

—Full Paper—

## Role of the Hyaluronan Receptor CD44 During Porcine Oocyte Maturation

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**Abstract.** Previous our studies have shown that CD44, the principal receptor for hyaluronan, is present on cumulus cells during oocyte maturation. Although hyaluronan-CD44 interaction has been implicated in cumulus expansion and/or oocyte maturation, the full significance of CD44 remains unknown. The objective of the present study was to further investigate the role of CD44 in cumulus expansion and oocyte maturation in pigs. We demonstrate here in that CD44 has a key role in oocyte maturation but not in cumulus expansion. Previous studies have reported the physiological significance of cumulus expansion in oocyte maturation. However, our results suggest that cumulus expansion is a necessary condition for oocyte maturation, but that it is not sufficient on its own. Furthermore, western blot analysis demonstrated that the CD44 of the *in vitro*-matured cumulus-oocyte complexes (COCs) had a larger molecular weight and more terminal sialic acid, which has been proven to inhibit the hyaluronan-binding ability of the receptor, than the CD44 of the *in vivo*-matured COCs, indicating that the hyaluronan-CD44 interactions during *in vitro* maturation might be insufficient compared with those *in vivo*. The insufficient interactions of hyaluronan-CD44 during *in vitro* maturation may cause the inferior capacity of fertilization and development of oocytes matured *in vitro*.

**Key words:** CD44, Hyaluronan, Oocyte maturation, Pig, Sialic acid

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In mammals, the cumulus-oocyte complex (COC) is a functional unit of the ovarian follicle that consists of several layers of compacted cumulus cells closely surrounding an oocyte. During the preovulatory period, the COC expands dramatically. This expansion is the result of the synthesis and accumulation of hyaluronan around

the cumulus cells [1, 2]. This process occurs *in vivo* after a gonadotropin surge and can also be achieved during *in vitro* maturation. It has been reported that the physiological significance of cumulus expansion is important in study of the developmental competence of mammalian oocytes matured and fertilized *in vitro* [3–6]. Furthermore, the degree of cumulus expansion is often cited as a major indicator in selection of oocytes for *in vitro* fertilization (IVF) protocols [3–7]. Insufficient oocyte maturation has been ranked as one of the

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reasons for failure of IVF of porcine oocytes, which most likely results from a deficient zona reaction due to insufficient oocyte cytoplasmic maturation (formation and alignment of the cortical granules), thus favouring the presence of lethal polyspermia. Polyspermia dramatically constrains the conduct of IVF in pigs [8].

In general, hyaluronan needs hyaluronan-binding protein(s), an important subset of proteins with highly homologous sequences for hyaluronan binding, to organize the hyaluronan-rich matrix [9]. CD44 is the principal cell-surface receptor for hyaluronan and is present in a number of isoforms with different molecular sizes in a wide variety of cell types [10–13]. It is a transmembrane protein consisting of extracellular and cytoplasmic domains linked through transmembrane segments in the cell membranes of a variety of cells [14]. The extracellular domain of CD44 contains the necessary motifs for binding hyaluronan [15], and glycosylation of its extracellular domain has been implicated in regulation of the hyaluronan-binding ability of CD44 [11]. In particular, enzymatic hydrolysis of sialic acid molecules augments the ability to bind hyaluronan, implying that the terminal sialic acids of CD44 have an inhibitory effect on the hyaluronan-binding ability of CD44 [16–18].

The binding of hyaluronan provides a physiologically important switch between the adhesive and signaling functions of CD44. Hyaluronan-CD44 interaction is responsible for cell-to-cell and cell-to-extracellular matrix interactions [19], inhibition of apoptosis [20], augmentation of tumor cell motility and metastasis [21], and stimulation of lymphocytes [22]. Recent studies indicate that CD44 may influence fertility and the quality of oocytes [23, 24]. Additionally, our previous studies demonstrated that CD44 expression on cumulus cells corresponds to cumulus expansion during oocyte maturation in pigs [25–27]. These findings suggest that the interplay between hyaluronan and CD44 may be one of the important requirements for cumulus expansion and/or oocyte maturation. However, the role of CD44 in cumulus expansion and oocyte maturation remains poorly understood.

Therefore, in the present study, we investigated the effects of CD44 on cumulus expansion and oocyte maturation in pigs and the differences in CD44 expression between *in vitro*-matured and *in*

*vivo*-matured COCs using western blot analysis.

## Materials and Methods

### *Collection and in vitro maturation of porcine COCs*

Offal gilt ovaries were obtained from a local slaughterhouse and transported to the laboratory within 30 min in a container kept at 38°C. Follicles 2–5 mm in diameter in the ovaries were aspirated with a 5-ml syringe and 20-G needle, and only COCs with uniform and compact cumulus cells were collected in 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, Inc., Kyoto, Japan), 0.1 mg/ml sodium pyruvate (Sigma, St. Louis, MO, USA), 10 mg/ml BSA (Sigma), 100 IU/ml penicillin (Meiji Seika, Tokyo, Japan), 100 µg/ml streptomycin (Meiji Seika), 10% (v/v) porcine follicular fluid, and 10 IU/ml eCG (Serotropin; Teikoku Zoki, Tokyo, Japan). The collected COCs were washed three times with the medium and were cultured in the same medium supplemented with 0–5.0 µg/ml rat anti-CD44 antibody (IM7; BD Biosciences Pharmingen, San Diego, CA, USA) and covered with paraffin oil (Nacalai Tesque) for 48 h at 37°C under 5% CO<sub>2</sub> in air. The antibody solution was ultrafiltrated through Microcon-10 (Millipore, Bedford, MA, USA) for solvent exchange into the culture medium. This antibody has been found to significantly reduce the hyaluronan-binding ability of CD44 [28–30] and to recognize porcine CD44. COCs cultured with 5.0 µg/ml normal rat IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as the control.

### *Morphologic assessment of cumulus expansion*

COCs in each treatment group were evaluated for the degree of cumulus expansion at 48 h of culture. Assessment of cumulus expansion was based on the size of the area occupied by the cumulus cell aggregate of the COC. The lengths and widths of COCs were measured with an eyepiece micrometer. The length was defined as the distance between the two most widely separated points, and the width was defined as the distance between the two closest points. The area occupied by a COC was calculated using the following formula: area=length × width × 0.7854 [31].

### *Assessment of nuclear maturation*

Oocytes were evaluated 48 h after maturation culture to assess their maturation rates. COCs were denuded by pipetting in 0.1% hyaluronidase (Sigma). The denuded oocytes were fixed for 48 h with aceto-ethanol (acetic acid:ethanol, 1:3, v:v), stained with 1% aceto-orcein, and examined under a phase-contrast microscope for evaluation of their chromatin configuration. The stage of meiotic progression was assessed as GV stage (germinal vesicle), metaphase I stage (diakinesis I, metaphase I, anaphase I and telophase I), or metaphase II stage (metaphase II).

### *Collection of *in vivo*-matured COCs*

Multiparous cross-bred (Swedish Yorkshire × Swedish Landrace) sows (n=4, parity 2–5) were recruited for experiments from a commercial farm on the day of weaning and were individually penned at the Department of Obstetrics and Gynaecology, SLU, Uppsala. The sows received water *ad libitum* and standard rations according to Swedish standards [32]. A fertile boar was always penned in the vicinity and the sows were checked twice daily by experienced personnel for spontaneous behavioural estrus. At the onset of estrus, the sows were scanned every 4 h by transrectal ultrasonography [33]. The time of ovulation was defined as 2 h before the first point in time when there was a significant reduction in the number of preovulatory follicles or when preovulatory follicles could no longer be seen. The sows were then immediately euthanized, and their ovaries and oviducts were promptly removed. The ovulated COCs were collected by flushing the oviductal ampulla. The experimental design has previously been reviewed and approved by the local Ethical Committee for Experimentation with Animals in Sweden.

### *Protein extraction*

Fifty COCs were transferred to microfuge tubes and treated with hyaluronidase by vortexing for 15 min at 37 °C. After treatment, the oocytes and cumulus cells were washed three times with phosphate-buffered saline (PBS) and vortexed with 10 µl of cell lysis buffer [50 mM Tris, pH 7.5, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 M 6-amino-n-caproic acid, 5 mM benzamidine HCl, 1% (v/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)]. These agents were

purchased from Sigma. After stirring at 4 °C for 1 h, the tubes were centrifuged at 10,000 × g for 30 min, and then the supernatants were collected. The protein extracts were frozen and stored at -20 °C until use.

### *Immunoprecipitation and de-sialylation of CD44*

Immunoprecipitation of CD44 was performed as described previously [26]. In brief, aliquots of 3 µg of anti-CD44 antibody (PORC24A; VMRD, Pullman, WA, USA) were precoupled to 10 µl of protein G-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ, USA) by incubation for 1 h at 4 °C. The COC extract was then added to the antibody-precoupled protein G beads and incubated for 2 h at 4 °C. After washing, the CD44 on the antibody-protein G beads was resuspended in 0.2 M acetate buffer (pH 5.0) with or without 0.05 U of sialidase (neuraminidase; Sigma) and incubated overnight at 37 °C with gentle shaking. After extensive washing, the CD44 was eluted from the beads by boiling in non-reducing SDS sample buffer and was then analyzed by western blotting, as described below.

### *Western blotting analysis of CD44*

Extracted proteins or CD44 immunoprecipitates were separated by 8% SDS-PAGE under non-reducing conditions [34] and electroblotted in a semidry blotting apparatus according to the method of Hirano and Watanabe [35]. Electroblotting was performed for 90 min with 0.8 mA/cm<sup>2</sup> constant current. After electroblotting, the membranes were blocked with 2% skim milk in PBS-T (0.05% [v/v] Tween 20, pH 7.5) for 1 h at room temperature. After washing three times with PBS-T, the membranes were incubated with anti-CD44 antibody (PORC24A) overnight at 4 °C and were then reacted with secondary antibody (horseradish peroxidase-labeled anti-mouse IgG; Sigma). Following washing three times with PBS-T, the peroxidase activity was visualized using an ECL western blotting detection system (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

### *Statistical analysis*

Experiments were repeated three times and the data was pooled for statistical analysis. The cumulus expansion data was analyzed using one-way ANOVA followed by the Bonferroni-adjusted

**Table 1.** Effect of anti-CD44 antibody on porcine oocyte maturation

Treatment	Concentration ( $\mu\text{g}/\text{ml}$ )	n	GV stage (%)	MI stage (%)	MII stage (%)
Anti-CD44 antibody	0	61	10 (19.4) <sup>a</sup>	6 (9.8) <sup>ab</sup>	45 (73.8) <sup>a</sup>
	0.5	64	20 (31.3) <sup>b</sup>	9 (14.1) <sup>ab</sup>	35 (54.7) <sup>b</sup>
	1.0	60	24 (40.0) <sup>b</sup>	12 (20.0) <sup>a</sup>	24 (40.0) <sup>b</sup>
	2.5	63	57 (90.5) <sup>c</sup>	6 (9.5) <sup>ab</sup>	0 (0) <sup>c</sup>
	5.0	61	55 (90.2) <sup>c</sup>	6 (9.8) <sup>ab</sup>	0 (0) <sup>c</sup>
	Normal IgG	5.0	11 (17.2) <sup>a</sup>	5 (7.8) <sup>b</sup>	48 (75.0) <sup>a</sup>

<sup>a,b,c</sup>Values within the same column with different superscripts are significantly different ( $P<0.05$ ).

GV: germinal vesicle. MI: metaphase I. MII: metaphase II.

significance test. Rates of oocyte maturation were compared by Chi-square test or, when expected values of  $<5$  were involved, Fisher's exact probability test. A probability of  $P<0.05$  was considered to be statistically significant.

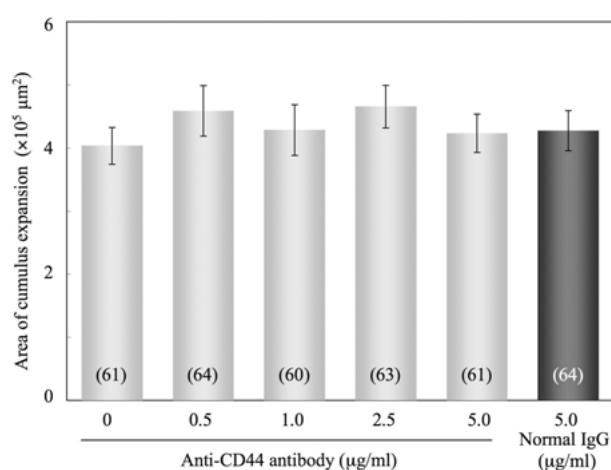
## Results

### Effect of anti-CD44 antibody on cumulus expansion and oocyte maturation

After culture of COCs for 48 h in the absence of antibody, 73.8% of oocytes reached the metaphase II stage. Addition of the antibody to the culture medium significantly suppressed oocyte maturation in a dose-dependent manner (Table 1). In contrast, addition of the antibody to culture media had no significant effect on cumulus expansion (Fig. 1 and Fig. 2). Meanwhile, addition of normal rat IgG to culture media had no significant effect on the rate of oocyte maturation or degree of cumulus expansion (Table 1 and Fig. 1).

### Western blot analysis for expression of CD44 protein in porcine COCs

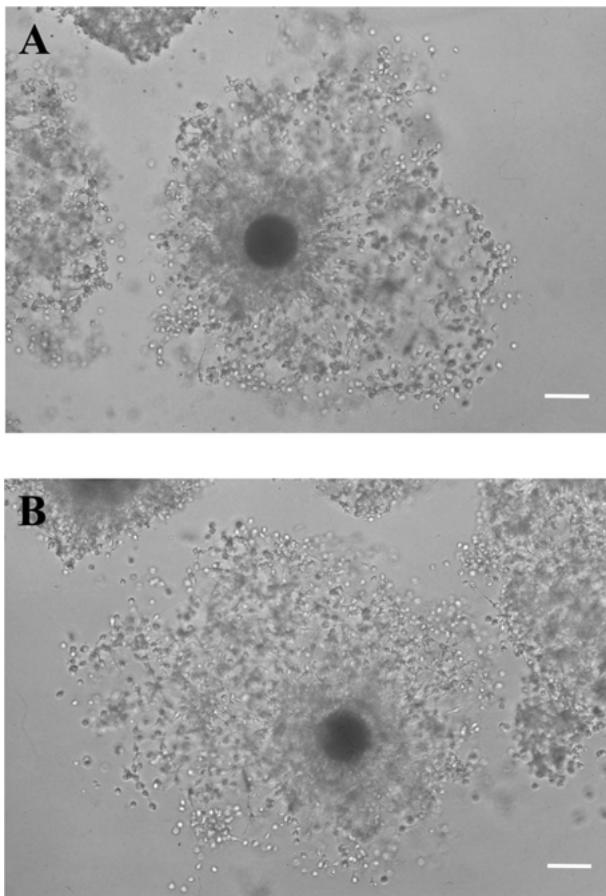
Although the CD44 band was absent from immature COCs collected from the ovarian follicles (Fig. 3, Immature), COCs matured *in vitro* for 48 h (Fig. 3, *In vitro*-matured) showed bands of CD44 ranging from 81 to 88 kDa. On the other hand, sialidase treatment reduced the size of the CD44 obtained from COCs matured *in vitro* [Fig. 3, Sialidase (+)]. We estimated that the size of the CD44 in the treated samples was 70–82 kDa and that it was clearly smaller than the size of the CD44 in COCs matured *in vitro*. There was no significant difference in the size of CD44 between the untreated and control samples; the control samples



**Fig. 1.** Effect of anti-CD44 antibody on cumulus expansion in pigs. COCs were cultured with anti-CD44 antibody (0, 0.5, 1.0, 2.5 or 5.0  $\mu\text{g}/\text{ml}$ ) for 48 h. COCs cultured with normal rat IgG were used as the control. The number of COCs is indicated in parentheses. The data is presented as the mean  $\pm$  SEM.

were *in vitro*-matured COCs extracted without sialidase [Fig. 3, Sialidase (-)].

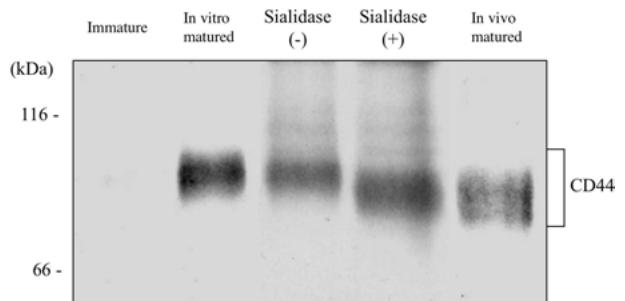
Furthermore, we also examined the molecular weight of CD44 in the COCs matured *in vivo*, which were collected from the oviductal ampulla, by western blot analysis. As shown in Fig. 3, western blot analysis indicated a difference in the size of CD44 between the *in vivo* and *in vitro* samples. The CD44 band of the *in vivo*-matured COCs was 73–83 kDa in size (Fig. 3, *in vivo* matured). The size of the CD44 in the COCs matured *in vivo* was clearly smaller than the band of CD44 from the COCs matured *in vitro* and was not significantly different from that of the sialidase treatment samples.



**Fig. 2.** Cumulus expansion in porcine COCs matured *in vitro* with (A) or without (B) anti-CD44 antibody (5.0 µg/ml) for 48 h. Bars=100 µm.

## Discussion

We showed here that CD44 plays an important role in porcine oocyte maturation, but not in cumulus expansion. We also showed differences in electrophoretic migration between *in vitro*-matured and *in vivo*-matured COCs, and *in vitro*-matured COCs contained more terminal sialic acid, which has an inhibitory effect on the hyaluronan-binding ability of CD44. Therefore, our results indicate that cumulus expansion is a necessary condition for oocyte maturation in pigs, but that it is not sufficient on its own, although previous studies have reported that the physiological significance of cumulus expansion is important for oocyte maturation. Furthermore, our results also indicate that sufficient hyaluronan-CD44 interaction during cumulus expansion is important for oocyte



**Fig. 3.** Western blot analysis for expression of CD44 protein in porcine COCs. COCs collected from follicles (immature), COCs cultured for 48 h (*in vitro* matured), and ovulated COCs (*in vivo* matured) were lysed with CHAPS. Extracts derived from COCs matured *in vitro* were immunoprecipitated with anti-CD44 antibody and then incubated with [Sialidase (+)] or without [Sialidase (-)] sialidase overnight at 37°C. Samples were subjected to 8% SDS-PAGE and western blot analysis for CD44 expression. Note the downshift of the size of CD44 after de-sialylation of COCs matured *in vitro*. The mobility of size standards is indicated.

maturity.

It has been reported that CD44 mRNA and protein are expressed in cumulus cells during *in vitro* maturation of COCs [23–27]. These results suggest that CD44 is important for cumulus expansion and oocyte maturation. However, there is no reported evidence about whether CD44 plays a role in cumulus expansion and oocyte maturation. In the present study, oocyte maturation was inhibited in a manner dependent on the concentration of the antibody when COCs were cultured with anti-CD44 antibody, which had a blocking effect on the hyaluronan-binding ability of CD44 [28–30]. This finding suggests that CD44 plays a key role in oocyte maturation. On the other hand, the antibody did not influence the degree of cumulus expansion. This result indicates that CD44 is not involved in cumulus expansion. In general, hyaluronan needs hyaluronan binding protein(s), an important subset of proteins with highly homologous sequences for hyaluronan binding, to organize the hyaluronan-rich matrix [9]. This is likely also true for organization of the hyaluronan-rich matrix of the cumulus cells during oocyte maturation. Recently, Zhuo *et al.* [36] reported that serum-derived hyaluronan-associated protein (SHAP), which corresponds to the heavy chain of plasma inter-alpha-trypsin

inhibitor and is bound to hyaluronan via a unique ester bond [37, 38], plays a key role in the formation and stability of the hyaluronan matrix during cumulus expansion. Moreover, it has been reported that SHAP is detected in porcine serum and follicular fluid [39]. These findings indicate that SHAP is the principal factor for cumulus expansion, and CD44 may not have as important a role in cumulus expansion as SHAP.

Previous studies have shown that cumulus expansion is closely related to oocyte meiotic progression [40–42]. In addition, the degree of cumulus expansion is often used as a parameter to predict the development of oocytes matured and fertilized *in vitro* [3–7]. In our present study, however, the addition of anti-CD44 antibody to the culture medium significantly inhibited oocyte maturation, in spite of the fact that cumulus expansion occurred adequately. This evidence demonstrates that cumulus expansion is a necessary condition, but that it is not sufficient in its own for oocyte maturation, and indicates that sufficient interaction between hyaluronan and CD44 during cumulus expansion plays an important role in oocyte maturation.

Katoh *et al.* [17, 18] reported that the terminal sialic acids of CD44 have an inhibitory effect on interaction between hyaluronan and CD44. In the present study, western blot analysis showed that CD44 in the COCs matured *in vitro* also has terminal sialic acids. Furthermore, we confirmed that the molecular weight of the CD44 in *in vitro*-matured COCs was larger than that *in vivo* and that the CD44 from *in vitro*-matured COCs treated with sialidase migrated in a manner similar to the CD44 of *in vivo*-matured COCs. Based on these results, the CD44 in *in vitro*-matured COCs contain more

sialic acids than the CD44 in *in vivo*-matured COCs during cumulus expansion. That is, although we did not measure the ability of CD44 to bind hyaluronan in the present study, this evidence indicates the possibility that interaction between hyaluronan and CD44 during *in vitro* maturation may not be sufficient for oocyte maturation compared with that *in vivo*. In general, oocytes matured *in vitro* have reduced capacity to be fertilized and a higher rate of abnormal fertilization and development as compared with their *in vivo* counterparts. In pigs, although oocytes matured *in vitro* can be penetrated by spermatozoa under appropriate conditions, *in vitro* maturation is associated with low rates of pronuclear formation and a high incidence of polyspermy [8]. Sun *et al.* [43] demonstrated that the rate of embryo development of *in vitro*-matured and fertilized COCs is significantly lower than that observed *in vivo*. Based on these observations, we speculate that insufficient interaction of hyaluronan-CD44 during *in vitro* maturation may cause inferior fertilization and developmental capacity in oocytes compared with those matured *in vivo*.

In conclusion, our results demonstrate that cumulus expansion is a necessary condition, but that it is not sufficient on its own, for oocyte maturation in pigs, although previous studies have reported that the physiological significance of cumulus expansion and the interaction of hyaluronan-CD44 during cumulus expansion are important for oocyte maturation. Moreover, our results suggest that further investigation of the insufficient interaction of hyaluronan-CD44 during *in vitro* maturation is important for improvement of the fertilization and developmental capacity of porcine oocytes matured *in vitro*.

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