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Induction of Oocyte Maturation by Hyaluronan-CD44 Interaction in Pigs

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Abstract. In most mammals, the oocyte is surrounded with compact multilayers of cumulus cells; these form cumulus-oocyte complexes (COCs). During oocyte maturation, the COCs dramatically expand and this is termed "cumulus expansion". We have previously demonstrated that cumulus expansion is the result of hyaluronan synthesis and accumulation in the extracellular space between cumulus cells in the COCs and that hyaluronan accumulation within the COCs affects oocyte maturation. We have also demonstrated that CD44, the principal hyaluronan receptor, is expressed in the COCs during cumulus expansion and that the interaction between hyaluronan and CD44 appears to be closely related to gap junctional communication of the COCs during the process of meiotic resumption. Based on our previous studies, we review herein that the physiological significance and the molecular mechanism of cumulus expansion for porcine oocyte maturation.

Key words: CD44, Cumulus expansion, Gap junction, Hyaluronan, Oocyte maturation

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In nearly all mammals, the oocyte is surrounded by compact multilayers of cumulus cells that form cumulus-oocyte complexes (COCs). During oocyte maturation, the COCs expand dramatically; this phenomenon is termed "cumulus expansion" and occurs after the pre-ovulatory surge of gonadotropins. It has been reported that cumulus expansion supports dissociation from the follicle wall and the expulsion of the oocyte through the ruptured follicle wall during ovulation. In addition to these effects, it has been reported that the expansion of COCs is essential for fertilization and the developmental potential of early embryos [1, 2]. Therefore, the degree of cumulus expansion is often cited as a major indicator in the selection of oocytes for *in vitro* fertilization (IVF) protocols [3–5]. Considering these observations, it is clear that an understanding of the physiological significance of cumulus expansion is important to the study of the developmental competence of mammalian oocytes that are matured and fertilized *in vitro*. Therefore, based on our previous studies, we describe herein the physiological significance and the molecular mechanism of cumulus expansion in porcine oocyte maturation.

Effects of Cumulus Expansion on Oocyte Maturation

Many researchers have documented that the formation of the COC matrix during cumulus expansion is characterized by the intercellular deposition of hyaluronan secreted from cumulus cells [6–8]. We first investigated whether the induction of cumulus expansion was due to the synthesis of hyaluronan during porcine oocyte maturation. COCs were cultured in maturation medium with or without 6-diazo-5-oxo-l-norleucine (DON; an inhibitor of hyaluronan synthesis) for 48 h. The degree of cumulus expansion increased gradually until 48 h in culture in the control medium. When the COCs were cultured with DON, they showed no evidence of cumulus expansion during the culture period. In addition, a remarkable accumulation of hyaluronan was confirmed in porcine expanded COCs by the immunostaining method, but hyaluronan accumulation was completely inhibited by treatment with DON (Fig. 1). These results indicate that the expanded cumulus mass of porcine COCs consists mainly of hyaluronan [9, 10].

Generally, serum and follicular fluid contain a factor(s) that stabilizes the hyaluronan-rich matrix. Previous studies have demonstrated that the inter-α-trypsin inhibitor (ITI) family plays an important role in the formation of the extracellular matrix of which hyaluronan is the predominant component [11]. Proteins of the ITI family are composed of a common light chain called "bikunin" and either one or two heavy polypeptide chains (HCs). In addition, serum-derived hyaluronan-associated proteins (SHAPs) have been isolated from the hyaluronan-rich extracellular matrix of mouse dermal fibroblasts cultured in the presence of serum [12], and these proteins were identical to the HCs of ITI [13]. It has been reported that the formation of a bond between SHAP and hyaluronan is also important for the stabilization of the hyaluronan-rich matrix [14, 15]. In our previous study, we documented that SHAPs were also present in porcine follicular fluid and serum as a single protein band at 70 kDa (Yokoo *et al.* unpublished data). In pigs, it has been reported that there is no apparent barrier to the transfer of SHAPs from the blood to the follicle [16]. Therefore, it is believed that SHAPs present in porcine follicular fluid are derived from serum.

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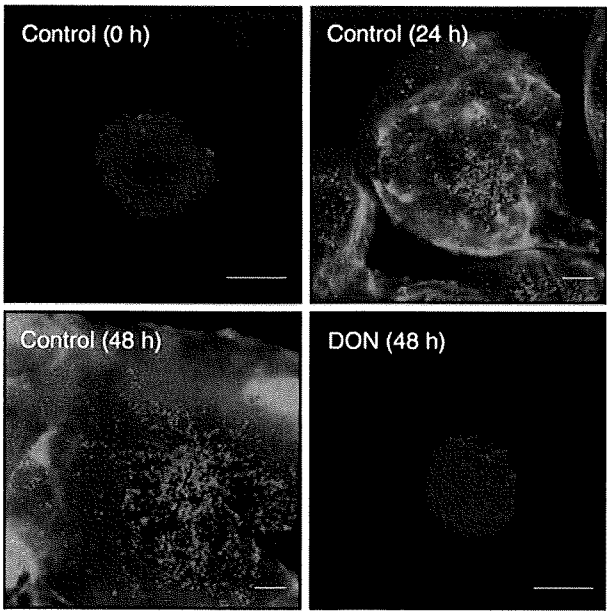

Fig. 1. Localization of hyaluronan in porcine cumulus-oocyte complexes (COCs) as detected by immunofluorescence using biotinylated hyaluronan-binding protein. Green=hyaluronan; red=nuclear; bars=100 μm.

Moreover, we demonstrated that the immunodepletion of SHAPs from follicular fluid produced not only incomplete cumulus expansion but also a decline in the oocyte maturation rates in a manner dependent on the antibody concentration against SHAPs. These results suggest that the retention and stabilization of hyaluronan within the COCs by the formation of the hyaluronan-SHAP complex during cumulus expansion is necessary for porcine oocyte maturation.

Expression of Hyaluronan-CD44 Interaction in COCs

Hyaluronan, which is the main component of cumulus expansion, is a linear glycosaminoglycan that is a high-molecular-weight polymer with repeating disaccharides linked by β 1–3 and β 1–4 glycosidic bonds. Despite its structural simplicity, hyaluronan is a biologically important biopolymer that is widely distributed in the extracellular matrix of connective tissues in the body. It plays important roles in diverse processes such as wound repair, cell motility, and cancer metastasis. Unlike others of the glycosaminoglycan family, hyaluronan is neither sulfated nor linked to a core protein. Hence, hyaluronan needs hyaluronan binding protein(s) for its biological functions. To obtain information regarding hyaluronan binding protein(s) during cumulus expansion, we investigated their expression in COCs during oocyte maturation by ligand blotting analysis with fluorescein isothiocyanate (FITC)-labeled hyaluronan. Interestingly, an 85-kDa hyaluronan binding protein was detected only in expanded COCs after maturation. Using immunoprecipitation assay, we showed that this protein was identical to CD44 [17].

CD44 is the principal cell-surface receptor for extracellular matrix hyaluronan and exists in a number of isoforms with different molecular sizes (approximately 80–250 kDa) on a wide variety of cell types [18–21]. It has been reported that the function of hyaluronan via CD44 is responsible for cell-to-cell and cell-to-extracellular matrix interactions [22], inhibition of apoptosis [23], augmentation of tumor cell motility and metastasis [24], and stimulation of lymphocytes [25]. Additionally, previous studies have indicated that the hyaluronan-CD44 interaction may influence fertility and the quality of oocytes [26, 27]. However, the role of CD44 in oocyte maturation remains poorly understood. To elucidate the role of hyaluronan-CD44 interaction in oocyte maturation, we examined the maturation-promoting factor (MPF) activity and the germinal vesicle breakdown (GVBD) rates in the COCs cultured in maturation medium containing with anti-CD44 antibody, which has been used for inhibition of hyaluronan binding [28, 29]. A low level of MPF activity was noted in oocytes from the COCs immediately after collection from follicles. After 24 h in culture, the MPF activity and the GVBD rates in oocytes cultured in the drug-free medium significantly increased compared to those of cumulus-oocyte complexes before culture. However, exposure of the COCs to anti-CD44 antibody during 24 h of culture significantly suppressed MPF activity and GVBD rates compared to those of the COCs cultured in the drug-free medium for 24 h (Table 1 and Table 2). Thus, these results clearly show that the hyaluronan-CD44 interaction is required for meiotic resumption in the oocyte maturation process.

We next examined the difference between the molecular size of CD44 expressed in the COCs matured *in vitro* and those matured *in vivo* [30]. The COCs matured *in vitro* showed bands of CD44 ranging from 81 to 88 kDa. However, the CD44 band in the *in vivo*-matured COCs was 73–83 kDa in size; thus, the size of the CD44 in the COCs matured *in vivo* was clearly smaller than the band of CD44 in the COCs matured *in vitro*. The amino acid sequence of CD44 predicts a polypeptide of <40 kDa, which contrasts with its apparent size on gel electrophoresis (approximately 80–250 kDa). This difference appears to be the result of extensive glycosylation of the extracellular domain [31]. It has been reported that CD44 glycosylation has been implicated in the regulation and function of CD44-mediated cell binding for hyaluronan [19]. Notably, Katoh *et al.* [32] reported that the terminal sialic acids on CD44 have an inhibitory effect on hyaluronan binding ability of CD44. Our results clearly demonstrated that the treatment with sialidase reduced the size of CD44 in the COCs matured *in vitro*, the size of which was not significantly different from that of the COCs matured *in vivo*. This evidence indicates the possibility that interaction between hyaluronan and CD44 during *in vitro* maturation may not be sufficient for oocyte maturation compared to that *in vivo*. In general, oocytes matured *in vitro* have a reduced capacity to be fertilized and a higher rate of abnormal fertilization and development as compared to 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 [33]. Sun *et al.* [34] demonstrated that the rates of embryo development rates of *in vitro*-matured and fertilized COCs

Table 1. Effects of 6-diazo-5-oxo-norleucine (DON) and anti-CD44 antibody on maturation-promoting factor (MPF) activity in porcine oocytes

	Relative MPF activity (0 h=1)			
	0 h	6 h	12 h	24 h
Control	1^{a}	1.09 ± 0.04^{a}	1.94 ± 0.10^{c}	5.87 ± 0.44^{d}
DON	1^{a}	1.32 ± 0.12^{a}	0.99 ± 0.08^{a}	2.51 ± 0.43^{b}
Anti-CD-44	1^{a}	1.01 ± 0.08^{a}	1.00 ± 0.01^{a}	2.16 ± 0.15^{b}

Cumulus-oocyte complexes were cultured for 0, 6, 12 or 24 h with 1.0 mM DON or 5.0 mg/ml anti-CD44 antibody. Data are expressed as fold increases of MPF activity in oocytes just after collection from follicles, defined as 1. Experiments were replicated four times at least. Data represent mean ± standard deviation (SD). Different superscripts denote significant differences ($P<0.05$).

Table 2. Effects of 6-diazo-5-oxo-norleucine (DON) and anti-CD44 antibody on germinal vesicle breakdown (GVBD)

	Rate of GVBD (%)			
	0 h	6 h	12 h	24 h
Control	0^{a}	2.5 ± 2.5^{a}	4.8 ± 4.8^{a}	63.8 ± 2.4^{c}
DON	0^{a}	2.4 ± 2.4^{a}	4.5 ± 4.5^{a}	19.0 ± 2.1^{b}
Anti-CD44	0^{a}	2.1 ± 2.1^{a}	6.8 ± 6.8^{a}	13.3 ± 7.0^{b}

Cumulus-oocyte complexes were cultured for 0, 6, 12 or 24 h with 1.0 mM DON or 5.0 mg/ml anti-CD44 antibody. Experiments were replicated three times at least. Data represent mean ± SD. Different superscripts denote significant differences ($P<0.05$).

is significantly lower than that observed *in vivo*. Based on these observations, we speculate that the insufficient interaction of hyaluronan-CD44 during cumulus expansion *in vitro* may cause inferior fertilization and developmental capacities in oocytes compared to those matured *in vivo*.

Molecular Mechanism of Hyaluronan-CD44 Interaction for Oocyte Maturation

Since our findings suggested that the hyaluronan-CD44 interaction is involved in the induction of meiotic resumption, it was thought that CD44 was expressed in/on the oocytes. However, CD44 has been shown to be localized in cumulus cells, not in the oocyte, of the COCs by using reverse-transcription polymerase chain reaction (RT-PCR), western blotting, and immnunohistological staining [17, 35, 36]. Considering these results, we conclude that the hualuronan-CD44 interaction might function to promote the meiotic resumption of porcine oocytes through the cumulus cells.

Generally, the coordination of function between the oocyte and cumulus cells is mediated by cell-cell communication via gap junctions [37]. Early studies have shown that oocyte growth and development are strictly dependent upon the supply of nutrients transmitted from the follicle cells [38, 39]. Later studies have demonstrated that the meiotic maturation of oocytes is also subject to regulation by the somatic compartment of the ovarian follicle. MPF activation at the onset of meiotic resumption is inhibited by intra-oocyte cAMP, which is transferred from cumulus cells via gap junctional communication within COCs [37]. Interruption of gap junctions in the COCs, which occurs in response to the preovulatory surge of gonadotropins, leads to a drop in the intra-oocyte concentration of cAMP, followed by MPF activation and meiotic resumption [40–42]. We demonstrated that the reduction of the intra-oocyte cAMP concentration was suppressed by the inhibition of the interaction between hyaluronan and CD44 (Yokoo *et al.* unpublished data). This result supports the concept that hyaluronan-CD44 interaction is involved in the regulation of gap junctional communication and the termination of the flux of cAMP flux from cumulus cells to oocytes.

Gap junctions are specialized regions in closely opposed membranes of neighboring cells that allow cells to exchange small molecules, thus coordinating their activities. Each gap junction channel comprises two symmetrical hemispheres (termed "connexons") derived from two neighboring cells. The connexon comprises a hexagonal arrangement of six subunits of a protein named "connexin" (Cx). Connexins are encoded by members of a multigene family; they are defined by their molecular weight and share high homology. At present, at least 15 connexin genes have been reported in mammals and 7 genes (*Cx26, Cx30, Cx32, Cx37, Cx43, Cx45 and Cx60*) have been identified in the ovary. We examined the effects of the hyaluronan-CD44 interaction on the expression of Cx43, which is the most abundant Cx found in the ovarian follicle and in the COCs. Exposure of COCs to DON and anti-CD44 antibody had no effect on the expression of total Cx43 in the COCs. Conversely, these treatments significantly inhibited the tyrosine-phosphorylation of Cx43 in the COCs (Fig. 2). Previous studies have shown that a tyrosine kinase, such as $pp60^{Src}$, induces the tyrosine phosphorylation of Cx43 and inhibits intercellular junctional communication [43–47]. Therefore, it is suggested that hyaluronan-CD44 interaction controls the inhibition of Cx43 gap junctional communication in COCs. These findings strongly suggest that the hyaluronan-CD44 interaction during cumulus

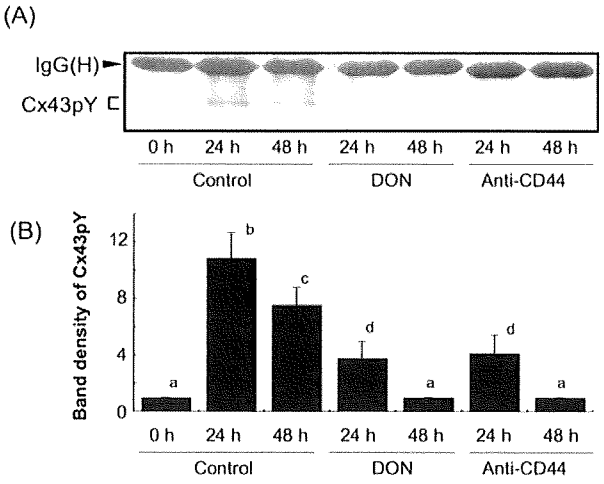

Fig. 2. Effects of 6-diazo-5-oxo-norleucine (DON) and anti-CD44 antibody on expression of Cx43. The cumulus-oocyte complexes (COCs) were cultured with 1.0 mM DON or 5.0 μg/ml anti-CD44 antibody. (A) Detection of tyrosine-phosphorylated Cx43 (Cx43pY). The extracts immunoprecipitated with anti-Cx43 antibody were probed with anti-phosphotyrosine antibody. Arrowhead means the band of heavy chain of immunoglobulin (IgG (H)). (B) Densitometric analysis of (A). Different superscripts denote significant differences ($P<0.05$). Data represent mean ± SD.

expansion induces disruption of the Cx43 gap junction in the COCs, inhibits the transport of cAMP from cumulus cells into oocytes, and leads to activation of MPF and meiotic resumption of oocytes (Fig. 3).

Concluding Remarks

Oocyte maturation is roughly divided into two types: nuclear maturation and cytoplasmic maturation. We have described herein the hyaluronan-CD44 interaction during cumulus expansion that concurrently controls the occurrence of meiotic resumption through the disruption of gap junctions in COCs. It has been demonstrated that volumetric expansion of COCs actively correlates, at least in the pig, with the progress of nuclear maturation. Although details of the mechanism controlling cytoplasmic maturation in mammals are still unclear, we believe that our findings as described here shed some light on the understanding of the cytoplasmic maturation process.

Recently, pigs have become increasingly important in the field of biomedical research and interest has grown in the use of transgenic pigs as potential xenograph donors. As most attempts to produce transgenic pigs by nuclear transfer/cloning techniques or pronuclear microinjection have used matured oocytes and early embryos, respectively, it is becoming more important to produce large numbers of developmentally competent oocytes and embryos. The developmental competence of porcine oocytes that are matured and fertilized *in vitro* has been enhanced by mimicking the active communication between the oocyte and follicular cells. Therefore,

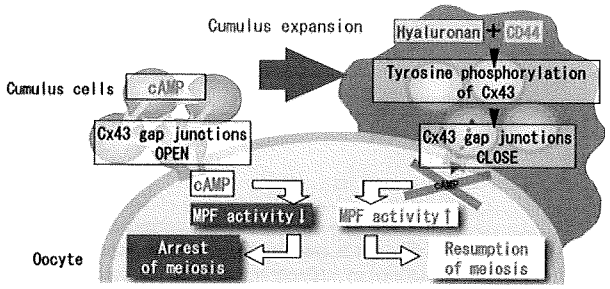

Fig. 3. Schematic representation of the oocyte maturation mechanism in the pig.

elucidation of the molecular mechanisms of oocyte maturation will enable substantial improvement of the quality of oocytes and embryos cultured *in vitro*.

During the past decade, new reproduction biotechnology based on molecular genetics, molecular biology, and embryology has rapidly developed. These techniques should enable us to increase the production of domestic animals. We now need to define issues and further develop the study of animal reproduction. The objective of our ongoing studies is to investigate in more detail the molecular mechanism of mammalian oocyte maturation and to develop an artificial technique for oocyte maturation *in vitro*.

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