—Original Article—

Short-term Cultivation of Porcine Cumulus Cells Influences the Cyclin-dependent Kinase 4 (Cdk4) and Connexin 43 (Cx43) Protein Expression—A Real-time Cell Proliferation Approach

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Abstract. The CC (cumulus cell) proliferation index in relation to the expression and distribution of Cdk4 and Cx43 proteins, which are crucial factors for oocyte maturation, was investigated. Cumulus-oocyte complexes (COCs) were recovered from pubertal crossbred Landrace gilts and treated with collagenase, and separated CCs were cultured in standard TCM199 medium for 44 h. At each step of *in vitro* cultivation (IVC) of CCs (0, 12, 24 and 44 h), a normalized proliferation index was assessed. Cdk4 and Cx43 protein expression and the CC-specific cellular distribution were analyzed by confocal microscopic observation. The normalized proliferation index (number of cells attached, measured by impedance) was increased in the first 12 h of IVC ($P<0.01$) and differed between 12 h and 24 h of cultivation ($P<0.001$). Later, between 24 h–44 h of IVC, the CC proliferation rate was stable, and no significant differences were observed. Based on the confocal microscopic observation, increased expression of both Cdk4 and Cx43 was found after 44 h of IVC compared with the expression of these proteins before IVC. Moreover, after IVC, a substantial translocation of Cdk4 and Cx43 was noted from the nucleus to the cytoplasm of CCs. In conclusion, it was demonstrated for the first time that CCs can be cultured *in vitro* separately without oocytes and that the proliferation index was significantly increased in the first 12 h of IVC, which may reflect the process of ordinary cumulus cell expansion. Furthermore, the expression of both Cdk4 and Cx43 in CCs suggested that these proteins may be regarded as markers not only of proper oocyte maturation but also of CC differentiation. Translocation of these proteins into the cytoplasm of CCs after 44 h of IVC may be related to the expansion process.
Key words: Connexin, Cumulus cells, Cyclin-dependent kinase, Oocyte, Pig

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The potential of oocytes to attain full maturation competence is characterized by an appropriate course of nuclear and cytoplasmic maturation, which finishes with attainment of the MII stage both *in vivo* and *in vitro*. Only mature oocytes can be successfully fertilized, undergo zygote formation and attain normal embryo development [1–3]. The maturation process is determined by several intrinsic and extrinsic factors, involving the storage of large amounts of mRNA and proteins as well as the transfer of low molecular weight compounds and substrates between oocytes and the surrounding somatic cumulus cells (CCs) [4–6]. The communication between these cells is associated with the formation of protein channels, also called *gap junction* connections (GJCs), permitting the transfer of small molecules of molecular weights less than 1 kD. It was clearly demonstrated that this "cross-talk" is critical for proper oocyte

maturation and resumption of meiosis [7–9]. Experiments based on knock-out models demonstrated that mutation in genes encoding proteins that form GJCs led to failed oocyte maturation and finally produced sterile females [10]. GJC channels are formed by connexins (Cx), including mainly Cx31, Cx37, Cx43 and Cx47. Although the presence of Cx molecules in cumulus cells has been reported [1, 11], no data are available on the profiling of differential expression of Cx genes and the distribution of the proteins in the course of real-time CCs proliferation. Furthermore, cyclin-dependent kinases (Cdks) have been shown to be also involved in proper oocyte maturation and attainment of the MII stage [12]. Using mouse knock-out models it was demonstrated that Cdks are the main regulators of cell divisions and represent a checkpoint of cell cycle progression [13]. Cdk4 belongs to the protein-serine kinase superfamily, which catalyzes phosphorylation of target proteins and promotes cell cycle progression. The formation of the complex between Cdk4 and type-D cyclin regulates cell proliferation during the G1 phase [14]. Since Cdk4 is the main target during the cell division, the expression of this protein in various phases of CC proliferation is examined in this study.

During the complex process of oocyte maturation *in vivo* and/or *in vitro*, cumulus cells undergo substantial expansion that is highly linked with the transfer of regulatory molecules and small substrates. However, it has not been fully recognized if this process is related to CC proliferation and at which steps of oocyte maturation it occurs. Since it was shown that CCs manifested adherence in monolayer culture systems, it is known that the proliferation of CCs should be associated with the expansion during maturation [15–17]. However, no evidence was provided for proliferation of COCs *in vivo*. The supposition of such proliferation comes from *in vitro* studies in which porcine COCs were cultivated for 44 h. Despite documented changes in CC abundance during maturation, there is no clear evidence that CCs proliferate during IVC and that the proliferation rate is determined by *in vitro* conditions. Therefore, the goal of this study was to show quantitative changes in CC proliferation in real time in relation to the Cdk4 and Cx43 mRNA expression profile and the distribution of encoded proteins during periods of *in vitro* maturation in separated porcine CCs.

Material and Methods

Animals

A total number of 41 pubertal crossbred Landrace gilts (age 170 days, body weight 98 kg) were used. The animals were kept under the same conditions. The experiments were approved by the local Ethics Committee.

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

The ovaries and reproductive tracts were recovered at slaughter and transported to the laboratory within 10 min at 38 C in 0.9% NaCl. In order to provide optimal conditions for subsequent oocyte maturation and fertilization *in vitro*, the ovaries of each animal were placed in 5% fetal bovine serum solution (FBS; Sigma-Aldrich, St. Louis, MO, USA) in PBS [18, 19]. Thereafter, individual large follicles (>5 mm) were opened by puncturing with a 20-G needle connected to a 5-ml syringe in a sterile Petri dish, and COCs were recovered. COCs were washed three times in a modified PBS supplemented with 36 µg/ml pyruvate, 50 µg/ml gentamicin, and 0.5 mg/ml BSA (Sigma-Aldrich). COCs were selected under an inverted microscope (Axiovert 35, Zeiss, Lübeck, Germany), counted and morphologically evaluated using the scale suggested by Jackowska *et al.* [20]. Only COCs of grade I with homogeneous ooplasm and uniform and compact cumulus cells were considered for use in the following steps of the experiment.

Assessment of oocyte developmental competence by BCB test

To perform the BCB staining test (brilliant cresyl blue), COCs were washed twice in a modified Dulbecco's PBS (DPBS) (Sigma-Aldrich), supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin (Sigma-Aldrich), 0.4% [w/v] BSA, 0.34 mM pyruvate, and 5.5 mM glucose (DPBSm). Thereafter, they were treated with 26 µM BCB (Sigma-Aldrich) diluted in DPBSm at 38.5 C in 5% CO_2 in air for 90 min. Next, the oocytes were transferred to DPBSm and washed two times. During the washing procedure, the oocytes were examined under an inverted microscope and classified as either stained blue (BCB$^+$)

or remaining colorless (BCB$^-$). After harvesting cumulus-granulosa somatic cells (CCs), only BCB$^+$-COCs were incubated with bovine testicular collagenase (50 to 200 units/ml in HBSS) (Sigma-Aldrich) for 10 min at 38 C. Thereafter, the cells were removed by vortexing and pipetting the BCB$^+$ oocytes in 1% sodium citrate buffer and by mechanical displacement, using a small-diameter glass micropipette. The oocytes were discarded, and the CCs were used for real-time cultivation procedures to determine the proliferation index and perform the confocal microscope analysis.

In vitro CC cultivation using a real-time cell analyzer (RTCA)

Harvested GCCs were transferred into a real-time cell analyzer (RTCA, E-Plates 48, Roche Applied Science, Penzberg, Germany) consisting of an RTCA Analyzer, RTCA SP Station and RTCA Software. The CCs were then cultured in 200 µl standard tissue culture medium (TCM-199) with Earle's salts and *L*-glutamine, (Gibco BRL Life Technologies, Grand Island, NY, USA) supplemented with 2.2 mg/ml sodium bicarbonate (Nacalai Tesque, Kyoto, Japan), 0.1 mg/ml sodium pyruvate (Sigma-Aldrich), 10 mg/ml BSA (bovine serum albumin), (Sigma-Aldrich), 0.1 mg/ml cysteine (Sigma-Aldrich), 10% (v/v) filtered porcine follicular fluid and gonadotropin supplements at final concentrations of 2.5 IU/ml hCG (Ayerst Laboratories, Philadelphia, PA, USA) and 2.5 IU/ml eCG (Intervet, Whitby, ON, Canada). The CCs were cultured for 44 h at 38 C under 5% CO_2 in air. After 44 h of cultivation, a set of CCs was treated with trypsin (0.25% trypsin in a balanced salt solution; Sigma-Aldrich), and the collected pool of proliferating cells was used for further confocal microscope analysis. After 12, 24 and 44 h of cultivation, the standardized cell index (CI) was used to evaluate quantitative changes in electrical impedance of cells. The cell status was determined using the RTCA software.

Confocal microscope analysis of Cdk4 and Cx43 expression and distribution in CCs

The CCs were analyzed before and after 44 h of IVC. After cultivation, CCs were collected and fixed using an acetone/methanol mixture (1:1) for 10 min at –20 C 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. CCs were incubated for 1 h at room temperature (RT) with rabbit polyclonal anti-Cdk4 antibody (Ab), H-22 and/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 were incubated with rabbit polyclonal anti-Cdk4 Ab and H-22 for 1 h at RT with fluorescent isothiocyanate (FITC)-conjugated anti-rabbit IgGAb (Santa Cruz Biotechnology) and diluted 1:500 in PBS/0.1% Tween 20. Samples with rabbit polyclonal anti-Cx43 Ab and (Ser 279/282)-R Ab were incubated for 1 h at RT with fluorescent isothiocyanate (FITC)-conjugated anti-rabbit IgG Ab and diluted 1:500 in PBS/0.1% Tween 20. Following washing in PBS/0.1% Tween 20, the CCs were stained with 0.1 µg/ml 4,6-diamino-2-phenylindole (DAPI; Santa Cruz Biotechnology) in mineral oil, mounted on glass slides in an antifade drop and observed under an LSM 510 confocal system microscope (Olympus FluoView 10i). FITC was excited at 488 nm from an argon laser, and emissions were imaged through a 505–530

nm filter. All confocal microscopic images were analyzed using the Imaris 7.2 (Bitplane, Zurich, Switzerland) software.

Statistical analysis

One-way ANOVA followed by a Tukey post hoc test was used to compare the results of real-time quantification of the proliferation index. The experiments were carried out with at least two replications. The cell proliferation index was quantified using an RTCA system. The differences were considered to be significant at P<0.05, P<0.01 and P<0.001 and evaluated by comparing the results of analyzes between four replicates of the same recovered cumulus-granulosa cells. The statistical calculations were applied to compare all investigated groups to the highest normalized proliferation index in each time ratio. The GraphPad Prism software, version 4.0 (GraphPad Software, San Diego, CA, USA) was used for the statistical calculations.

Results

In this study, we analyzed the cumulus cell proliferation index between 0–44 h of IVC and assessed the expression and cellular distribution of Cdk4 and Cx43 using confocal microscope observation. The normalized cell index was assessed between 0–12 h, 12–24 h, 0–24 h, 24–44 h and 0–44 h of IVC (Fig. 1A–E), respectively. Differences in the proliferation index were found at 0–12 h and 12–24 h of IVC (P<0.01, P<0.001, respectively) (Fig. 1A and B). The proliferation index assessed at 0–24 h, 24–44 h and 0–44 h did not vary between groups (Fig. 1C–E).

Using confocal microscopic observation, we analyzed Cx43 (Fig. 2A–C) and Cdk4 (Fig. 2D–F) expression and cellular distribution after 12 h of IVC. The Cx43 and Cdk4 cellular expression was determined in the first 12 h of CC proliferation during cultivation. Expression of Cdk4 protein was significantly higher in CCs after 44 h of cultivation compared with its expression before IVC (Fig. 3A–F). Furthermore, Cdk4 was localized in the nucleus of CCs before IVC, whereas after IVC this protein was distributed in the cytoplasm of CCs (Fig. 3C and F). Similarly, a substantially increased expression of Cx43 was observed after IVC as compared with CCs analyzed before IVC (Fig. 4A–F). Moreover, a translocation of Cx43 from the cell nucleus before IVC to the cytoplasm after IVC was observed (Fig. 4C and F).

Discussion

Cumulus cells undergo substantial morphological changes during maturation of COCs *in vivo* and *in vitro*, which are recognized as CC expansion. This process is accompanied by the transfer of large amounts of small molecules and substrates between the mature oocyte and surrounding somatic cells [15, 21–23]. However, the molecular changes, visible as differential expression profiles of genes and proteins responsible for COC maturation, are still not fully recognized. It was shown in several studies that the achievement of a fully mature stage of oocytes is regulated via an appropriate communication between the oocyte and the surrounding cumulus-granulosa cells, and it is assumed that attainment of the MII stage by the oocyte is accompanied by CC expansion [8, 18, 19, 24, 25]. The mechanisms responsible for this process are not entirely known. In our study, we

postulated that the expression and cellular distribution of Cdk4 and Cx43 regulate not only oocyte maturation but also the mechanisms responsible for cumulus cell differentiation.

Since the bidirectional communication between the oocyte and CCs is acknowledged, analysis of the expression of specific proteins in oocytes and/or CCs may determine important molecular factors regulating oocyte-CC maturation and differentiation. Regassa *et al.* [26] investigated the global expression profile of genes in separated bovine oocytes and CCs. They found several hundred genes that were differentially expressed when CCs were cultured with or without oocytes. A bidirectional effect of cultivation on CCs and oocytes was observed because several genes were expressed in oocytes or CCs only or in both cell types, respectively. Likewise, we observed an expression of Cdk4 and Cx43 proteins in porcine CCs that has previously been described in oocytes [1, 27]. This indicates that both proteins might play a key role not only during oocyte maturation but that they also might regulate processes of CC differentiation. The assumption that CC expansion is associated with attainment of the MII stage in oocytes is supported only by biochemical data of the transfer of substances between oocytes and CCs [8, 23, 28]. Our observation of Cdk4 and Cx43 translocation from the nucleus to the cytoplasm of CCs during IVC provides clues regarding mechanisms of CC differentiation. Translocation of Cx43 and Cdk4 proteins into cytoplasm after IVC might be associated with an increased transport of molecules between the oocyte and CCs and with acquisition of the MII stage. However, in our experiment, we cultured CCs without oocytes, and the results indicate that even in a separated culture system both Cdk4 and Cx43 exert an effect on CC maturation. Almost all previously published data described oocytes enclosing cumulus cells and the possible association between oocyte and CC communication during IVC procedures [29–31]. In addition to the analysis of protein expressions after short-term cultivation of separated CC, we recorded in our study the CC proliferation index. Differences in the proliferation index were observed only between 0 h and 24 h of IVC. This period may be interpreted as the adherent phase and the pre-proliferation stage. During the subsequent period, no differences in the proliferation index were observed, and it can be recognized as a stabile period. Besides the translocation of Cdk4 and Cx43 into the cytoplasm of CCs during 44 h of IVC, the time window of 0–20 h of IVC may be crucial for CC growth. Only a few studies have shown that CCs may proliferate *in vitro*, but in all cases, this was based on the culture of CC-enclosed oocytes. Gilchrist *et al.* [23] stated that the process of CC expansion may occur without oocytes, and this was demonstrated in three out of four examined mammalian species. They used porcine and mouse COCs and oocyte-free complexes (OOXs) for evaluation the SMAD signaling pathway, which contributes to the efficiency of CC expansion. They found that inhibition of the ERK1/2 and p38 MAPK pathways affects porcine COC expansion. Although they did not culture separated CCs, as in our study, it can be concluded that porcine oocyte-free CCs may expand during IVC. Our results demonstrated that oocyte free-CCs are able to proliferate *in vitro*, which is presumably related to *in vitro* CC expansion.

For the first time, we could demonstrate the proliferation ability of separated oocyte-free CCs *in vitro*. Moreover, substantial changes in the expression and translocation of Cdk4 and Cx43 in CCs during IVC
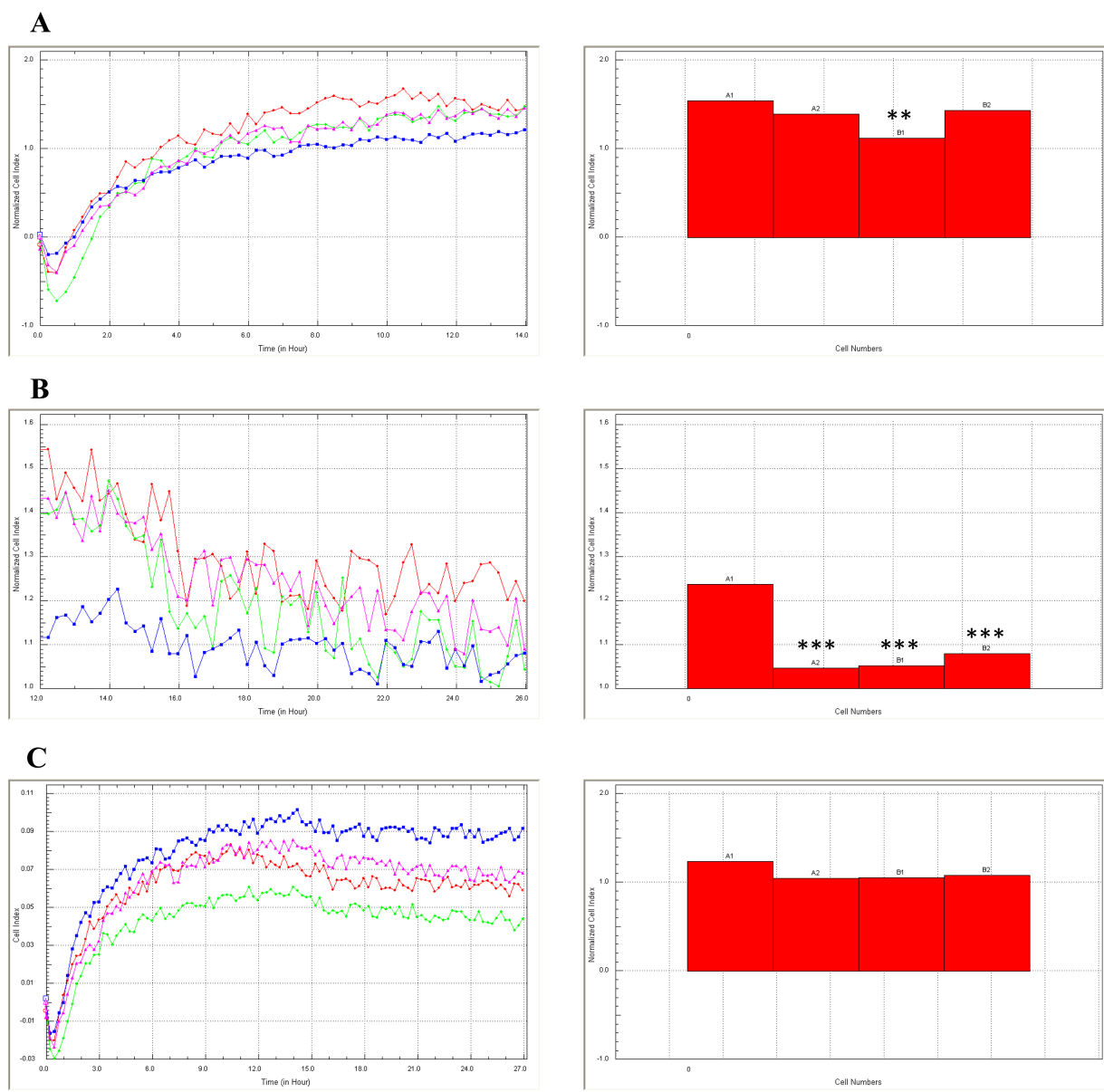
Fig. 1.   Normalized proliferation index of cumulus cells cultivated for 44 h. The cumulus cells were recovered from porcine COCs after collagenase treatment for 10 min at 38.5 C. Then, the CCs were immediately transferred into a real-time cell analyzer (RTCA, E-Plates 48, Roche Applied Science, Penzberg, Germany). The experiment consisted of four replications involving cultivation of the same population of collected CCs. At every step of the experiment, the normalized proliferation index was assessed in real-time *in vitro* cultivation for time periods of 0–12 h (A), 12–24 h (B), 0–24 h (C), 24–44 h (D) and 0–44 h (E).

were observed. The results of our study show that the concentration levels of both analyzed proteins were higher after IVM. Moreover, similarity was also observed in the distribution of proteins, with the localization of proteins before IVM being mainly nuclear and after IVM being mainly cytoplasmic. Both processes are thought to be crucial for full maturation of porcine oocytes.
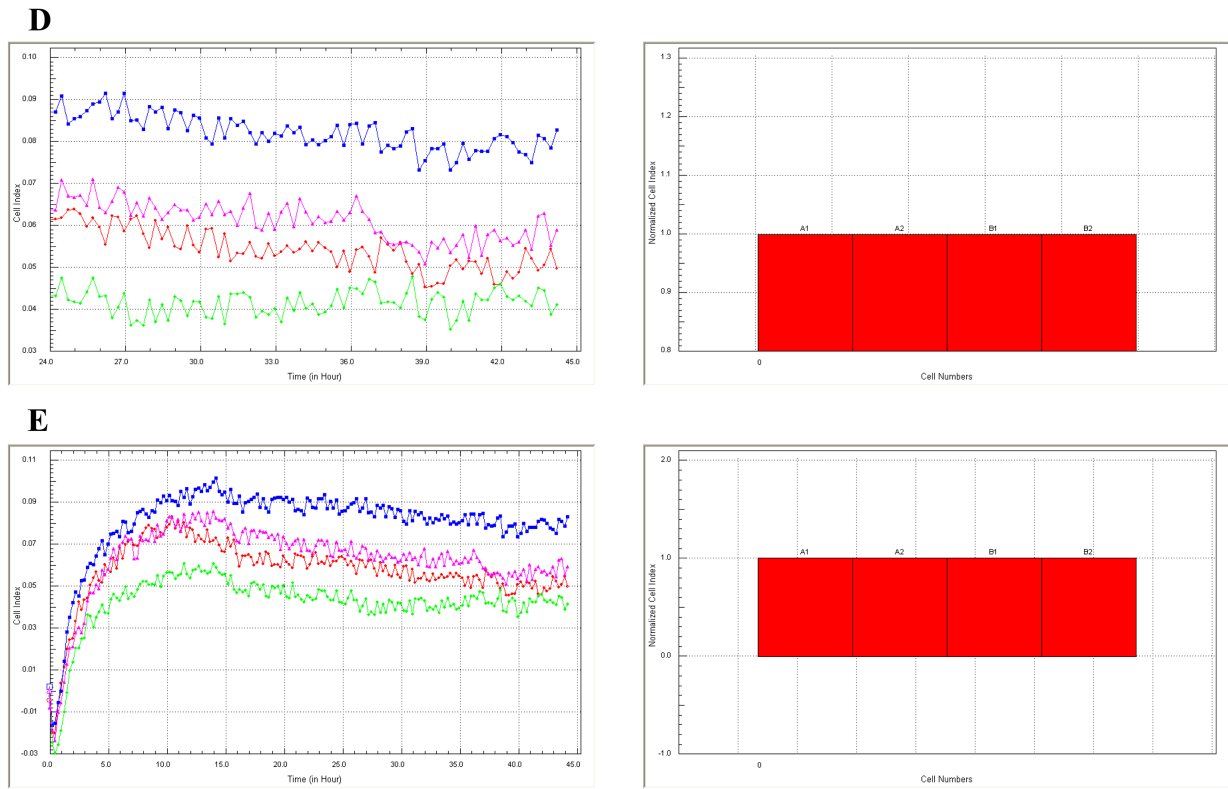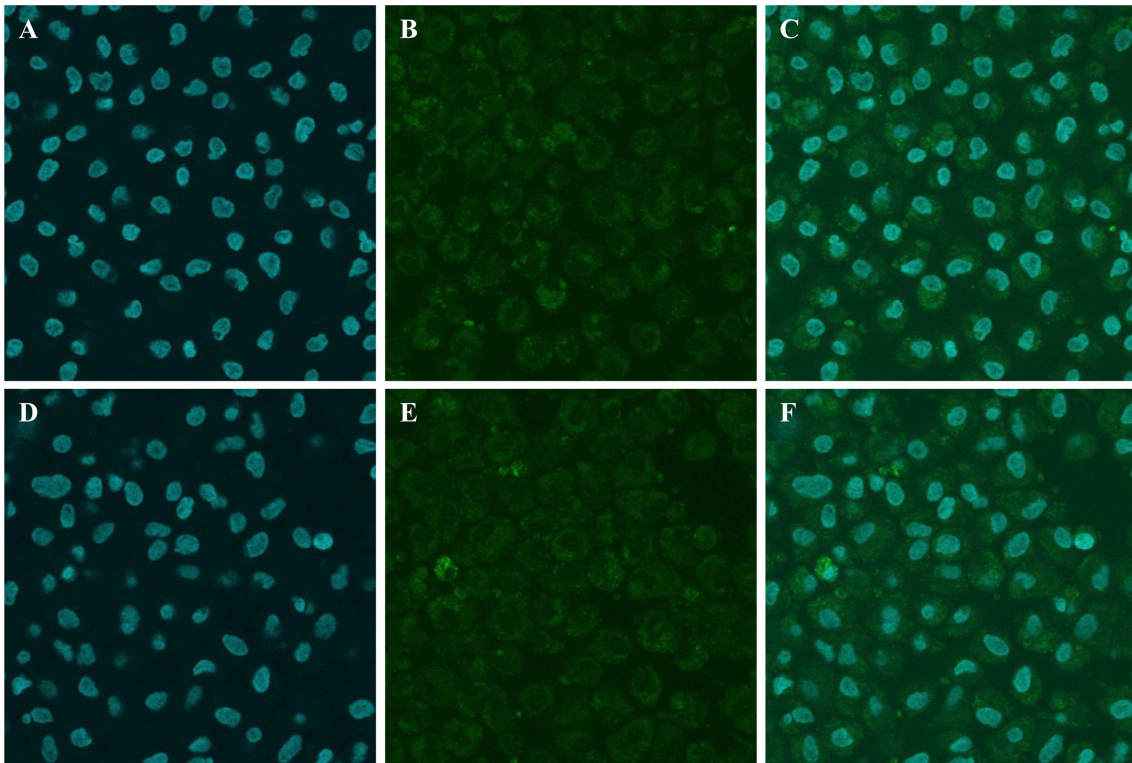
D

E

Fig. 1. (Continued)

Fig. 2. Confocal microscopic observation of Cdk4 and Cx43 expression and cellular distribution in porcine CCsat 0–12 h of IVM. Expression and localization of Cx43 (Fig. 2A–C) and Cdk4 (Fig. 2D–F) at the 0–12 h of IVM. CCs were stained with 0.1 µg/ml 4,6-diamino-2-phenylindole (DAPI; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in mineral oil (Fig. 2A and D) following staining with porcine Cx43 (rabbit polyclonal anti-Cx43 Ab) (Fig. 2B) and Cdk4 (rabbit polyclonal anti-Cdk4 Ab) (Fig. 2E). Secondary antibodies were labeled with FITC (fluorescence isothiocyanate), which emits a green fluorescent signal after excitation at 488 nm. Double staining of CCs is presented for Cx43 (Fig. 2C) and Cdk4 (Fig. 2F).
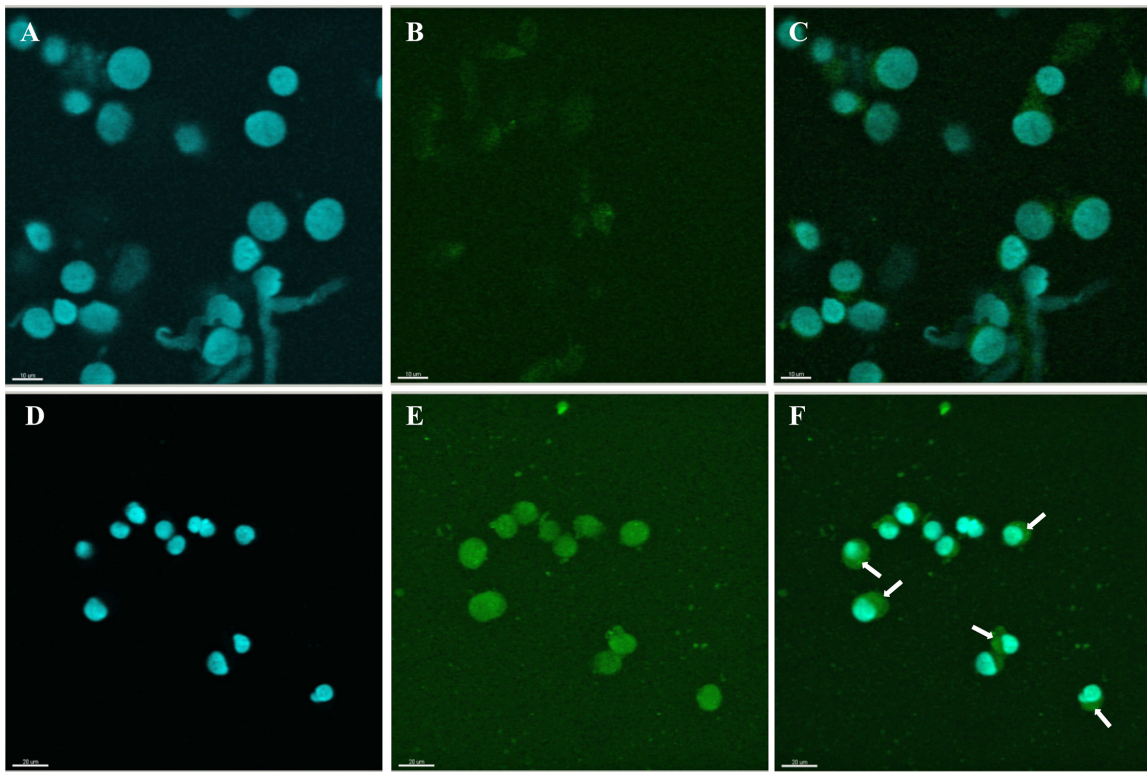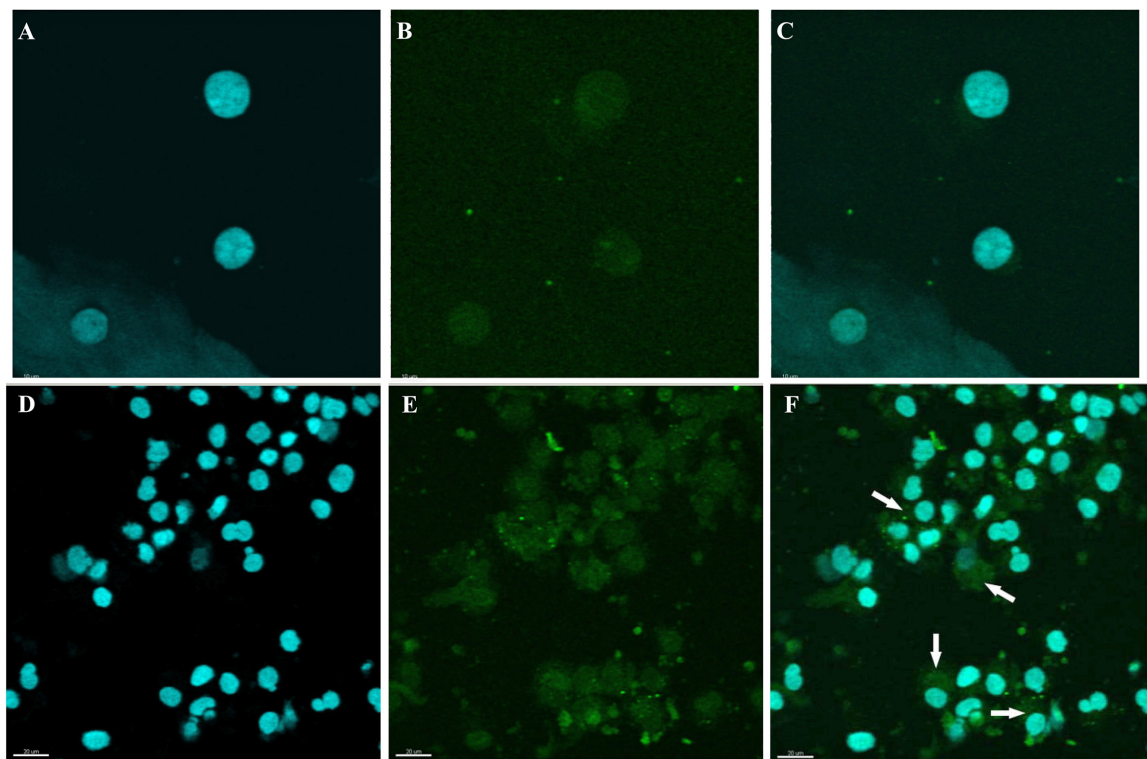
Fig. 3.

Fig. 4.

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Fig. 3. Confocal microscopic observation of Cdk4 expression and cellular distribution in porcine CCs before and after IVC. The collected CCs before (Fig. 3A–C) and after IVC (Fig. 3D–F) were stained with 0.1 µg/ml 4,6-diamino-2-phenylindole (DAPI; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in mineral oil (Fig. 3A and D), following staining with porcine Cdk4 (rabbit polyclonal anti-Cdk4 Ab), (Fig. 3B and E). Secondary antibodies were labeled with FITC (fluorescence isothiocyanate), which emits a green fluorescent signal after excitation at 488 nm. Double staining of CCs is presented as before (Fig. 3C) and after (Fig. 3F) IVC. The arrows point to the cytoplasmic localization of Cdk4 in CCs after IVC. Scale bars represent 10 µm (Fig. 3A–C) and 20 µm (Fig. 3D–F).

Fig. 4. Confocal microscopic observation of Cx43 expression and cellular distribution in porcine CCs before and after IVC. The collected CCs before (Fig. 4A–C) and after IVC (Fig. 4D–F) were stained with 0.1 µg/ml 4,6-diamino-2-phenylindole (DAPI; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in mineral oil (Fig. 4A and D), following staining with porcine Cx43 (rabbit polyclonal anti-Cx43 Ab), (Fig. 4B and E). Secondary antibodies were labeled with FITC (fluorescence isothiocyanate), which emits a green fluorescent signal after excitation at 488 nm. Double staining of CCs is presented as before (Fig. 4C) and after IVC (Fig. 4F). The arrows point to the cytoplasmic localization of Cx43 in CCs after IVC. Scale bars represent 10 µm (Fig. 4A–C) and 20 µm (Fig. 4D–F).