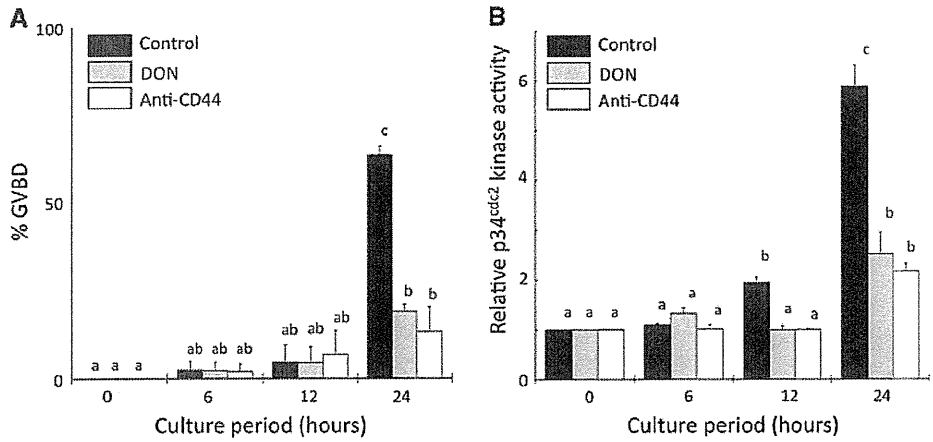

Fig. 2 Effects of hyaluronan–CD44 interaction on meiotic resumption. **a** Effects of 6-diazo-5-oxo-L-norleucine (DON) and anti-CD44 antibody on germinal vesicle breakdown (GVBD) in porcine oocytes. Cumulus-oocyte complexes (COCs) were cultured for up to 24 h in 1.0 mM DON or 5.0 μg/mL anti-CD44 antibody. **b** Effects of DON and anti-CD44 antibody on $p34^{cdc2}$ kinase activity in porcine oocytes. COCs were cultured for up to 24 h in 1.0 mM DON or 5.0 μg/mL anti-CD44 antibody. Data are expressed as fold strength of $p34^{cdc2}$ kinase activity relative to the activity in oocytes immediately after collection from follicles (0-h culture), which was set at a value of 1. *Lowercase letters* denote significant differences ($p < 0.05$). Data represent the mean ± SEM

We also observed a remarkable accumulation of hyaluronan in porcine-expanded COCs by immunostaining, but hyaluronan accumulation was completely inhibited by treatment with 6-diazo-5-oxo-1-norleucine (DON) (an inhibitor of hyaluronan synthesis) (Fig. 1) [27]. These results show that cumulus expansion of COCs is dependent on hyaluronan synthesis and accumulation. Moreover, we demonstrated that inhibition of cumulus expansion by DON resulted in a decrease in the rate of oocyte maturation in a dose-dependent manner. These results imply that hyaluronan accumulation is not only involved in the expansion of COC volume but also in the induction of oocyte maturation. The most recent studies indicated that inhibition of cumulus expansion by DON inhibited the activation of maturation promoting factor (MPF) and GVBD of the oocytes (Fig. 2) [28]. In summary, these data imply that hyaluronan synthesis during cumulus expansion plays an important role in the progression of meiotic resumption.

Hyaluronan-associated proteins on cumulus expansion

Hyaluronan synthesis is not sufficient for organizing an extracellular matrix. In general, hyaluronan needs extracellular hyaluronan-associated protein(s), an important subset of proteins with highly homologous sequences for hyaluronan binding, to organize the hyaluronan-rich matrix [29]. It has been reported that three proteins have been identified as essential for proper formation and stability of the hyaluronan matrix of COCs: inter-α-trypsin inhibitor (ITI), tumor necrosis factor-induced protein 6 (TSG6), and pentraxin 3 (PTX3) [14, 30, 31].

The ITI family isolated from serum reportedly plays an important role in the formation of the extracellular matrix, of which hyaluronan is a predominant component [32]. Proteins of the ITI family are composed of a common light chain called bikunin and 1 or 2 heavy polypeptide chains (HCs). The hyaluronan–HC complex is found in the hyaluronan-rich matrix of expanded COCs; hyaluronan covalently binds to the HCs released by the proteins of the ITI family during cumulus expansion [14, 33, 34]. Moreover, in bikunin-null mice, COCs exhibited defective formation of extracellular hyaluronan-rich matrix because of impaired synthesis of the ITI family proteins, and the mice developed severe infertility [14]. TSG6 is synthesized by cumulus cells and mural granulose cells of antral follicles after the gonadotropin surges. This protein has the ability to specifically bind to hyaluronan and to interact with the ITI family and the HCs of the ITI family are covalently transferred to hyaluronan by catalysis of TSG6 [35]. PTX3 synthesis also increases in mouse and human cumulus cells during the time preceding ovulation and localizes in the COC extracellular matrix [30, 31]. Although PTX3 does not bind to hyaluronan, the N-terminal domain of PTX3 interacts with the HCs of the ITI family and this portion of the molecule is necessary and sufficient for organizing hyaluronan and for enabling matrix formation of the COCs [36]. Both TSG6- and PTX3-deficient mice synthesize a normal amount of HAS2 expression, but they are infertile due to their inability to organize hyaluronan into a stable matrix [31, 37]. Although it is not clear whether these hyaluronan-associated proteins affect the progression of oocyte maturation, these reports suggest that these proteins may modulate the hyaluronan function during cumulus expansion.

Hyaluronan receptor (CD44) on cumulus expansion

CD44 is the most studied cell–surface receptor for hyaluronan and is present in a number of isoforms with different molecular sizes in a wide variety of cell types [38–41]. It is a transmembrane protein consisting of extracellular and cytoplasmic domains linked through transmembrane segments in the cell membranes of a variety of cells [42]. The form and function of CD44 can change depending on the cell type. CD44 expression has also been found in COCs during oocyte maturation [16, 43–45]. Immunoblotting analysis revealed that a single band of CD44 was detected between 85 and 90 kDa in COCs and the increase in its expansion was dependent on the degree of cumulus expansion [45]. The expression of CD44 in COCs appears to be controlled by FSH/eCG stimulation [16].

Moreover, when COCs were cultured with anti-CD44 antibody, oocyte maturation was inhibited in an antibody concentration-dependent manner, which had a blocking effect on the hyaluronan-binding ability of CD44 [46–49]. The most recent results indicated that the binding between hyaluronan and CD44 during cumulus expansion is highly significant for MPF activity and meiotic resumption of the oocytes (Fig. 2) [28]. Interestingly, this antibody does not affect the degree of cumulus expansion. Although previous studies have reported that the physiological significance of cumulus expansion is oocyte maturation, these results indicate that cumulus expansion is a necessary condition for oocyte maturation; however, cumulus expansion only is insufficient [50]. The results of these studies suggest that sufficient interaction between hyaluronan and CD44 is essential for meiotic resumption of the oocytes.

Function of hyaluronan in meiotic resumption

We demonstrated that hyaluronan played an important role in meiotic resumption of oocytes via CD44; however, because CD44 was expressed in cumulus cells and not in oocytes [45, 49], details of the mechanism by which hyaluronan regulates the progression of meiotic resumption has remained unclear. The critical component of meiotic resumption is MPF, a serine/threonine protein kinase composed of a regulatory unit, cyclin B, and a catalytic subunit, $p34^{cdc2}$ [51, 52]. In mammals, MPF activation is controlled by the concentration of cAMP in the oocyte. cAMP is synthesized in the cumulus cells and transferred into the oocytes via gap junctions in the COCs. Therefore, meiotic resumption of oocytes is regulated by gap junctional communication between the oocyte and the cumulus cells following the preovulatory gonadotropin surges. Therefore, we hypothesized that the effects of hyaluronan on oocyte maturation are related to the mechanism of gap junction gating in COCs. Connexin 43 (Cx43) is the most abundant gap junction protein; it is expressed by the ovarian follicles of many species [53–56] and also expressed in COCs as phosphorylated forms (Fig. 3a, b). We evaluated the effects of hyaluronan–CD44 interaction on the expression of Cx43. The results showed that exposure of COCs to DON or anti-CD44 antibody from 24 to

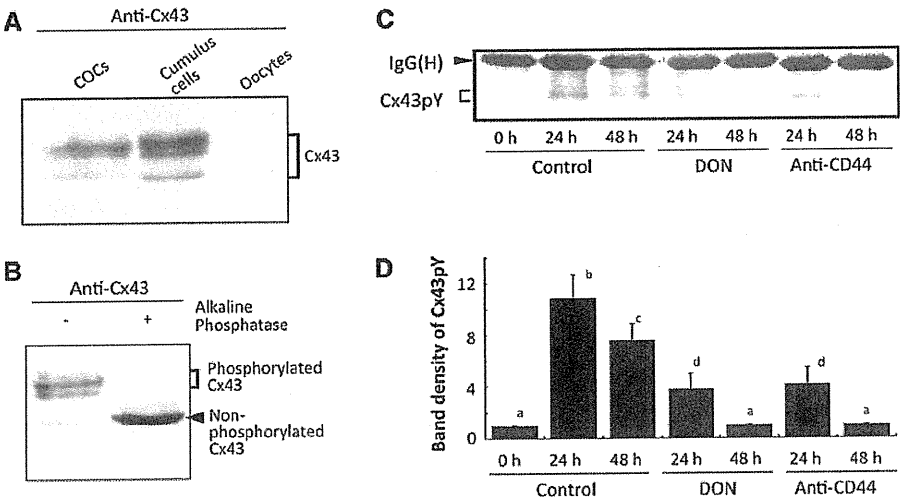

Fig. 3 Immunoblotting analysis of connexin 43 (Cx43) in cumulus–oocyte complexes (COCs). **a** Extracts of COCs, cumulus cells, and denuded oocytes were analyzed by immunoblotting with anti-Cx43 antibody. **b** Two samples of cultured COCs were treated with or without alkaline phosphatase. At the end of the treatment, the samples were subjected to immunoblotting analysis with anti-Cx43 antibody. **c** Detection of tyrosine-phosphorylated Cx43 (Cx43pY). The extracts immunoprecipitated with anti-Cx43 antibody were probed with anti-phosphotyrosine antibody. The *arrowhead* indicates the immunoglobulin heavy chain band (IgG (H)). **d** Densitometric analysis of (**c**). *Different superscripts* denote significant differences ($p < 0.05$). Data represent mean ± SD

48 h of incubation had no effect on the expression of total Cx43. Conversely, these treatments significantly inhibited the tyrosine phosphorylation of Cx43 in COCs (Fig. 3c, d) [57]. A previous study showed that Cx43 is phosphorylated at multiple residues, and that gap junction function is regulated by several kinases [58, 59]. The closure of Cx43-containing gap junctions is induced by phosphorylation of this protein on the tyrosines at positions 247 and 265 [60–62]. Therefore, our results revealed that hyaluronan induced the closure of Cx43-containing gap junctions in COCs via tyrosine phosphorylation, and meiotic resumption subsequently occurred in the oocytes. Previous studies showed that Src tyrosine kinase induces tyrosine phosphorylation of Cx43 and inhibits intercellular gap junctional communication [61–65]. In addition, the interaction between hyaluronan and CD44 has been shown to stimulate the activation of tyrosine kinases such as Src kinase [66–68]. Although the function of Src kinase in the cumulus cells during oocyte maturation is unclear, we speculate that hyaluronan induces the tyrosine phosphorylation of Cx43 via Src kinase.

Interestingly, we observed these results using a maturation medium without luteinizing hormone (LH). Several previous studies have demonstrated that LH is a key factor in the initiation of oocyte maturation in vivo and in vitro, and it is involved in the regulation of closure of the Cx43-containing gap junctions in COCs. Various studies have investigated the signal transduction pathway and LH-stimulated maturational processes. Cx43 levels are reduced in response to the preovulatory surge of LH [69, 70]. In addition, LH stimulates the phosphorylation of this gap junction protein [59, 71, 72]. Recent studies showed that LH causes mitogen-activated protein kinase-dependent serine phosphorylation and closure of Cx43-containing gap junctions, allowing meiotic resumption [73]. In addition, it has been shown that progesterone induces reduction in Cx43 expression via the progesterone receptor, which is induced by stimulation with LH, also resulting in meiotic resumption [74]. Moreover, the LH-induced decrease in Cx43 permeability is reportedly not due to phosphorylation at tyrosine sites [75, 76]. Considering that hyaluronan induces tyrosine phosphorylation of Cx43, the meiosis stimulatory function of hyaluronan is likely to be independent of the meiosis stimulatory pathway of LH. The relationship between hyaluronan and LH with regard to meiotic resumption of the oocytes is undocumented and needs to be addressed.

Hyaluronan-binding ability of CD44 and oocyte maturation

In general, oocytes that have 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. Although oocytes that have 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 [77]. Furthermore, the rate of embryo development of in vitro-matured and fertilized COCs is significantly lower than that observed in vivo [78]. The results of our experiments suggest that the binding between hyaluronan and CD44 during cumulus expansion is important for oocyte maturation. Therefore, we focused on the role of the CD44 molecule in cumulus expansion for the in vitro and in vivo comparisons in our study [49, 79]. Immunoblotting analysis indicated a difference in the size of CD44 between the in vivo and in vitro samples. While the CD44 band of the in vitro-matured COCs was 81–88 kDa in size, the CD44 band of the in vivo-matured COCs was 73–83 kDa in size. Sialidase treatment reduced the size of the CD44 obtained from the COCs matured in vitro to a size similar to that of the CD44 from the COCs matured in vivo. These results suggest that during cumulus expansion, the CD44 in the in vitro-matured COCs contains more sialic acids than the CD44 in the in vivo-matured COCs. The extracellular domain of CD44 reportedly contains the necessary motifs for binding hyaluronan [80], and glycosylation of its extracellular domain has been implicated in the regulation of the hyaluronan-binding ability of CD44 [39]. 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 [81–83]. Although we did not measure the ability of CD44 to bind hyaluronan, our results indicated the possibility that the interaction between hyaluronan and CD44 during in vitro maturation may not be sufficient for oocyte maturation compared to in vivo maturation. Based on these observations, we speculate that insufficient interaction of hyaluronan with CD44 during in vitro maturation may cause inferior fertilization and developmental capacity in the oocytes compared to those matured in vivo. These results may represent a new target for controlling oocyte maturation and oocyte quality in the in vitro culture systems.

Concluding remarks

In conclusion, we propose a model wherein hyaluronan serves a previously unrecognized role in inducing meiotic resumption (Fig. 4). Hyaluronan secreted from the cumulus cells is synthesized by HAS2, whose level of expression is controlled by FSH/eCG. Hyaluronan secreted from the cumulus cells is linked to the hyaluronan-associated proteins (ITI, TSG6 and PTX3) and forms an hyaluronan-rich

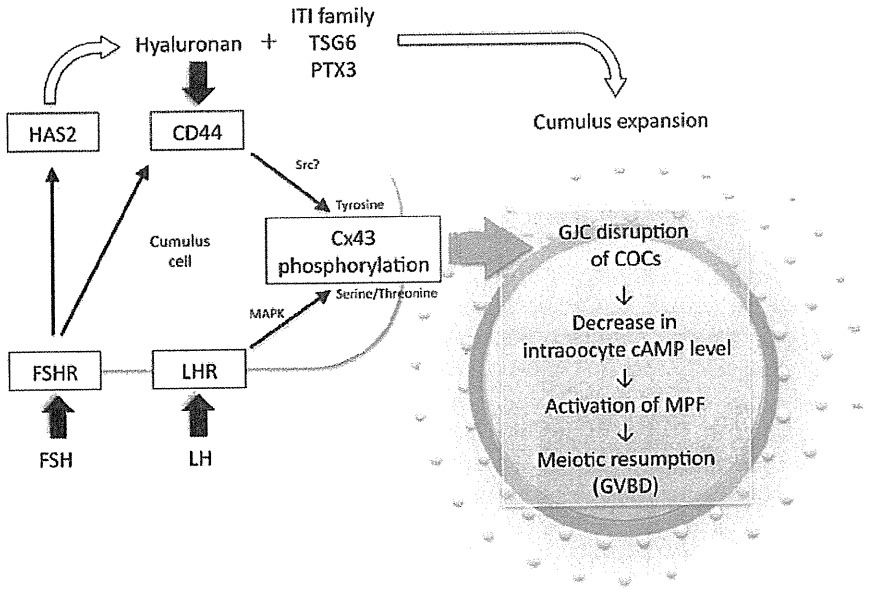

Fig. 4 A proposed model for hyaluronan-induced mammalian oocyte meiotic resumption. *FSH* follicle-stimulating hormone, *LH* luteinizing hormone, *FSHR* FSH receptor, *LHR* LH receptor, *HAS2* hyaluronan synthase 2, *ITI* inter-α-trypsin inhibitor, *TSG6* tumor necrosis factor-induced protein 6, *PTX3* pentraxin 3, *Cx43* connexin 43, *MAPK* mitogen-activated protein kinase, *GJC* gap junctional communication, *cAMP* cyclic adenosine 3′,5′-monophosphate, *MPF* maturation promoting factor, *GVBD* germinal vesicle breakdown

matrix in the COCs, resulting in cumulus expansion. FSH/eCG stimulation also results in the expression of hyaluronan receptor CD44 on cumulus cells. The hyaluronan accumulated during cumulus expansion binds to CD44 and induces the phosphorylation of Cx43 at tyrosine residues. The hyaluronan–CD44 interaction during cumulus expansion induces disruption of the gap junctional communication in the COCs, inhibits the transport of cAMP from the cumulus cells into the oocytes, and leads to MPF activation and meiotic resumption of oocytes. On the other hand, LH also induces meiotic resumption of the oocytes, and it is quite likely that LH interrupts the gap junctional communication in the COCs by another pathway involving hyaluronan.

Our studies provide the evidence that the hyaluronan–CD44 interaction during cumulus expansion of COCs acts to induce the progression of meiotic resumption in the oocytes. In mice, it is known that CD44 is not essential to induce the ovulation process and that CD44 mutant mice are fertile; however, it is also known that spontaneous maturation occurs in mouse oocytes and that a normal percentage develop to live offspring when fertilization does occur in these oocytes [84]. Thus, the function of the hyaluronan–CD44 interaction may be not necessarily essential for mouse oocyte maturation that is not critically dependent on gonadotropin. Although the dependence of the hyaluronan–CD44 interaction on the oocyte maturation process in other species is poorly understand, we speculate that the gonadotropin-induced meiotic resumption process of mammalian oocytes requires sufficient interaction between hyaluronan and CD44 during cumulus expansion. In vitro maturation of oocytes for ART in humans has not yet been completely established. In particular, cumulus expansion during in vitro maturation of human COCs has failed. We hope that this review will lead to an improvement in the in vitro oocyte maturation techniques for ART.

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