Expression of CD44 in Porcine Cumulus-Oocyte Complexes during In Vitro Oocyte Maturation

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Abstract: *In this study we sequentially investigated the expressions of CD44, the principal hyaluronan (HA) receptor, in porcine cumulus-oocyte complexes (COCs) during in vitro maturation. The mRNA expressions of CD44 were analyzed in porcine cumulus cells and oocytes. In reverse transcription-polymerase chain reaction (RT-PCR) analysis, the expression of CD44 was identified only in cumulus cells, indicating that cumulus cells express CD44 mRNAs. The transcript of CD44 was weakly detectable in fresh (0 h) cumulus cells, but was clearly detected after 12 h of culture. In immunostaining, CD44 was distributed on the cytoplasm along the perimembrane of cumulus cells and at the junctions between cumulus cells and oocytes. The level of CD44 expression reached a peak at 24 h of culture although its expression was very weak at 0 h. These findings implied that the level of CD44 expression depends on the extent of cumulus expansion. These results suggest that CD44 may be involved in HA retention in the extracellular matrix of COCs during oocyte maturation.*

Key words: *Cumulus expansion, CD44, Oocyte maturation, Porcine COCs*

After the preovulatory gonadotrophin surge, cumulus cells surrounding the oocyte begin to synthesize and secrete a large amount of hyaluronan (HA) [1–4]. HA, a major constituent of the extracellular matrix, is high molecular weight glycosaminoglycan (GAG) comprised alternating units of D-glucuronic acid and *N*-acetylglucosamine [5]. Proteins derived from serum [6, 7], and synthesized by cumulus cells [8–10] or granulosa cells [11], stabilize HA into an intercellular elastic network of cumulus-oocyte complexes (COCs). The deposition of HA in the cumulus-oocytes matrix causes “cumulus expansion”, a volumetric enlargement of the COCs (about 20–40 times the initial volume). The loss of gap junctions and cytoskeletal modification accompanying cumulus expansion in COCs are closely related to oocyte meiotic progression [12–15]. The cumulus expansion promotes dissociation of COCs from the follicle wall, accompanied by meiosis resumption of the oocyte [16]. Moreover, this cumulus-oocyte structure composed of HA-enriched matrix has been shown to positively support its extrusion at ovulation, it is picked up by oviduct fimbria, the signaling in spermatozoa through the binding of HA to sperm membrane protein PH-20, fertilization, and the subsequent development of zygotes [17–19].

Hyaladherins (hyaluronan-binding proteins) comprise the link module superfamily (containing a common structural domain which interacts with HA to form complexes that stabilize the extracellular matrixes) and other hyaluronan-binding proteins [20]. The ubiquitous HA receptor CD44 [21], a member of the link module superfamily, exists in numerous isoforms because of alternative splicing of 10 variant exons in different combinations [22, 23]. The hyaluronan binding properties of CD44 are determined by the isoform and cell type on which it is expressed [24]. HA-CD44 interactions have been reported to result in the activation of signaling cascades that contribute to cell adhesion, proliferation, migration and angiogenesis [25–27]. We previously showed expression of hyaluronan synthase (has) and CD44 mRNAs in porcine COCs during *in vitro* maturation (IVM) [28]. In cumulus cells, eCG and porcine follicular fluid (pFF) increased

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expression of has2 that is involved in HA synthesis leading to cumulus expansion, and the cell surface glycoprotein CD44. CD44 is present on mature oocytes and preimplantation embryos in man [29]. The addition of HA or chondroitin sulfate A, a ligand of CD44, to culture medium improves the development of *in vitro* matured/fertilized porcine embryo to the blastocyst stage [30]. These facts suggest that CD44 interacts with these GAGs and plays a positive role in oogenesis and embryogenesis.

In the present study we investigated sequentially expressions of CD44 mRNA and protein in porcine COCs during IVM. In RT-PCR analysis, the expression of CD44 mRNA was detected only in cumulus cells during IVM. Immunoreaction with CD44 was observed on the cytoplasm along the perimembrane of cumulus cells, at the junctions between oocytes and cumulus cells, on the surface of oocytes. It appeared that the level of CD44 expression reached a peak at 24 h of culture, although its expression was very weak at 0 h.

Materials and Methods

Chemicals and supplies

Chemicals and other supplies were obtained from the following sources: Dulbecco's phosphate buffered solution (PBS)-minus from Nissui Pharmaceutical Co. (Tokyo, Japan); medium 199 and the oligonucleotide primers from Gibco BRL Life Technologies Inc. (Grand Island, NY, USA); bovine serum albumin (BSA), sodium pyruvate, mineral oil, polyvinyl-pyrrolidone (PVP) and hyaluronidase from Sigma Chemical Co. (St. Louis, MO, USA); penicillin G potassium and streptomycin sulfate from Meiji Seika Co. (Tokyo, Japan); eCG from Teikoku Zouki Pharmaceutical Co. (Tokyo, Japan); An RNeasy mini-kit to isolate total ribonucleic acid (RNA) from QIAGEN K. K. (Tokyo, Japan); Ready-To-Go™ reverse transcription-polymerase chain reaction (RT-PCR) beads from Amersham Pharmacia Biotech Inc. (Piscataway, NJ, USA); CD44 monoclonal antibody; i.e., anti-porcine CD44 mouse IgG (cell line, PORC24A) from VMRD Inc. (Pullman, WA, USA); fluorescein isothiocyanate (FITC)-conjugated goat affinity purified F(AB') fragment to mouse IgG as the secondary antibody from ICN Pharmaceuticals Inc. (Aurora, OH, USA); and all other reagents from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). PBS-minus containing 3 mg/ml BSA, 100 IU/ml penicillin and 100 μg/ml streptomycin (PBS-BSA) was prepared for washing COCs, and for dilution of antibodies for immunofluorescence. PBS-minus containing 3 mg/ml PVP and antibiotics (PBS-PVP) was prepared for fixing samples. The arrangement of porcine follicular fluid (pFF) was as follows. Collected ovaries from prepubertal gilts were transported at 37°C from a local abattoir to the laboratory within 1 h. Follicular contents were aspirated from antral (2–5 mm in diameter) follicles and centrifuged at 1500 $\times$ g at 10°C for 20 min. The supernatant was stored at –20°C.

In vitro maturation of porcine COCs

Collected ovaries from prepubertal gilts were transported at 37°C from a local abattoir to the laboratory within 1 h. Follicular contents were aspirated from antral follicles (2–5 mm in diameter), and then only oocytes with compact, multi-layered cumulus cells and a uniformly granulated cytoplasm were collected as cultivable COCs. After a brief wash in PBS-BSA, COCs were placed in medium 199 with Earle's salt and L-glutamine containing 2.2 mg/ml sodium bicarbonate, 0.1 mg/ml sodium pyruvate, 10 mg/ml BSA, 100 IU/ml penicillin and 100 μg/ml streptomycin, 10 IU/ml eCG and 10% (v/v) pFF. The COCs were incubated at 37°C for 0 h, 12 h, 24 h, 36 h and 48 h in a humid atmosphere of 5% CO_2 in air. Each droplet of medium (200 μl) was overlaid with mineral oil and contained approximately 20 COCs.

Detection of CD44 mRNA by reverse transcription-polymerase chain reaction (RT-PCR)

For extraction of total RNA, the cumulus cells and the oocytes from COCs cultured for 0 h, 12 h, 24 h, 36 h and 48 h were collected after treatment with 0.1% (w/v) hyaluronidase. Each sample of total RNA was isolated according to the instructions supplied with the RNeasy mini-kit. RT-PCR was performed with Ready-To-Go™ RT-PCR beads that were optimized to allow the first-strand cDNA synthesis and PCR reactions to proceed sequentially as a single-step reaction, and a PCR Thermal Cycler TP2000 (TaKaRa Co., Kyoto, Japan). Each primer was designed according to published mouse cDNAs, including regions highly conserved between the mouse and humans. For CD44, the sense primer (5'-GTACATCAGTCACAGACCTAC-3') and the antisense primer (5'-CACCATTTCCTTGAGACTTGCT-3') generated a 598-bp cDNA fragment correspondent to mouse CD44 nucleotides (accession number:M27129). For β-actin (as an internal positive control), the sense primer (5'-GACCCAGATCATGTTTGAGACC-3') and the antisense primer (5'-ATCTCCTTCTGCATCCTGTCAG-3') generated a 593-bp cDNA fragment correspondent to mouse β-actin nucleotides (accession number: X03672).

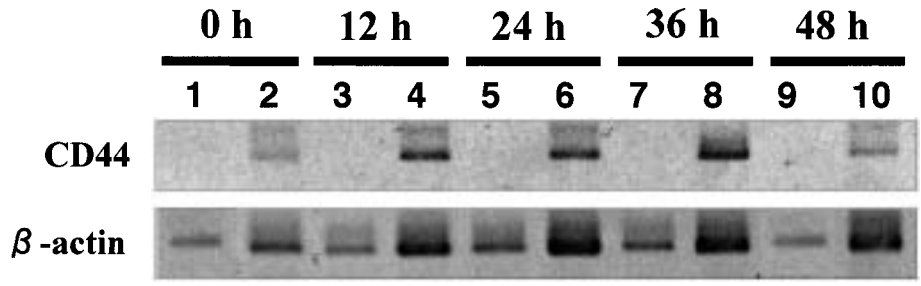

Fig. 1. Expression of CD44 and β-actin mRNAs in porcine cumulus cells and oocytes after culture for 0 h, 12 h, 24 h, 36 h and 48 h in the presence of eCG and pFF. The equivalent total RNA of 30 oocytes per sample and 50 ng of total RNA extracted from cumulus cells were subjected to RT-PCR analysis. Lanes 1, 3, 5, 7 and 9: oocytes; lanes 2, 4, 6, 8 and 10: cumulus cells.

Fifty nanograms of total RNA extracted from cumulus cell mRNA, the equivalent of total RNA from 30 oocytes, was reverse-transcribed and then PCR amplified, in a total reaction volume of 50 μl containing 10 pmol of each sense and antisense primer, 0.5 μg of oligo d $(T)_{12\text{-}18}$ primer, ~2.0 U of Taq deoxyribonucleic acid polymerase, 10 mM Tris-HCl (pH 9.0), 60 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, Moloney Murine Leukemia Virus reverse transcriptase (FPLCpure™), and RNAguard™ ribonuclease inhibitor (porcine) and stabilizer, including ribonuclease/deoxyribonuclease-free BSA. This mixture overlaid with mineral oil was incubated at 42°C for 20 min for the RT reaction. PCR amplification proceeded after inactivation of the reverse transcriptase by heating at 95°C for 5 min. PCR cycling conditions were as follows: 30 cycles of 1 min denaturation at 95°C, 1 min annealing at 55°C and 1 min extension at 72°C. Total RNAs were subjected to RT-PCR without reverse transcriptase by using β-actin primer pairs to detect genomic DNA contamination. The amplified products were electrophoresed on 2% agarose gel and visualized by ethidium bromide staining.

CD44 localization by immunofluorescence

COCs cultured for 0 h, 12 h, 24 h, 36 h and 48 h were fixed in 2% (v/v) formaldehyde in PBS-PVP at 4°C for 30 min and then washed three times with PBS-BSA. The specimens were permeabilized in 0.25% (v/v) Tween 20 in PBS-BSA at room temperature for 2 min and washed three times in PBS-BSA, and then stored in PBS-BSA containing 0.02% sodium azide at 4°C for 0–7 days. Fixed samples were incubated with primary antibody diluted 1:50 in PBS-BSA at 37°C for 2 h. After several washes with PBS-BSA, these samples were exposed to FITC-conjugated goat anti-mouse antibody diluted 1:200 in PBS-BSA at 37°C for 1 h. To detect nuclear localization, propidium iodide (PI) staining was followed by several washes with PBS-BSA. Stained samples were incubated in 10 μg/ml PI at 37°C for 1 h, washed with PBS-BSA, and then mounted under a coverslip with PBS-BSA. Whole mounted specimens were placed on an Axioplan Zeiss microscope and examined with a Biorad MRC-1024 laser scanning confocal microscope (Bio-Rad Laboratories Co, Hercules, CA, USA).

Results

Expression of CD44 mRNA in cumulus cells by RT-PCR

Amplified product of CD44 was detected near the predicted size (598 bp). In a previous study, we verified that the partial porcine cDNAs amplified with mouse CD44 primers conformed to porcine CD44 [28]. The transcript of CD44 was weakly detectable in fresh (0 h) cumulus cells, but more clearly detected after 12 h of culture (Fig. 1). The expression of CD44 was not found in any group of oocytes during IVM.

CD44 localization by immunofluorescence

Although there was no apparent staining in fresh COCs, positive staining was observed after 12 h of culture (Fig. 2). An especially strong signal was detected in COCs cultured for 24 h. The expression of CD44 in cumulus cells which closely surround an oocyte after 48 h of culture, tends to be reduced as compared with that after 24 h of culture (Fig. 3B and C). It was difficult to confirm expression of CD44 in the outer layer of COCs cultured for 48 h because the outer layer of the COCs was torn off in the course of immunostaining. Positive staining was found on the cytoplasm along the

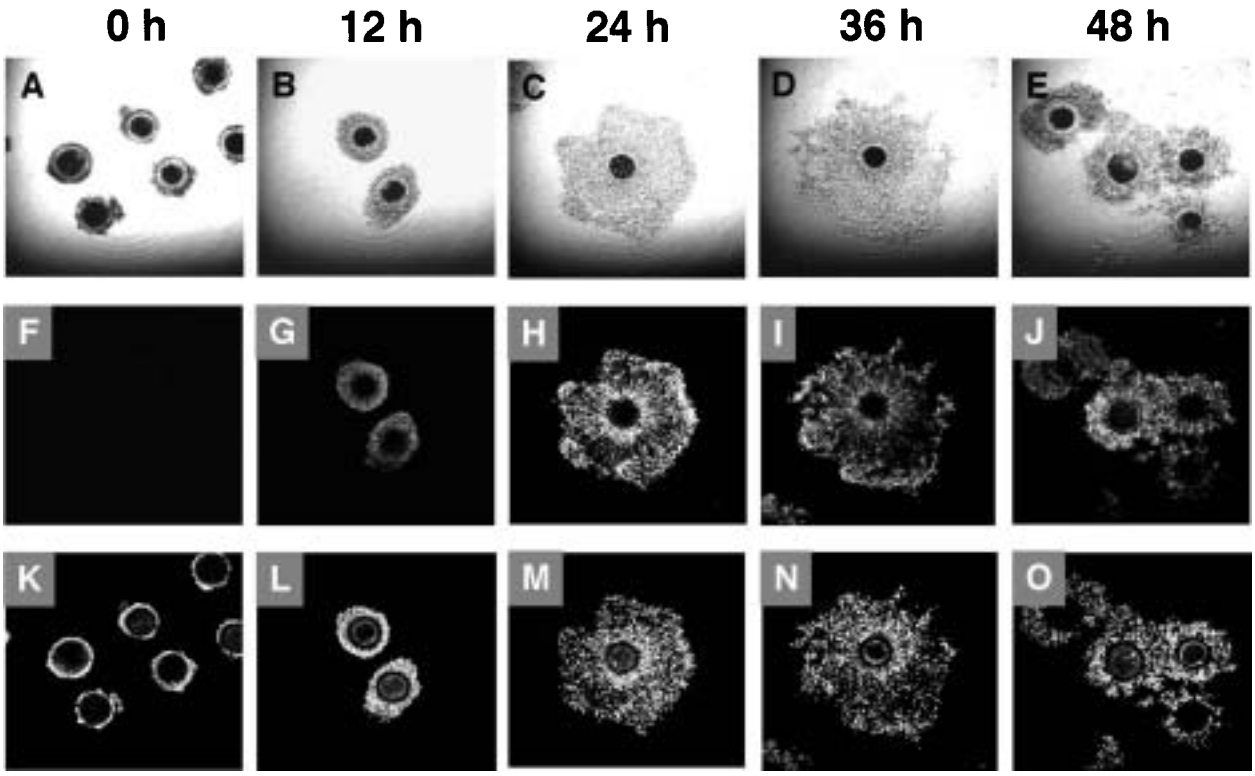

Fig. 2. Detection of CD44 by double staining with immunofluorescence (FITC and PI) in porcine COCs cultured with eCG and pFF for 0 h, 12 h, 24 h, 36 h and 48 h. Selected sections obtained by laser scanning confocal microscopy. A-E: under normal light; F-J: localization of CD44; K-O: localization of nuclei.

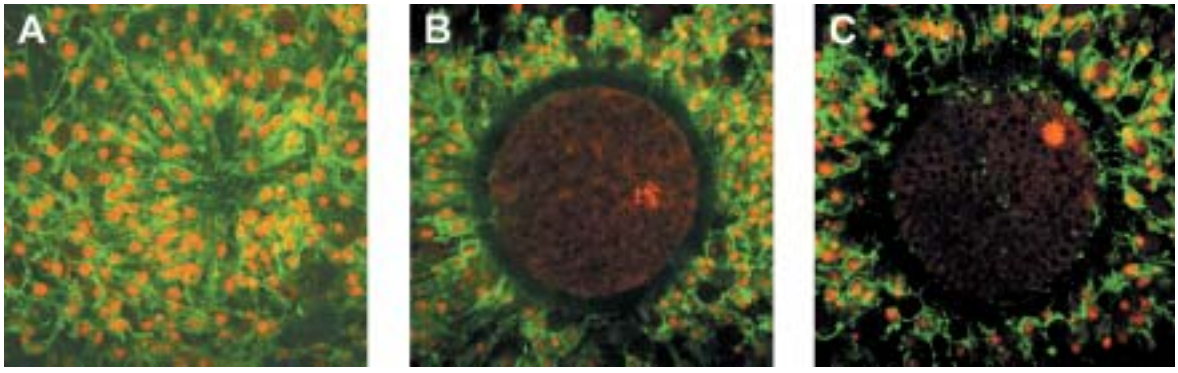

Fig. 3. Localization of CD44 by double staining with immunofluorescence (FITC and PI) in cumulus expanded COCs after culture with eCG and pFF. Selected sections obtained by laser scanning confocal microscopy. A: surface of COCs cultured for 24 h; B: cross section of COCs cultured for 24 h; C; cross section of COCs cultured for 48 h.

perimembrane of cumulus cells (Fig. 3A) and at the junctions between cumulus cells and oocytes (Fig. 3B and C). Staining of heterogeneous patches, such as at the bottom of junctions between cumulus cells and oocytes, was also found in the surface of oocytes or in inside zona.

Discussion

In this study we investigated sequential expressions of CD44 mRNA and protein in COCs, or cumulus cells and oocytes during cumulus expansion stimulated *in vitro*. The expression of CD44 mRNA was detected

only in cumulus cells but not in oocytes, in RT-PCR analysis. We detected heterogeneous immunoreactivity of CD44 on the surface of oocytes or inside zona, but CD44 mRNA was not found on oocytes. Possible reasons for this contradiction are that 1) CD44 produced by cumulus cells enters through the zona with connections between the oocyte and cumulus cells and 2) we did not verify the expression of the alternative spliced exons that are involved in the functional heterogeneity of CD44 by using RT-PCR. Genomic analysis of CD44 showed the existence of 20 exons, which by alternative RNA splicing, code for the CD44 standard/hemopoietic form (CD44s/CD44H) and numerous variant isoforms (CD44v) [22, 23]. CD44s is composed of the following exons: exons 1s to 7s encoding the extracellular domain, exon 8s (the transmembrane region), and exons 9s and 10s which can be alternatively spliced resulting in the generation of a cytoplasmic tail [31]. The variant exons 1v to 10v located between exons 5s and 6s give enormous variability to the CD44 family of molecules. The part of the CD44 cDNA amplified was restricted to exons 3s to 10s in the present study. It has been reported that the CD44 expressed in cumulus cells and mural granulosa cells is mainly the standard form (CD44s) [32]. The positive reactivity to CD44 antibody in oocytes may indicate the expression of CD44 variants.

Campbell *et al.* [29] demonstrated by indirect immunofluorescence that CD44 is present on the surface of human oocytes and cumulus cells and continues to be expressed on the embryonic surface until the pre-hatched blastocysts. We also detected immunoreactivity of CD44 on the surface or the cytoplasm of cumulus cells and also in patches on the surface of oocytes. Notably, cumulus cells of COCs at 24 h of culture expressed CD44 strongly, but only moderately at 12 h of culture and almost never expressed it at 0 h. In the previous study we confirmed the effects of eCG and pFF on cumulus expansion and expression of CD44 mRNA [28]. The pattern of CD44 mRNA expression seemed to be related to the state of the expansion area after 24 h of culture. These results showing that the level of CD44 expression depends on the extent of cumulus expansion imply that CD44 is involved in HA retention in the extracellular matrix of COCs during oocyte maturation.

The role of the HA/CD44 system in oocyte maturation is not known. Ohta *et al.* [32] demonstrated that cumulus cells showed stronger CD44 expression than mural granulosa cells in human patients after superovulation with human menopausal gonadotrophin and human chorionic gonadotrophin. Therefore, a high level expression of CD44 in cumulus cells during IVM compared with immature cumulus cells in our results may indicate that the signaling cascade of HA-CD44 interaction has an important effect on oocyte maturation. There is also a report that apoptotic granulosa cells with fragmented condensed nuclei were reduced by HA via CD44 [33]. Sato *et al.* [34] showed that the culture of mouse oocytes with specific GAGs purified from pFF, the fraction with a retention time nearly coincident with that of HA, promotes the viability of oocytes. These results indicate that HA also positively participates in the viability of granulosa cells, cumulus cells and oocytes.

There is evidence suggesting that the size of HA molecules may determine the signaling response in target cells. For example, the binding of high molecular weight polymers of HA to CD44 isoforms facilitates cell adhesion, whereas binding to HA fragments results in activation of cell signaling event [35]. It has been shown that the degradation product of HA (3 to 10 disaccharides) induces the phosphorylation of the CD44 receptor, leading to the activation of a cytoplasmic kinase cascade, that is, Raf-1 kinase, mitogen-activated protein kinase (MAPK) kinase (MEK-1), and extracellular signal-regulated kinase (ERK-1) which is subsequently translocated to the nucleus [36]. This cascade is important for mitogenic signal transduction and sufficient for the induction of cell proliferation through the stimulation of proto-oncogenic transcription factors (such as ATF-2, c-Fos, c-Jun, c-Myc, ELK-1 and NF-I16) [37]. The MAPK cascade also plays a crucial role in the M-phase promoting factor activation and stabilization during oocyte maturation [38, 39]. Recently, Su *et al.* [40] demonstrated that MAPK activity in cumulus cells, rather than in the oocyte, is required for gonadotropin-induced meiotic resumption. The activation of MAPK in cumulus cells required at least one signaling pathway, e.g. cAMP-PKA.

In conclusion, the results of current study indicate that CD44 is expressed in cumulus cells during IVM and may play important roles in HA retention to form cumulus expansion and oocyte maturation. Further investigation will be necessary on the involvement of the signal of HA/CD44 in oocyte maturation during cumulus expansion and the maintenance of oocyte viability.

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