—Review—

Significance of Mammalian Cumulus-Oocyte Complex Matrix in Oocyte Meiotic Maturation: Review of the Synthetic Control and Possible Roles of Hyaluronan (HA) and HA-binding Protein

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Abstract: *In most mammals, the growth and development of the oocyte and its surrounding somatic cell compartment in the follicle occur in a highly coordinated and mutually dependent manner. Oocytes acquire developmental competence sequentially during follicle growth, finally gaining the ability to undergo complete meiotic and cytoplasmic maturation at the final stage of the preovulatory follicle. Fully-grown immature oocytes are tightly surrounded by compact layers of specialized granulosa cells called cumulus cells that form the cumulus-oocyte complex (COC). After a preovulatory surge of gonadotrophin, the cumulus cells organize a special muco-elastic extracellular matrix (ECM) that requires synthesis and deposition of a large amount of hyaluronan (HA) and HA-binding matrix glycoproteins. Many studies have reported that the formation of the COC matrix mass plays important roles in a variety of reproductive phenomenons: oocyte meiotic maturation with changes of junctional communication and cytoskeletal modification in COC, ovulation, fertilization and early embryo development. Recently, we identified the expressions of HA synthases and the HA receptor, CD44, in the porcine COC matrix. The interaction of HA and CD44 appears to be closely related to gap-junctional communications and meiotic resumption during oocyte maturation. This review describes the recent findings on the regulation and the presumptive mechanism of COC matrix molecules, and physiological features in COC expansion.*

Key words: *Cumulus-oocyte complex, Hyaluronan, CD44, Cumulus expansion, Oocyte meiotic maturation*

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Introduction

In nearly all mammals, the development of ovarian follicles, ovulation, and the formation of the corpus luteum are complex processes accompanied by dramatic changes in follicular cells under the specific and strict regulation of pituitary gonadotrophins, steroids, and growth factors. The growth and development of the oocyte and its companion somatic cell compartment in the follicle take place in a highly coordinated and mutually dependent manner [1–4]. In the perinatal stage, once the meiotic process is started by a meiosis-initiating factor derived from the cell of the in-growing mesonephiric tissue, the oogonial germ cells are defined as primary oocytes [5]. Owing to the meiotic initiation, mitosis in the oogonial germ cells is terminated and the number of oocytes that a female will be endowed with is finally set. Oocytes that enter the diplotene stage, a prolonged dictyate stage of prophase I (first meiotic division) become surrounded by a single layer of somatic cells to form non-growing primordial follicles (Fig. 1). With the formation of the primordial follicles, oocytes suddenly arrest their meiotic progress at the dictyate stage. This large population of resting primordial follicles serves as the source of developing follicles and oocytes until the end of a female's reproductive life. Initial growth from primordial follicles

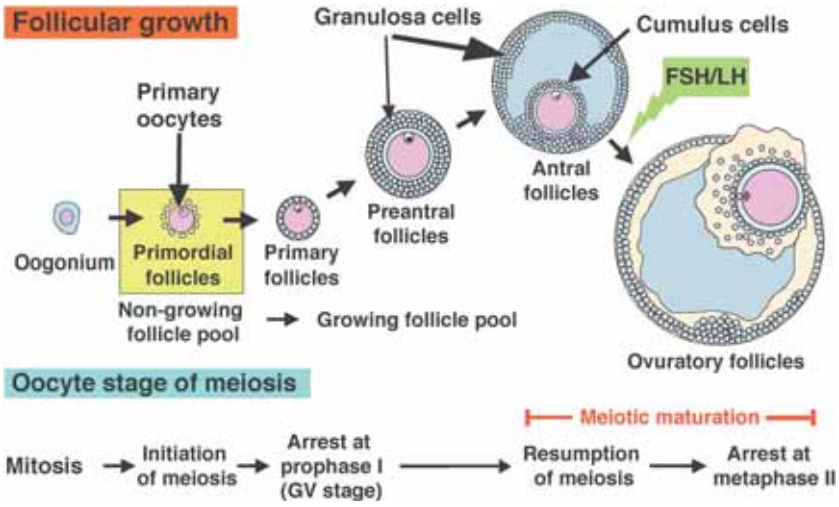

Fig. 1. Follicular growth and oocyte meiotic maturation.

to pre-antral or secondary follicles is a gonadotrophin-independent process occurring in the fetal stage and continuing to the prepubertal stage. Throughout prepuberty, the resting pools of primordial follicles are continuously recruited into the growing follicle pool. After puberty, gonadotrophin-dependent growth occurs in a cohort of antral follicles, selected from the growing follicle pool, which will supply the dominant follicles. While very few follicles develop to the ovulatory stage (<1% of primordial follicles present at the time of an animal's birth), the majority of non-ovulatory follicles undergo atresia through fetal life to adult life. Studies on animals have shown that apoptosis is the underlying mechanism of oocyte depletion and follicular atresia during folliculogenesis.

Ovarian follicles, the basic functional units of an ovary, start their development as primordial structures that consist of an oocyte arrested at the dictyate stage of prophase I. They are encompassed by a basal lamina and a single layer of flattened epithelial cells (pre-granulosa cells) [6]. Once a restricted number of primordial follicles are activated in response to an unknown signal, enlargement of the oocyte commences, and the epithelial cells become cuboidal in shape and begin to divide. As the granulosa cells proliferate, the number of cell layers around the oocyte increases, and the basal lamina expand. Outside the basal lamina, the pre-theca cells are organized concentrically to form the theca layer. As the follicular maturation proceeds, zona pellucida (ZP) develops on the surface of oocyte. Later in development, a fluid-filled cavity called the antrum develops within the granulosa cell layers (antral stage). Antrum formation forces the oocyte into a more eccentric site and leads granulosa cells to differentiate into two specialized cell subpopulations: cumulus cells, which are closely connected with the oocyte, and mural granulosa cells, which are organized as a stratified follicular epithelium encapsulated by a basal lamina. Specialized stromal layers, theca interna and externa, develop. Bidirectional communication occurs between the granulosa cells and theca cells as well as between granulosa cells and oocytes, via both paracrine and gap-junctional signaling. These exchange of small regulatory molecules, play critical roles in the normal growth and development of follicles [7–10]. It also requires appropriately-timed endocrine signals, pituitary gonadotrophins and metabolic hormones, which act on receptors in the each of the cell types and interact with local autocrine/paracrine signaling pathways.

The cumulus cells-oocyte complex (COC), a structural unit of the antral follicle, includes several layers of cumulus cells (approximately 1,000–3,000 cells/COC) around the oocyte in the mouse [11]. The compact COCs maintain an extensive communication between cumulus cells (known as corona radiata) and the oocyte thorough transzonal projections in which cumulus cells traverse the ZP and arrest on on the oocyte plasma membrane forming both adhesive and gap-junctional contacts. Intercellular communication is also kept among cumulus cells via gap junctions composed of the connexin family proteins [8, 12, 13]. Just before ovulation, following the endogenous gonadotrophin surge, a Graafian follicle rapidly

increases in volume by the accumulation of follicular fluid and intrafollicular pressure. Concomitantly, the COCs form muco-elastic extracellular matrix (ECM) containing a large amount of hyaluronan (HA) and specific HA-binding proteins, causing a radical volumetric enlargement called cumulus expansion [11, 14–16]. The expanded COCs become detached from membrane granulosa and float free in the follicular fluid. The follicular wall becomes thin and is broken down by proteolytic enzymes (collagenase and plasmin) produced by mural granulosa cells. As a result, the COC matrix mass is expelled from the follicle and reaches the oviductal ampulla [6, 17].

The HA-enriched COC matrix seems to provide the oocyte with an essential vehicle for its transfer to the oviductal ampulla in the process of ovulation, pick-up by the oviductal fimbria and sperm penetration. Besides, the junctional networks between cumulus cells and mural granulosa cells, and between cumulus cells and oocyte during cumulus expansion, are retracted or become modified. These changes have physiological roles not only in supplying metabolites but also in controlling oocyte meiotic and cytoplasmic maturation. Appropriate oocyte maturation is important for successful fertilization and subsequent embryo development. This review describes recent developments in the understanding of the molecular regulation of the COC matrix and its possible role in oocyte maturation.

Components of the COC Matrix Mass and Factors Inducing Cumulus Expansion

HA, the major material of the COC matrix

Many investigators have documented that the formation of the COC matrix is characterized by the intercellular deposition of HA derived from cumulus cells [15, 18, 19]. In mice treated with pregnant mare serum gonadotrophin (eCG), preovulatory Graafian follicles, before injection of human chorionic gonadotrophin (hCG), have very little HA-enriched matrix in the compact COCs and mural granulosa cells. Five hours after hCG injection, the COC matrix is partially organized. Also, HA is found in the mural granulosa cells closest to the COC and in those cells adjacent to the antrum. Shortly before ovulation, the COC is fully expanded (20 to 40-fold the initial volume) and occupies most of the antral cavity. In addition, HA is abundantly localized in the rim of the antrum in contact with the mural granulosa cells, however, it is not present in the mural granulosa cells closest to the basement membrane [11]. In other species, studies of the drastic enlargement of the COC matrix have been performed both *in vivo* and *in vitro* (Fig. 2). HA and other glycosaminoglycans (GAGs) linking to a core protein named proteoglycan have also been identified in the follicular fluid, implying that they may have roles in follicular development and atresia [20].

HA is widely distributed as the hydrophilic material of ECMs in nearly all vertebrate tissues. It is characterized by a linear macromolecule GAG, ranging in molecular mass from 10^5 to 10^7 Da, composed of the repeating disaccharide units of D-glucuronic acid $\beta 1\text{–}3$ linked *N*-acetylglucosamine $\beta 1\text{–}4$ [21]. Different from other GAGs, HA is not sulfated nor assembled on core proteins for a proteoglycan. It is identified not only in cartilage, synovial fluid, vitreous humor of the eye, and skin, but also in tumor tissues of the prostate, bladder, colon, liver, etc. [22]. In addition to its structural role maintain the hydration and physical properties of tissues, HA influences cell shape and behavior, e.g. adhesion migration, proliferation, differentiation, cell death and cell anchorage via binding to specific cell surface receptors, such as CD44 and RHAMM, in many biological processes of tissue organization, tissue morphogenesis, cancer metastasis, wound healing, inflammation and angiogenesis [23–25].

HA biosynthesis is basically distinct from that of other GAGs, which are synthesized in the Golgi network. HA is synthesized at the plasma membrane and directly extends into the ECM [26, 27], and growing HA chains elongate by the addition of sugar residues derived from UDP-glucuronate and UDP-*N*-acetylglucosamine. HA synthase (HAS), the enzyme responsible for HA biosynthesis, was not identified until the Group A *has* gene was cloned in 1993 [28], even though HA was discovered as long ago as 1934 [21]. Many investigators expected that HA biosynthesis would require at least two different enzymes for the glycosyltransferase reactions. After its expression, the encoded protein was shown to synthesize high molecular weight HA, thus establishing that a single protein utilizes both sugar substrates to synthesize HA [29]. Several groups have since succeeded in cloning and characterizing three separate genes for vertebrate *HAS* genes: *HAS1*, *HAS2*, *HAS3* [30]. They have a predicted molecular mass of approximately 63 kDa, and the transfection of cells with expression vectors for each of these isoforms is able to induce the synthesis of hyaluronan and the formation of a pericellular coat. The mammalian HAS proteins share between 55% and 71% amino acid identity, and are encoded by distinct genes located on separate autosomes. Recent studies have

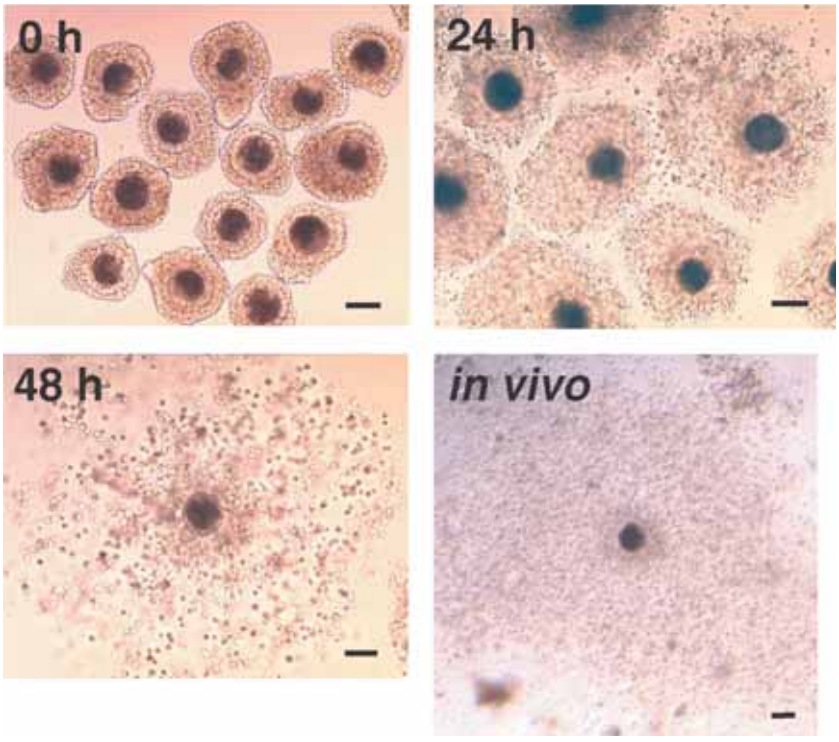

Fig. 2. Typical cumulus expansion in porcine COCs cultured in the presence of eCG and porcine follicular fluids for 0–48 hours (h), and an *in vivo* superovulated COC. The degree of COC expansion *in vitro* maturation is smaller than that *in vivo*. Bar = 100 μm.

demonstrated the properties of three kinds of HAS isoforms: 1) these expression patterns distinguished during embryonic development and distribution in adult tissues; 2) HAS3 or HAS1 deficient mice are viable and fertile, and exhibit no obvious abnormalities while HAS2 deficiency results in embryonic lethality; and 3) HAS3 appears to be more active than either HAS2 or HAS1, and drives the biosynthesis of significantly shorter HA polymers *in vitro* [31, 32].

Expression of HAS genes in COCs

Studies in mice, pigs and cattle have demonstrated that HAS2 mRNA is expressed in COCs with cumulus expansion during oocyte maturation *in vivo* and *in vitro* [33–35]. We have characterized two kinds of porcine cDNA, *shas2* expressed in cumulus cells and *shas3* expressed in oocytes. The cDNA encoding the open reading frame (ORF) of *shas2* contains 1,656 nucleotides (GenBank accession No.: AB050389) coding for 552 amino acids. The ORF of *shas3* contains 1,659 nucleotides (GenBank accession No.: AB159675) coding for 553 amino acids. Each sequence of the *shas2* and *shas3* cDNAs indicates exceedingly high similarity with the HAS family of other mammals. Stock *et al*. [36] have shown that HAS2 in equine mural granulosa cells is specifically expressed in the ovulatory process after hCG injection. Therefore, it is now generally accepted that HA secreted by cumulus cells and mural granulosa cells is attributed to HAS2. On the other hand, HAS3 in oocytes is expressed maternally from the germinal vesicle (GV) stage up to the 8-cell stage (Kimura N., unpublished data).

Whereas the preovulatory luteinizing hormone (LH) surge triggers the cumulus expansion of preovulatory follicles *in vivo*, follicle-stimulating hormone (FSH) is a key factor *in vitro* [18]. FSH increases intracellular second messenger cAMP, activators of cAMP-dependent protein kinase, and promotes maturation of epidermal growth factor (EGF) receptors which in turn activates the tyrosine kinase cascade [14, 37, 38]. Therefore, it seems likely that this sequential response is mediated by cyclic adenosine monophosphate (cAMP) or the tyrosine kinase cascade. The positive effects of EGF, transforming growth factor (TGF)-α and growth hormone on cumulus expansion in FSH-free culture medium, have been demonstrated *in vitro* in

several species [39–41]. Expression of HAS2 in cumulus cells and granulosa cells is stimulated by eCG, FSH and porcine follicular fluid *in vitro* [34, 35]. In porcine COCs, oocytectomy slightly reduces the level of HAS2 mRNA in the presence of eCG and porcine follicular fluid, suggesting that the oocyte up-regulates HAS2 expression during cumulus expansion ([34], Fig. 3). Oocytectomized mouse COCs synthesize very little HA and fail to expand in the presence of FSH or EGF [42, 43]. Incubation of mouse cumulus cells with isolated oocytes (co-culture), or culture in denuded-oocyte conditioned medium, stimulates FSH-dependent HA synthesis and expansion [42, 44]. These facts suggest that the secretion of a soluble factor from oocytes is involved in HA synthesis and cumulus expansion *in vitro*. On the other hand, oocyte factor does not influence the production of other GAGs, dermatan sulfate and chondroitin sulfate in cumulus cells [45, 46]. Porcine and bovine oocytes also secrete a cumulus expansion-enabling factor, though cumulus expansion progresses steadily without this factor [47–49]. It is well known that growth differentiation factor-9 (GDF-9), bone morphogenetic protein 15 (BMP-15, also called GDF-9B) and BMP-6 are likely candidate molecules for oocyte-secreted factor [50]. These growth factors are members of the TGF-β superfamily that shows a high level of expression in mammalian oocytes. Elvin *et al*. [51] reported that recombinant GDF-9 induces HAS2 expression and cumulus expansion *in vitro* in the presence of FSH, indicating that GDF-9 interacts with FSH and modulates downstream target gene HAS2. However, the expression of porcine HAS3 in oocytes is not remarkably affected by gonadotrophins [34, 35].

It has been established that cumulus cells express of FSH and LH receptors in [52, 53]. While the expression of the LH receptors are present at a very low level in cumulus cells isolated from preovulatory follicles, that of the FSH receptors are abundantly present [52]. Meduri *et al*. [53] have documented the presence of FSH (but not LH) receptors in oocytes during follicular development from the primary stage to the preovulatory stage in human and porcine ovaries. Also, Patsoula *et al*. [54] have found both FSH and LH receptor mRNAs in mice oocytes, zygotes and preimplantation embryos. The porcine HAS3 expression is not considerably affected by gonadotrophins (Kimura N., unpublished data) regardless of whether oocytes have both FSH and LH receptors, however, the porcine HAS2 expression in cumulus cells abundant in FSH receptors is stimulated by FSH. This evidence may imply that 1) there is different signaling for transcriptional induction between HAS2 and HAS3; and 2) HAS3 is independent of HA synthesis for cumulus expansion.

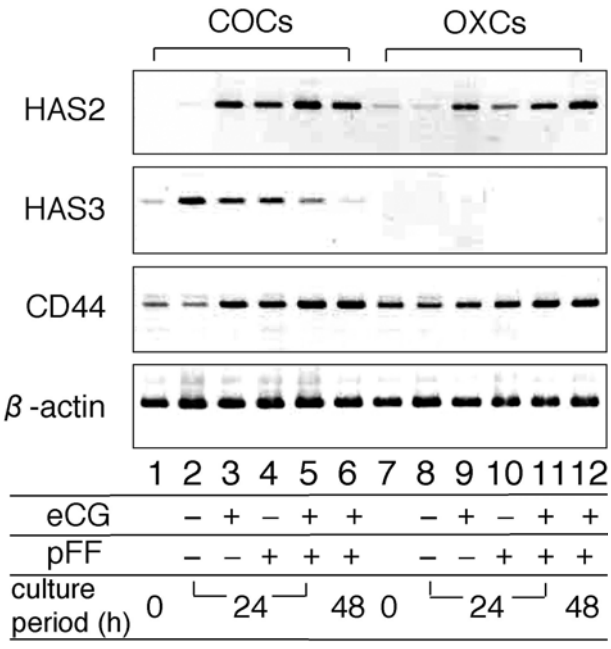

Fig. 3. Effects of eCG and pFF on has2, has3, CD44 and β-actin mRNA expressions in porcine COCs and OXCs. Total RNA was isolated from COCs and OXCs at 24 and 48 hours (h) after culture in medium supplemented with 10 U/ml eCG and 10% (v/v) pFF, and then subjected to RT-PCR. Lanes 1–6, COCs; lanes 7–12, OXCs; lanes 1 and 7, 0 h (fresh); lanes 2–5 and 8–11, 24 h after culture; lanes 6 and 12, 48 h after culture; lanes 2 and 8, medium199 (m199) alone; lanes 3 and 9, m199 supplemented with eCG; lanes 4 and 10, m199 supplemented with pFF; lanes 5, 6, 11 and 12, m199 supplemented with eCG and pFF.

Proteoglycans and HA-associated proteins are retained in COC matrix

Volumetric COC matrix is organized not only by synthesis of HA and mucoid materials, but also by their retention within the COC mass. A number of proteoglycans and HA-binding proteins secreted by cumulus cells and granulosa cells contribute to stabilization of the matrix and may provide functional characteristics through cell-matrix and cell-cell interactions [9, 20, 55]. Proteoglycans are macromolecules composed of a core protein with covalently attached variable GAG chains [20]. While producing a large amount of HA, cumulus cells also synthesize moderate quantities of chondroitin sulfate,

dermatan sulfate and heparan sulfate, all of which associate with a core protein to form proteoglycan. However, these GAGs except for HA do not appear to be directly involved in the organization of the COC matrix [19, 46].

Camaioni *et al*. [56] have shown that a dermatan sulfate proteoglycan of large hydrodynamic size (>1 million Da and a core protein of about 280 kDa) and a protein approximately 46 kDa in molecular mass are secreted by cumulus cells and are accumulated into the COC matrix. The properties of this proteoglycan are similar to these of aggrecan and versican both of which interact specifically with HA. The 46 kDa protein is the same size as the cartilage link protein that is found in all hyaline cartilage and in the aortic intima [57, 58], and it interacts with HA to stabilize binding of proteoglycan monomers to HA for the formation of a stable structure. Link protein markedly localizes in the COC matrix and mural granulosa cells in large preovulatory follicles after hCG injection [59, 60]. *In vitro* studies have suggested that the synthesis of link protein in cumulus cells requires gonadotrophin stimulation and oocyte-derived factor. It is speculated that fetal bovine serum improve the production of link protein and proteoglycans, and their deposition of these molecules into COC matrix [56, 59]. Sun *et al*. [61] have indicated that link protein enhances cumulus expansion by stabilizing the heavy chains (HCs) of an inter-α-inhibitor (IαI) family HA complex. Besides, versican isoforms are predominantly expressed in cumulus cells and mural granulosa cells in the ovulation stage [62].

Tumor necrosis factor-induced protein-6 (TNFIP-6; also known as tumor necrosis factor-stimulated gene-6) is a member of the matrix proteins [63] that is essential for formation of the normal COC matrix assembly [64, 65]. TNFIP-6, an approx 35 kDa glycoprotein, is synthesized by cumulus cells and mural granulosa cells of antral follicles after the LH surge, and is incorporated into the expanded COC matrix [66, 67]. There are two types of TNFIP-6: 1) a monomer binding HA through its link module, and 2) a covalent complex of approximately 125 kDa with the heavy chains of the Iαl family [68–70]. The heavy chains of the Iαl family are covalently transferred to HA by catalysis of TNFIP-6. HA oligosaccharides with eight or more monosaccharide units are potent acceptors in the heavy chain transfers [71]. Serum and follicular fluid also contain factors that stabilize the organization of the COC matrix. It is well known that the serum-derived glycoprotein Iαl family is pivotal for the formation of the COC matrix both *in vivo* [72, 73] and *in vitro* [74, 75]. After gonadotrophin stimulation, the Iαl family flows into the predominant antral follicles from the plasma due to increased permeability of the blood-follicle barrier, and is accumulated in the follicular fluids [76, 77]. The Iαl family consists of two homologous heavy chains (HCs), HC1 and HC2 (approximately 65 kDa and 70 kDa, respectively), that are covalently linked to one light chain of about 30 kDa (named bikunin) and to a chondroitin sulfate chain. Additionally, inter-α-like inhibitor (IαLI) and pre-α-inhibitor (Pαl) are complexes of one HC (HC2 or HC3 (about 90 kDa)) and a bikunin associated with a chondroitin sulfate chain [78–80]. These major members of the Iαl family are synthesized by hepatocytes and circulate in the blood (0.15 to 0.5 mg/ml plasma) [81]. One or two HCs are connected to the chondroitin sulfate chain of bikunin via an ester bond that forms between the C-terminal aspartic acid residues of HCs and the *N*-acetylglucosamine residues of the chondroitin sulfate chain. On the other hand, the covalent linkage between HCs of the Iαl family and HA, which is referred to as the serum-derived HA-associated protein (SHAP)-HA complex, has been found in the HA-rich matrix of cultured mice dermal fibroblasts supplemented with serum and in pathological synovial fluid from human arthritis patients [82–84]. These findings suggest that the transesterification model of covalent binding between HCs of the Iαl family and HA involves the exchange of the chondroitin sulfate chain with HA at the HCs/chondroitin sulfate junction, followed by the release of chondroitin sulfate-bikunin. Bikunin with chondroitin sulfate moiety is indispensable for the arrangement of the SHAP-HA complex [73]. In mice studies, Chen *et al.* [85, 86] have demonstrated that HCs of the Iαl family are covalently linked to HA and are bound to the COC matrix in ovulated COCs while the Iαl family is incorporated into the HA-enriched matrix by a non-covalent mechanism in *in vitro* expanded COCs, when serum was added to the medium. Conversely, Nagyova *et al*. [87] showed covalent transfer of HCs of the Iαl family to HA in both *in vivo* and *in vitro* expanded porcine COC matrices that were cultured in porcine serum and follicular fluid. Granulosa cell secreted factor and follicular fluids may contain the essential components that catalyze the covalent binding of HCs and HA [86, 88, 89]. On the cumulus cell surface, the interaction among HA, proteoglycans such as aggrecan and versican, extracellular matrix HA-binding proteins including link protein, TNFIP-6, and the Iαl family, and additional unknown factors appears to induce and strengthen COC expansion and lead to ovarian follicle maturation.

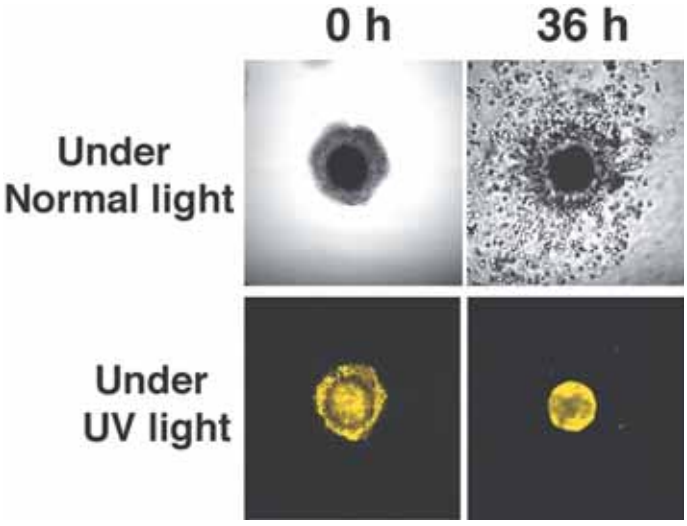

Fig. 4. Lucifer yellow (LY) diffusion in porcine COCs matured *in vitro*. The oocyte-cumulus junctional communications decline with COC expansion. 0 hour (h), LY is transferred to all layers of cumulus cells surrounding oocytes. 36 h, LY is not transferred to cumulus cells.

Physiological Roles of Cumulus Expansion

A number of studies have been undertaken on the physiological importance of cumulus expansion leading to oocyte maturation, ovulation, fertilization and the subsequent embryo development.

Before COC expansion, each cell in the COC is interconnected by intricate networks called gap junctions that are specialized regions in closely opposed membranes of neighboring cells [90, 91]. In the process of cumulus expansion, the rearrangement of microfilaments in cumulus cell cytoplasm precedes the acceleration of ECM synthesis, the redistribution of microtubules and intermediate filaments and the change of the gap junctional pathway. Ultimately, it results in the withdrawal of microfilament-filled transzonal projections by the cumulus cell [92–95]. Therefore, it is generally accepted that intercellular communication does not occur in expanded COCs (Fig. 4). The loss of gap junctions between cumulus cells and the oocyte during cumulus expansion is closely related to oocyte meiotic progression [96–98]. We describe in detail the presumptive role of ECM molecules in oocyte meiotic maturation in the following section.

The structure of the expanded COC promotes its dissociation from the follicle wall. Moreover, it supports the expulsion of the oocyte through the ruptured follicle wall during ovulation, and also facilitates its capture by oviduct fimbria and transfer to the fertilization site [99]. Chen *et al*. [100] have demonstrated the roles of preovulatory synthesis of HA within the COC matrix in mice using 6-diazo-5-oxo-1-norleucine (DON), an inhibitor of glucosamine synthesis. The suppression of COC expansion by DON not only considerably inhibits ovulation and reduces the rate of fertilization, but also may be correlated with oocyte viability. Besides, Hess *et al.* [72] have shown that intravenous administration of excess HA oligosaccharides inhibits the binding of Iαl to endogenous HA, resulting in a reduction in the size of the COC matrix. Consequently, the rate of ovulation and the developmental potential of early embryos in HA-treated mice are markedly decreased. Functional studies using knockout mice models have described the crucial roles of ovarian-expressed genes through neonatal abnormal expression or lethality. The female mice of null mutations of cyclooxygenase-2 (COX-2), TSG-6, are infertile and exhibit reduced ovulation and fertilization rates [64, 101]. Similarly, prostaglandin E2 receptor, bikunin knockout mice also show severe in female fertility due to a decreased fertilization rate [73, 102]. Also, pentraxin-3 (PTX-3) null mice are subfertile and show reduced ovulation and fertilization rates [103]. The sterility in these null mutants is due to an impaired cumulus expansion. Collectively, these facts demonstrate that the normal formation of the COC matrix is essential for normal ovulation and fertilization.

The presumptive roles of interplay between HA derived from the COC matrix and HA-binding proteins on the sperm surface have been demonstrated. In fertilization, capacitated sperm pass through a layer of cumulus cells enveloped with HA-enriched matrix and bind to the oocyte ZP. This passage must depend on the hyaluronidase activity of a glycosylphosphatidylinositol-anchored membranous protein, PH-20, which was originally identified as a protein binding to the ZP in guinea pig sperm [104]. On acrosome-intact sperm, PH-20 acts during two different stages: hyaluronidase activity for sperm penetration through the cumulus cell layer, and sperm-zona binding [105]. Signaling via the binding of HA to PH-20 is likely to induce the acrosome reaction or to increase intracellular calcium, as has been demonstrated in humans and macaques [106, 107]. On the other hand, Baba *et al*. [108] have demonstrated that mice lacking PH-20 are still fertile, indicating that PH-20 is not essential for the sperm penetration into cumulus ECM. Furthermore, analyses by SDS-poly acrylamide gel electrophoresis and western blot have shown the presence of other hyaluronidases but not PH-20 in mice sperm. These facts may suggest a species specificity in the importance of PH-20 on fertilization. For monitoring the functional competence and maturation of human sperm, sperm penetration into a HA polymer and HA binding intensity are used as indices [109, 110]. The matrix mass of COC appears to be a physiological barrier, impeding penetration by functionally incomplete sperm.

HA is one of the most abundant GAGs in the uterine, oviductal and follicular fluids [111, 112]. It has been demonstrated that GAGs effectively prevent fragmentation of porcine oocytes, and the most active factor of GAGs purified from pFF is closely related to HA [113, 114]. The addition of HA to culture medium improved sperm motility, the rate of normal fertilization and the developmental potential of *in vitro* produced embryos [115-118]. This evidence indicates that HA positively participates in the development or viability of oocytes and embryos, and thus, it is tempting to speculate that oocytes produce HA to maintain their development and viability.

Presumptive Roles of Cumulus Expansion in Oocyte Meiotic Maturation

Generally, the process of oocyte maturation can be broadly divided into two aspects, nuclear and cytoplasmic. In order to generate haploid cells, immature oocytes undergo two meiotic divisions, meiosis I and II, in which the number of chromosomes are reduced to half the original number. Fully-grown immature oocytes are arrested at the dictyate stage of prophase I, commonly known as the germinal vesicle (GV) stage. The traits of nuclear maturation also called meiotic maturation are: 1) following stimulation by pituitary LH, initiation of meiotic resumption is recognized morphologically by germinal vesicle breakdown (GVBD), chromosome condensation and spindle formation; 2) transition from meiosis I to meiosis II with the skipping S-phase; and 3) arrest in metaphase II until fertilization. Cytoplasmic maturation is referred to as other maturational events required for complete fertilization and full developmental potential. Maturational progression including nuclear and cytoplasmic features occurs simultaneously, and so it is commonly accepted that a metaphase II oocyte has obtained normal developmental potential.

In comparison with *in vivo* meiotic resumption by LH signaling, it can also be stimulated by liberation of COC from the follicle into a suitable culture medium [119, 120]. This impies that the follicular environment is accountable for maintaining meiotic arrest at the GV stage. It is considered that some factors produced by granulosa cells such as oocyte-maturation inhibitor are accumulated in follicular fluid and sustain meiotic arrest in preovulatory follicles [121, 122]. The other potential mechanism is that meiosis inhibitory factors such as cAMP are transmitted from the follicular cells to the immature oocytes efficiently through the gap junctions [123–125]. Hence, the uncoupling of cumulus cells from the oocytes by interruption of gap junctions accompanied by cumulus expansion could block elevation of intra-oocyte cAMP concentration and permit reinitiation of meiotic maturation [97, 98, 126]. However, it has been found that cell-cell communications in COCs are remained until after GVBD [96, 127, 128]. Moreover, recent studies have shown that an increase, rather than a decrease, in cAMP facilitates the induction of meiotic maturation [129, 130, 131]. Also, *in vitro* studies indicate that first FSH induces an increase in cAMP in the cumulus cells resulting in an increase in cAMP in the oocyte via diffusion from the somatic cells to the oocyte through the gap junction [132]. Thus, it is hypothesized that a certain concentration of cAMP is maintained in GV oocytes, while a transient increase in cAMP induced by hormonal stimulation is likely the trigger of GVBD [133]. A drastic change in cAMP may be an important stimulus for reinitiation of meiosis, but not important in the absolute level of cAMP in the oocyte. The action of

cAMP within oocytes is mediated by cAMP-dependent protein kinase (PKA). Downs and Hunzicker-Dunn [134] have demonstrated that two major isozymes of PKA are involved in opposing functions of meiotic regulation in COCs. They suggest that elevation of type I PKA within the oocyte is related to maintaining meiotic arrest while type II PKA mediates cAMP-stimulated cumulus expansion and meiotic resumption. Additionally, cAMP phosphodieserase (PDE) is an important enzyme controlling PKA activity. This enzyme is present within oocytes and keeps low cAMP levels and tolerates spontaneous maturation [135, 136]. The accumulation of 5'-AMP that is a product of PDE activity, stimulates AMP-activated protein kinase (AMPK), leading to meiotic resumption [137].

Gap-junctional communication between the oocyte and the innermost layer of cumulus cells surrounding the oocyte and also among each of the cumulus cells, manage bidirectional transfer of the regulatory molecules concerned with oocyte maturation such as cAMP, organic ions, small nutrients and metabolites. Each gap junction channel comprises two symmetrical hemispheres named connexons derived from two neighboring cells. Each connexon is a hexamer of protein subunits called connexins (Cxs) [138, 139]. Cxs are members of a large family that are defined by their molecular weight and shared high homology. Numerous kinds of Cxs are expressed in ovarian follicular cells, and the presence of Cx32, Cx37, Cx43 and Cx45 have been identified in mouse COCs [8, 13, 140]. Cx37 is detected in the gap junction-like structure on the surface of oocytes, beneath the ZP. Cx32, Cx43 and Cx45 are found in corona radiata cell projections close to the oocyte surface. Particularly, Cx43 contributes through transzonal projections from the cumulus cell, coupling with oocyte Cx37 to form heterotypic junctions. Also, Cx43 is predominantly expressed in cumulus-cumulus gap junctions. Studies of knockout mice have demonstrated that Cx37 and Cx43 seem to be essential at each step of normal folliculogenesis [141–143]. In Cx37 deficient mice, follicular growth fails at the preantral-antral transition and oocytes are unable to initiate meiotic maturation [141, 142]. As disruption of the gene encoding Cx43 results in neonatal lethality because of a severe heart abnormality [144], to investigate the folliculogenesis in Cx43 deficient mice, neonatal ovaries were grafted into the kidney capsules of ovariectomized, immunocompromised adult mice and allowed to develop for up to 3 weeks [143]. However, most mutant follicles failed to become multilaminar and arrested at an early preantral stage. Correspondingly, the mutant oocyte failed to undergo meiotic maturation and could not be fertilized. Vozzi *et al*. [145] have shown the physiological role of Cx43 in meiotic maturation by inhibiting Cx43 expression with a recombinant adenovirus expressing the antisense Cx43 cDNA (Ad-asCx43). The rate of GVBD is decreased by about 50% in COCs infected with Ad-asCx43. On the contrary, 89% of COCs infected with Ad-GFP (positive control) matured to metaphase II. This body of evidence suggests that the completion of oocyte growth and the acquisition of meiotic competence are wholly supported by gap-junctional communication between the oocyte and its cumulus cells.

Recently, our studies have focused on the physiological functions of COC matrix molecules on meiotic maturation, especially the effect of HA-CD44 interaction on the modification of gap junctional channels during cumulus expansion [146]. The ubiquitous HA receptor, CD44, is a member of the link module superfamily containing a common structural domain which interacts with HA to form complexes that stabilize the extracellular matrices [147], and displays numerous isoforms because of alternative splicing of 12 variant exons in different combinations [148, 149]. HA-CD44 interaction has been reported to result in the activation of signaling cascades that contribute to cell adhesion, proliferation, migration and differentiation [23, 150, 151]. The expression of CD44 in porcine COCs during *in vitro* maturation has been identified [34, 146, 152, 153]. Immunostaining revealed that CD44 is distributed on the cytoplasm along the perimembrane of cumulus cells and at the connections between cumulus cells and oocytes ([34, 152], Fig. 5). Only a single band (about 85 to 90 kDa) of CD44 standard was detected in numerous isoforms of different molecular sizes from 80 to 250 kDa, using western blotting analysis [146, 153]. CD44 was identified in cumulus cell extracts, but not in oocytes extracts. The level of CD44 expression reached a peak at 24 h of culture, although its expression was very weak at 0 h [34, 152], implying that the level of CD44 expression depends on the degree of COC expansion. Furthermore, Yokoo *et al*. [154] have investigated the effect of HA-CD44 interaction on maturation-promoting activity (MPF), and GVBD. MPF, a complex formed by cyclin B and $p34^{cdc2}$ kinase, is a key regulator of M phase, resulting in meiotic resumption [155, 156]. In Yokoo's study, porcine COCs were cultured with DON or anti-CD44 antibody for 24 hr. After culture for 24 hr, the MPF activity and the rate of GVBD were significantly decreased by treatment with DON and anti-CD44 antibody, suggesting that HA-CD44 interaction might

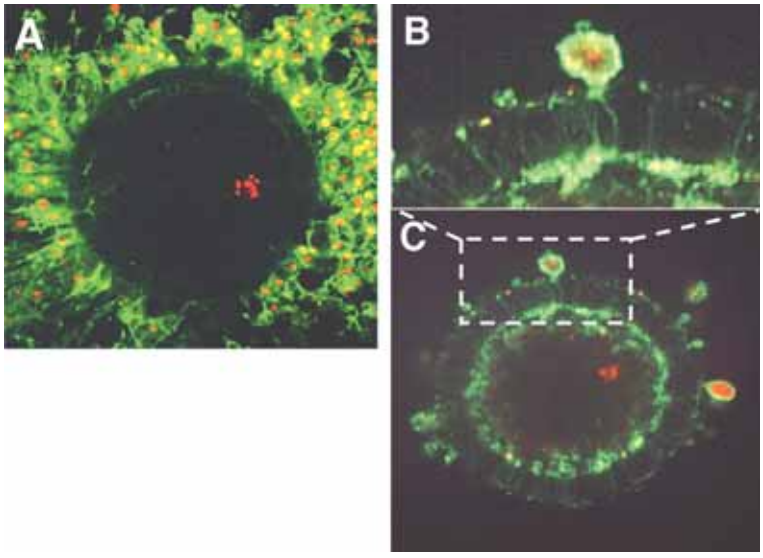

Fig. 5. Localization of CD44 in expanded porcine COCs by double staining with immunofluorescence (green: CD44 localization, red: nuclear localization) after 24 hours culture with eCG and pFF. Selected sections obtained by laser scanning confocal microscopy. A, cross-section of a COC; B, junctional communications between cumulus cells and the oocyte (magnification of the dotted line area); C, cross-section of a denuded oocyte.

have promoted the meiotic resumption. Also, HA-CD44 interaction is involved in the phosphorylation of mitogen-activated protein kinase (MAPK) in oocytes (Fig. 6). The treatment with DON or anti-CD44 antibody down-regulated MAPK phosphorylation . Furthermore, HA-CD44 interaction might affect the expression of Cx43 [154]. Exposure of COCs to DON and anti-CD44 antibody had no effect on the expression level of Cx43, however, the tyrosine phosphorylation of Cx43 was markedly inhibited. When Cx43 is phosphorylated on tyrosine residues, and intercellular junctional communication is inhibited. Cx43 is abundantly present in cumulus-cumulus gap junctions and is also found in cumulus-oocyte heterotypic gap junctions. LH, a stimulator in meiotic maturation *in vivo*, induces down-regulation of Cx43 expression, and phosphorylation through the PKA and MAPK cascades [157–159]. This indicates that HA-CD44 interactions are likely to be involved in the interruption of Cx43-derived gap junctional channels and may lead to inhibition of the transfer of cAMP from the cumulus cells to the oocyte.

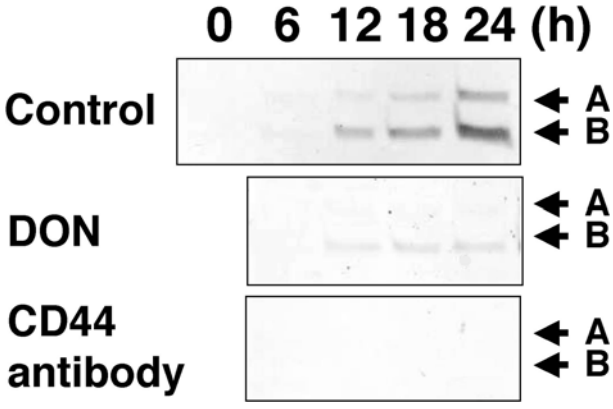

Fig. 6. Effects of DON and CD44 antibody on the phosphorylation of MAPK in porcine oocytes. The porcine COCs were cultured with 1.0 mM DON or 5.0 μg/ml anti-CD44 antibody for 24 hour. A hundred denuded oocytes per lane were examined by western blotting using anti-phospho-MAPK antibodies. A, phospho-Erk1; B, phospho-Erk2.

Conclusion

Establishing a successful *in vitro* technique for oocyte maturation and fertilization in farm animals could provide a sufficient supply of oocytes and embryos for use in basic research and biotechnology. The degree of cumulus expansion seems to be an useful parameter for predicting the development of an oocyte matured and fertilized *in vitro* [160, 161], suggesting that the

morphology of the COC matrix mass is important for nuclear and cytoplasmic maturation. Synthetic regulation of COC matrix molecules and the presumptive roles of cumulus expansion during meiotic maturation are summarized as follows: 1) HA secretion by cumulus and mural granulosa cells is attributable to HAS2, on the other hand, has3 expression in oocytes is maternal; 2) cumulus expansion plays important physiological roles leading to oocyte maturation, ovulation, fertilization and the subsequent embryo development; and 3) HA-CD44 interaction may be involved in the interruption of Cx43-derived gap junction channels, resulting in the inhibition of transfer of cAMP from cumulus cells to the oocyte.

Note: We described each of the hyaluronan synthases as follows: human hyaluronan synthase, HAS; porcine hyaluronan synthase, shas; hyaluronan synthases protein and hyaluronan synthases in general, HAS, according to the decision by the meeting of gene mapping.

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