in vitro* culture.

In vitro maturation of porcine COCs

The BCB⁺ 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 the 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% CO₂ in air.

Real-time quantitative PCR (RQ-PCR) analysis of Cdk4 and Cx43 mRNAs expression

Total RNA was isolated from oocytes before (n = 40) and after IVM (n = 40), using an RNeasy mini column (Qiagen GmbH, Hilden, Germany) (Kempisty et al., 2008; Kempisty et al., 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 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 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β -actin were amplified as references for mRNA quantification.

In order 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 mRNAs were not differentially expressed 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 β -actin did not vary when normalised against 18S rRNA (results not shown).

Confocal microscopic observations

COCs were isolated from follicles as described above. Oocytes were fixed using 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 (0.2%). In order to block nonspecific binding, samples were incubated in 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 antibody (Ab), H-22 or rabbit polyclonal anti-Cx43 Ab and (Ser 279/282)-R Ab (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 with rabbit polyclonal anti-Cdk4 Ab and H-22 were incubated for 1 h at RT with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG Ab produced in goat and diluted 1:500 in PBS/0.1% Tween-20. Samples with rabbit polyclonal anti-Cx43 Ab and (Ser 279/282)-R Ab were in-

cubated for 1 h at RT with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG Ab produced in goat and diluted 1:500 in PBS/0.1% Tween-20. Following washing in PBS/0.1% Tween-20, the oocytes were mounted on glass slides in antifade drop and observed under LSN 510 confocal system in Olympus microscope, Fluoview 10i. FITC was excited at 488 nm from an argon laser, and emissions were imaged through a 505–530 nm filter. All 3D confocal microscopic images were analysed using Imaris 7.2 (BitPlane, Zurich, Switzerland) software (Kempisty et al., 2012).

Statistical analysis

One-way ANOVA followed by Tukey's *post hoc* test was used to compare the results of real-time RT-PCR quantification. The experiments were carried out in at least three replications. The results quantifying relative abundance (RA) of investigated mRNAs are expressed as the mean of the transcript: GAPDH/ β -actin/18S rRNA ratio. The differences were considered to be significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$. The software programme GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA, USA) was used for the statistical calculations.

Results

RQ-PCR assays revealed both an increased Cdk4 mRNA level ($P < 0.001$) and Cx43 mRNA expression ($P < 0.05$) in oocytes after IVM (Figs 1A and 1B).

Confocal microscopic observations demonstrated both cytoplasmic (65%) and zona pellucida distribution of Cdk4 in oocytes before IVM (Fig. 2A). After IVM, a significantly higher expression of this protein was noted in the cytoplasm of oocytes in approximately 90% of all investigated gametes (Figs 2B, 3A and 3B). The Cx43 protein showed high zona pellucida localisation in 85% of the oocytes before IVM, whereas a less intense expression of the protein was observed in the cytoplasm (15%; Fig. 2C). The oocytes analysed after IVM revealed a strong cytoplasmic distribution of the Cx43 protein in 97% of the oocytes (Figs 2D, 3C and 3D). The zona pellucida localisation of Cx43 was almost not detectable. The confocal microscopic observation in 3D projection showed a similar expression pattern and distribution of both Cdk4 and Cx43 proteins (Figs 3A, 3B, 3C and 3D). The Cdk4 protein was localised both in the zona pellucida and cytoplasm of oocytes in a similar manner (Figs 3A and 3B).

Discussion

It has been demonstrated that the maturation of mammalian COCs differs significantly under *in vivo* and *in vitro* conditions (Polanski, 1986; Kempisty et

al., 2012). These differences are strongly influenced by the experimental methods used (Kempisty et al., 2011). Although standard *in vitro* culture media attempt to mimic the *in vivo* environment, the efficiency of IVM is still unsatisfactory. In many species, e.g. in dogs, the number of oocytes that reach the MII stage after IVM is less than 10–20% (Chastant-Maillard et al., 2011). Considering that the maturation process is complex and regulated by several factors, the relationship between the surrounding somatic cells and the oocyte plays a predominant role. This cross-talk is determined by small cell-to-cell connections, i.e. gap junctions, which are formed by connexins. It has been found that both connexins and cyclin-dependent kinases are involved in the regulation of important steps of development and maturation of COCs by paracrine activation of the transport of small substrates and/or by the induction of G2/M transitions, respectively (Valdimarsson et al., 1993; Pant et al., 2005).

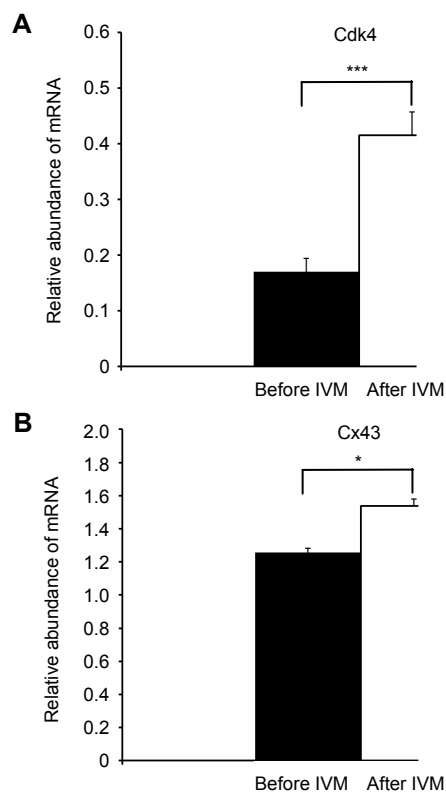


Fig. 1. Relative abundance of Cdk4 and Cx43 mRNAs in porcine oocytes before and after IVM. RNA from porcine oocytes was isolated immediately after recovery of oocytes (before IVM; n = 40), and after *in vitro* maturation (after IVM; n = 40). The RNA was reverse-transcribed into cDNA. RQ-PCR was used to evaluate the presence and quantity of Cdk4 (A) and Cx43 (B) transcripts. Each sample was assayed in triplicates. Results are presented as mean \pm SEM with the level of significance: *P < 0.05, ***P < 0.001

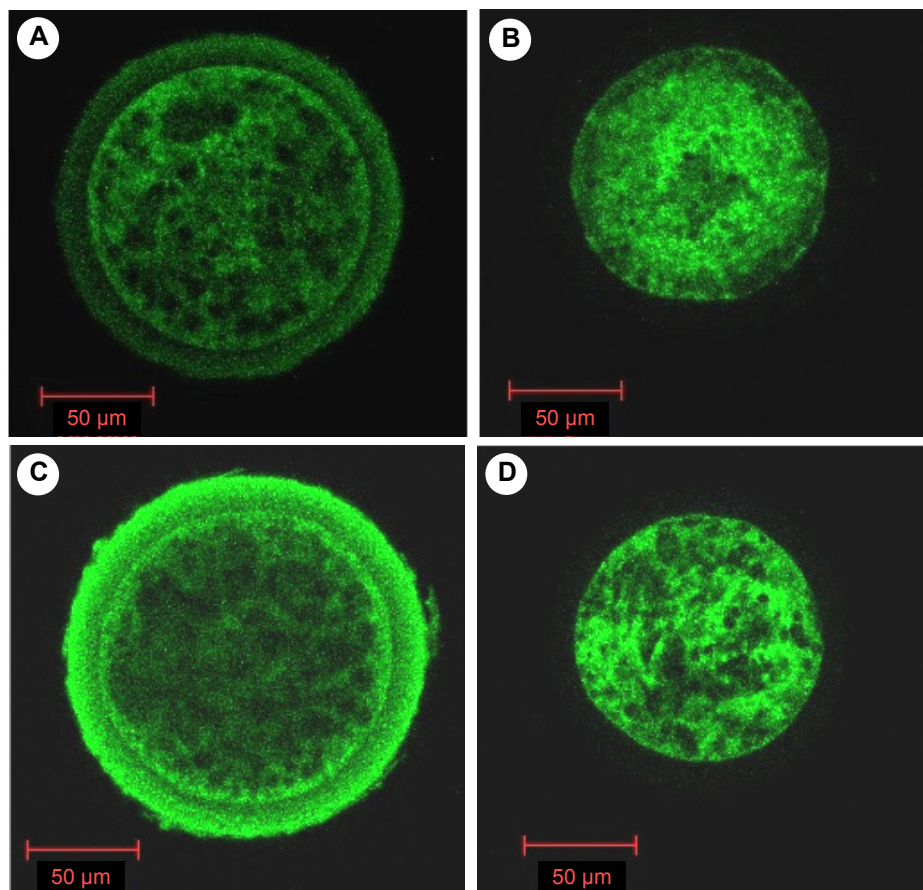


Fig. 2. Localisation of Cdk4 and Cx43 proteins in porcine oocytes before and after IVM. Porcine oocytes were stained with (A) porcine Cdk4 (rabbit polyclonal anti-Cdk4 Ab, H-22; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary antibodies were labelled with fluorescein isothiocyanate (FITC) conjugated with goat anti-rabbit antibody (Ab), which emits a green fluorescent signal after excitation at 488 nm, or with (B) porcine Cx43 (rabbit polyclonal anti-Cx43 Ab, Ser 279/282)-R; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary antibodies were labelled with FITC conjugated with goat anti-rabbit Ab. Scale bars represent 50 µm before and after IVM (C and D, respectively)

In several studies using stimulators and/or inhibitors it was shown that connexins induce cumulus cell proliferation and enhance oocyte maturation (Nitta et al., 2010). In a previous study, Kölle et al. (2003) analysed the effect of growth hormone (GH) on the maturation of bovine oocytes and on apoptosis of cumulus cells. They found that GH inhibits the expression and cellular localisation of Cx43 protein within the cumulus cells. Moreover, the effect of GH on oocyte maturation was stimulated via gap junction activation, which involves Cx43 as the main protein forming these connections. The expression of Cx43 mRNA

was significantly marked in COCs compared to denuded oocytes, and the abundance of Cx43 mRNAs was increased at 0–6 hours of IVM and decreased subsequently (Pandey et al., 2010). These results are in accordance with our findings, where the expression of Cx43 and of Cdk4 was higher in oocytes after IVM as compared to oocytes analysed before. Additionally, our results from confocal microscopic observations revealed similar protein expression patterns compared to mRNA profiles. The localisation of Cx43 was displaced from the zona pellucida in oocytes before IVM to a strong cytoplasmic distribution in oocytes analysed thereafter. These observations suggest that Cx43 may be differentially localised in porcine oocytes dependent on the stage of maturation. Bouvier et al. (2009) described an interaction between Cx43 and Cx40, which led to the formation of connexin heterodimers with one protein loop anchored in the cytoplasm. It is suggested that Cx43 forms a heterodimer structure with Cx40 in porcine oocytes, which is manifested by a distinct cytoplasmic distribution of Cx43 after IVM. The significant localisation of Cx43 in the zona pellucida before IVM may be associated with an increased activity of gap junction transport in not fully grown oocytes which did not reach the MII stage. Contrary to these results, Gittens and Kidder (2005) found that another connexin, Cx37, is involved in the formation of fully competent gametes and has a more important function than Cx43. They have shown that mouse COCs do not need Cx43 to grow to developmentally competent cells, but Cx37 seems to be necessary for proper communication between surrounding cumulus-granulosa cells and oocytes during normal oogenesis. However, as opposed to that and in agreement with our results, Teilmann (2005) found in mice COCs that Cx43 may be translocated between cumulus-granulosa cells and oocyte in follicles during the process of preantral to antral transition. Furthermore, the localisation of Cx43 within the zona pellucida suggests its trans-zonal projection, which would point to new intracellular communications between somatic and germ cells during early folliculogenesis.

The role of cyclins and cyclin-dependent kinases in the cell cycle progression has been investigated in several studies using mammalian oocytes and embryos as well as carcinogenic cell lines (Lequarre et al., 2004; Quetglas et al., 2010; Yue et al., 2012). Among others, the role of cyclins Cdk2 and Cdk4 was analysed in the cell cycle progress during meiotic and mitotic divisions in mouse embryos (Moore et al., 1996). It was found that the abundance of Cdk2 and Cdk4 was increased during the S and/or G2 phases of the first mitotic division. Additionally, it was stated that the higher level of Cdk4 mRNA during the first mitotic division was related to polyadenylation, whereas the increased level of Cdk2 and Cdk4 transcripts during the second mitotic division was due to the activation of the embryonic genome. In another study (Zhang et al., 1999), the expression pattern of cyclin D3, Cdk4 and p27 was investigated in the murine testis and ovary. It was suggested that all of these genes and proteins are expressed in these tissues during early development, but are dependent on the stage of maturation and are cell spe-

cific. However, the expression of cyclin D3 related kinases was detected only in growing tissues and was absent in the fully developed testis and ovary. Our results indicate that Cdk4 expression significantly increased when comparing oocytes before and after maturation *in vitro*, which underlines the importance of this protein during oocyte maturation. Like Cx43, Cdk4 was localised both in the zona pellucida and the cytoplasm of oocytes, which implies a possible protein translocation between these two cell structures, depending on the stage of oocyte maturation.

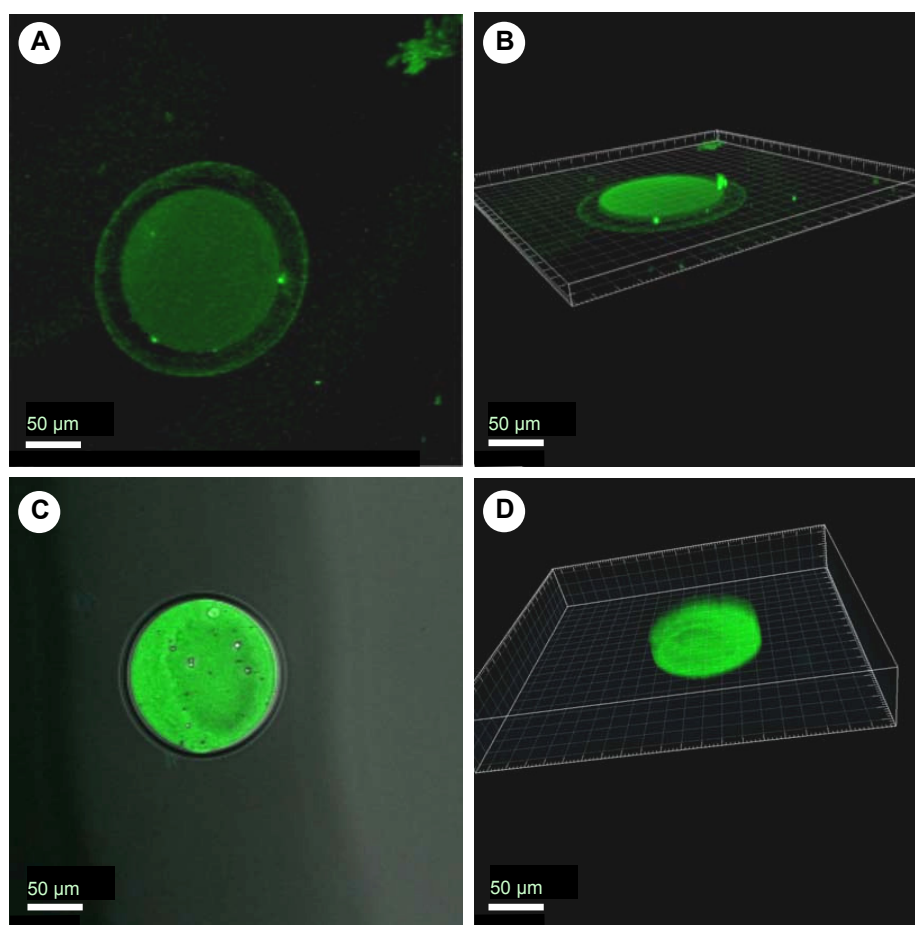


Fig. 3. 3D-confocal microscopic observation of Cdk4 and Cx43 distribution within porcine oocytes after IVM. Porcine oocytes ($n = 50$) were stained after IVM (A, B) for porcine Cdk4 (rabbit polyclonal anti-Cdk4 Ab, H-22; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and labelled for 1 h with FITC-conjugated goat anti-rabbit IgG Ab at a 1:500 dilution in PBS. Other porcine oocytes ($n = 50$) after IVM (C, D) were stained with rabbit polyclonal anti-Cx43 Ab (Ser 279/282-R; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and labelled with FITC-conjugated goat anti-rabbit IgG Ab at a 1:500 dilution in PBS. The confocal microscopic images were analysed by the use of Imaris 7.2 (BitPlane, Zurich, Switzerland) software and presented in 3D projection (B, D). Scale bars represent 50 μm

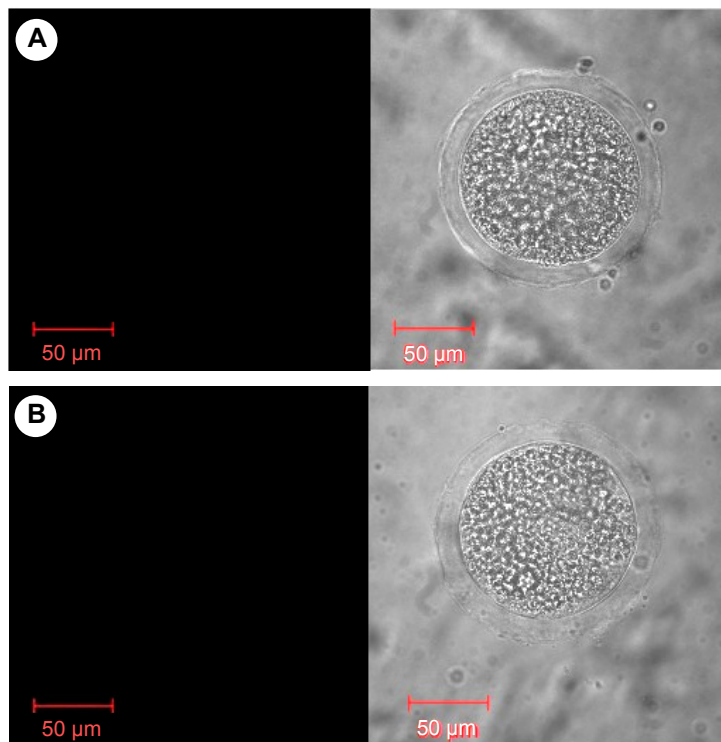


Fig. 4. Confocal microscopic observation of control oocytes. The negative controls, i.e. porcine oocytes with an intact zona pellucida (Figs 4A and 4B), were incubated for 1 h at room temperature with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG Ab at a 1:500 dilution in PBS/0.1% 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. Scale bars represent 50 μm

In summary, Cdk4 and Cx43 mRNA expression increases in porcine oocytes during the *in vitro* maturation process and the respective proteins translocate from the oocyte periphery into the cytoplasm. Both Cdk4 and Cx43 can be used as ‘checkpoints’ of oocyte maturation.

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