Molecular organization and mechanical properties of the hyaluronan matrix surrounding the mammalian oocyte

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Abstract

Successful ovulation and oocyte fertilization are essential prerequisites for the beginning of life in sexually reproducing animals. In mammalian fertilization, the relevance of the protein coat surrounding the oocyte plasma membrane, known as zona pellucida, has been widely recognized, while, until not too long ago, the general belief was that the cumulus oophorus, consisting of follicle cells embedded in a hyaluronan rich extracellular matrix, was not essential. This opinion was based on *in vitro* fertilization procedures, in which a large number of sperms are normally utilized and the oocyte can be fertilized even if depleted of cumulus cells. Conversely, *in vivo*, only very few sperm cells reach the fertilization site, arguing against the possibility of a coincidental encounter with the oocyte. In the last two decades, proteins required for HA organization in the cumulus extracellular matrix have been identified and the study of fertility in mice deprived of the corresponding genes have provided compelling evidence that this jelly-like coat is critical for fertilization. This review focuses on the advances in understanding the molecular interactions making the cumulus environment suitable for oocyte and sperm encounter. Most of the studies on the molecular characterization of the cumulus extracellular matrix have been performed in the mouse and we will refer essentially to findings obtained in this animal model.

Introduction

In most mammals, the release of mature oocyte at ovulation requires tight control of extracellular matrix (ECM) remodeling in different regions of the ovarian follicle. At this stage, three somatic cell compartments can be identified in the follicle that must cooperate and cross-talk for accomplishing the final task: the epithelium of granulosa cells lining the follicular fluid-filled cavity, the epithelium of cumulus cells (CCs) surrounding the oocyte and a specialized and vascularized connective tissue, namely theca cells, which is separated from the granulosa cells by a basal membrane (Fig. 1). An inflammatory-like process is triggered in the follicle by the physiological surge of luteinizing hormone (LH) or by the injection of an ovulatory dose of human chorionic gonadotropin (hCG). This event results in increased permeability of theca vasculature, massive production of prostaglandin E_2 and selective degradation of perifollicular matrix by granulosa cells leading to the formation of a thinner area at the follicular apex [1]. Conversely, CCs synthesize a large amount of a hyaluronan (HA) matrix, under the combined action of paracrine stimuli produced by both granulosa cells and the enclosed oocyte [2,3]. The cells lose epithelial arrangement, move away from each other and become dispersed in a highly hydrated extracellular matrix (ECM) with viscoelastic characteristics. As a result, the space between cells enlarges and consequently the total volume of the cumulus increases, a process for this reason named cumulus expansion. As ovulation approaches, the follicle wall breaks, and a hole is formed on the ovarian surface sufficiently large to allow part of cumulus mass to pass through. A transient deformation of the cumulus

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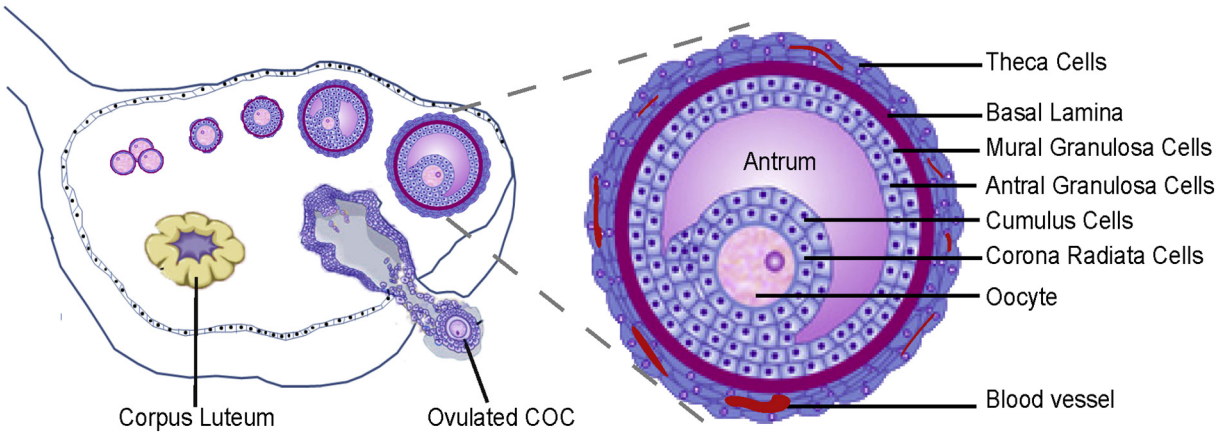

Fig. 1. Schematic representation of the ovary containing follicles at the different stages of folliculogenesis. A magnification of the pre-ovulatory follicle is reported, and the localization of all the different cell compartments discussed in the review are indicated.

ECM subsequently drives the egg out of the follicle. Cumulus cells and the oocyte remain firmly bound within the ECM so that they are not dispersed during their extrusion and transfer to the fertilization site [4]. Electron microscopy analyses shows a continuum of a mesh-like network of filaments and granules anchored to the surface of the CCs and extending up to the zona pellucida (ZP) [5]. Indeed, the innermost cell layer of the cumulus, the corona radiata cells, lose the contact with the oocyte and retract the cytoplasmic projections crossing the ZP, leaving the synthesized HA matrix entangled in pores of this internal oocyte envelop [5–7]. Cumulus ECM is required by the sperm for successful *in vivo* oocyte fertilization, but it is stable just for few hours after ovulation. The dispersion of the cumulus matrix progressively occurs, paralleling oocyte aging [8]. Thus, this provisional HA matrix has the function to favor the fertilization of oocytes with high embryo developmental capacity.

Assembly and disassembly of the hyaluronan matrix

The observation, reported in 1942, that the viscoelastic ECM embedding the CCs around the ovulated oocyte is quickly dispersed by hyaluronidase digestion led to the suggestion that HA is the major structural macromolecule in this matrix [9]. HA is a very large, polyanionic glycosaminoglycan which expands to a highly solvated coil in free solution attracting water and increasing the space between cells. Isolation of mouse cumulus cell-oocyte complexes (COCs), and identification of culture conditions able to induce its expansion *in vitro* provided an invaluable tool for understanding the complex cell interactions and changes in gene expression required for HA synthesis and organization in the ECM. Follicular stimulating hormone (FSH) or epidermal growth factor (EGF), as well as EGF-like growth factors amphiregulin, betacellulin and epiregulin, are potent stimulators of *in vitro* COC expansion [10–12]. All these factors induce the phosphorylation of mitogen-activated protein kinase kinase- (MEK) activated kinases, extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2), which is the intracellular signaling pathway obligatory for COC expansion, as well as for triggering all the events preceding ovulation [13,14]. Beside FSH/EGF, expansion of the mouse COC requires the concomitant stimulation by oocyte-secreted paracrine factor belonging to the transforming growth factor beta (TGFβ) superfamily [15], likely growth differentiation factor 9 (GDF9) or its variant growth differentiation factor 9 heterodimer (GDF9/BMP15) [16].

As a result of this interplay, the expression of hyaluronan synthase 2 (HAS2) dramatically increases within 2–3 h from the stimulus both *in vitro* and *in vivo* [17]. Hyaluronan synthesis is transient and is maximal between 3 and 12 h, and then declines toward basal levels at about 15–18 h [18]. Staining of HA in the follicle showed that HA synthesis is extended to the granulosa cells nearby the COC and to the innermost layers of granulosa cells adjacent to the follicular antrum, named antral granulosa cells, while little or no HA was synthesized in the outermost layers of granulosa cells, named mural granulosa cells. Likely, regional differences in follicular HA production reflect the formation of a concentration gradient of the oocyte factor due to its binding or inactivation by the responding cells [19]. It is well known that heparan sulfate proteoglycans (HSPGs) bind the latent form of TGFβ family members regulating their signaling and extracellular location. The HSPG Glypican 1 has been proposed

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as a candidate for restricting the diffusion and enhancing the localized signal of GDF9 in the cumulus. This proteoglycan is rapidly and highly expressed by CCs during expansion and is associated to the HA matrix [20].

Antral and the more proximal mural granulosa cells become incorporated into the COC and are released from the follicle as a single mucified mass. In optimal culture conditions the net production of HA is about 4 pg per cumulus cell (about 4 ng/COC), and yield a final concentration of approximately 0.5 mg/ml at the completion of expansion (based on the estimate volume of a COC), which is nearly the same as *in vivo* [18,19]. Expanded COCs are only temporarily deformed when subjected to shearing forces, and proteins are required to organize and maintain HA in such a highly structured gel, conferring to the COC ECM unique physical properties. Disassembly of the cumulus ECM begins soon after the completion of expansion, and continues thereafter in parallel with a progressive apoptosis of CCs, leading to COC dispersion in about 15 h [8,21]. Hyaluronan is released from the ECM into the medium without any significant variation in size, and protein synthesis inhibitors prevent ECM disassembly, suggesting that this process does not depend on HA cleavage, but rather on degradation of proteins involved in its organization [21,22]. In agreement, the expression of several proteases, such as urokinase plasminogen activator and a disintegrin and metalloproteinase with thrombospondin motifs 1 and 4 (ADAMTS1 and ADAMTS4) are upregulated or activated at this time [8,23]. A decrease of the intracellular cyclic adenosine monophosphate (cAMP) level appears to be functional in the execution of this program because treatment of *in vivo* and *in vitro* expanded COCs with cAMP analogues or adenylate cyclase activators inhibits HA release and cumulus cell apoptosis for several days [21]. Exogenous HA oligomers (decasaccharides or larger), which compete for the interaction of HA binding proteins, do not accelerate the physiological disaggregation [22] neither displace HA from the matrix preserved by cAMP analogue treatment (personal communication). These findings suggest that assembly of such elastic ECM requires cooperation of several structural proteins in order to stably and tightly crosslink HA strands. Presently, three proteins meeting these criteria have been identified.

It has been demonstrated that HA organization in the cumulus ECM requires the participation of a serum protein, inter-α-trypsin inhibitor (IαI or ITI), and other two proteins, both synthesized by CCs under oocyte influence: pentraxin 3 (PTX3 or TSG14) and tumor necrosis factor alpha-induced protein 6 (TNFAIP6 or TSG-6). Knock out mice of each of these proteins produce the same phenotype consisting in defective COC expansion, failure of oocyte fertilization and sterility [24–27].

Cooperation between IαI and TSG-6 in cumulus matrix formation

IαI family proteins are mainly secreted by the liver and present in the blood at considerably high concentrations (0.15–0.5 mg/ml). Indeed, they are peculiar proteoglycans formed by an about 40 kDa light chain protein carrying a single chondroitin 4-sulfate chain, named bikunin, to which 1 or 2 (out of 3) accessory proteins with high homology sequence, named heavy chains (HC1, HC2, HC3), are covalently linked [28,29]. In the trans-Golgi network, the large C-terminal extension (240–280 amino acid residues) of HCs is released and HCs are coupled to bikunin chondroitin sulphate chain *via* an ester bond between the C-terminal aspartate residue of the HCs and an internal *N*-acetylgalactosamine in the chondroitin sulphate [30,31]. The linkage of one HC brings to the formation of pre-α-trypsin inhibitor (PαI) (~130 kDa) while the linkage of two HCs forms IαI (~220 kDa). These molecules are specifically associated to the HA matrix formed during several inflammatory diseases and accumulate within inflamed tissues [32]. Pioneering studies, performed to assess the culture condition able to induce COC expansion, demonstrated that serum was indispensable to form the muco-elastic ECM. In its absence, FSH-stimulated CCs synthesized HA at normal rate and size but they failed to organize it in the intercellular space, so that most of the HA diffused into the culture medium, and COC disaggregated [10,18,22,33]. Vessel permeability allows the diffusion of IαI into the follicular fluid [34–36], and its co-immunolocalization with HA in the preovulatory follicle supported its physiological role [37]. The direct participation of these serum derived molecules in cumulus ECM formation was first provided by the evidence that they are necessary and sufficient for substituting serum in retaining HA within the cumulus, and that IαI depleted serum is inactive [37,38]. Purified bovine PαI and IαI as well as human IαI are almost identical in their ability to stabilize the expanding mouse cumulus ECM *in vitro* [37,39,40] although they differ in HCs combinations. IαI in human and mouse is composed of HC1 and HC2, while in cow the HC3 and HC2 are present in IαI and the HC2 is present in PαI [41]. The high sequence homology among the species and between the three types of HCs likely account for their comparable efficacy.

It has been proven that HCs are transferred from IαI CS to HA during COC expansion, forming a HC-HA covalent complex [39]. Analogous complexes were found in synovial fluid of arthritic patients [42], and mass spectrometry showed that an ester bond was formed between HC and HA, suggesting that the transfer of HCs from IαI CS to HA occurs by a transesterification reaction accompanied by the release of bikunin proteoglycan [43]. The importance

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of this reaction in cumulus ECM formation has been proven by the deletion of bikunin gene. Bikunin-null mice exhibited female sterility due to fertilization failure, associated with abnormal COC matrix. After an ovulatory stimulus, CCs became scattered in the antral cavity of the follicle and during ovulation most of them were dissociate from the oocyte. HCs were not covalently linked to HA even though unprocessed free HCs were circulating in the blood. The expansion process could be rescued by intraperitoneal administration of intact IαI but not bikunin alone [24]. These results suggest that the construction of the cumulus ECM requires the formation of the HC-HA complex and that bikunin plays an indirect role by transporting the HCs to the ovarian follicle, thereby allowing the transesterification reaction. TSG-6 is the co-factor and catalyst of this reaction.

TSG-6 is a ~35 kDa secreted glycoprotein known as a natural modulator of inflammatory responses [44]. This protein consists of an *N*-terminal link module and a C-terminal CUB domain. It binds to HA with high affinity *via* the link module, which is a key feature of the members of the hyaladherins family.

The expression of TSG-6 is strongly upregulated both in granulosa and CCs in the preovulatory follicle and temporally correlated with HA matrix deposition around the oocyte [26,45,46]. Immunostaining of ovarian sections showed colocalization of TSG-6 with HA and IαI in the cumulus ECM. Western blot analyses of ovulated COC revealed that TSG-6 is present as a free protein (35 kDa) and as a species of ~120 kDa that is immunoreactive with both anti-TSG-6 and anti-IαI antibodies [47,48]. Mass spectrometry of peptides derived from the ~120 kDa species demonstrated that it contains TSG-6, HC1 and HC2 (but not bikunin) [47]. These complexes were cleaved by treatment with alkali, suggesting the presence of an ester bond. The indispensable role of TSG-6 for HC-HA complex formation and COC expansion has been established by the study of transgenic mice [25]. *Tsg-6* null female mice are infertile due to their inability to form the cumulus ECM, a phenotype that correlates with the total absence of HC-HA complexes in the ovaries of these animals, similar to bikunin-null mice. Accordingly, serum was not sufficient to support ECM organization by COC isolated from *Tsg-6* null mice unless recombinant TSG-6 was added to the culture medium. It was then proposed that TSG-6-HC complexes could act as intermediates in the transfer of HC to HA. Biochemical analysis using purified human IαI and recombinant human TSG-6 demonstrated that HC and TSG-6 in the complexes are linked through ester bonds and that TSG-6 acts as a co-factor and catalyst in the translocation of HC from CS of bikunin to HA *via* two sequential transesterification reactions [49,50].

Although HCs of IαI lack overall sequence homology to the characterized HA binding proteins, they show some ability to bind to HA, likely through basic amino acid residue-rich regions [38,51]. In addition, agarose gel electrophoresis and gel filtration analyses of HC-HA complexes from synovial fluids of patients with rheumatoid arthritis suggest that they tend to form aggregates likely through HC to HC interaction [52]. However, although HC to HA and HC to HC interaction might crosslink separate HA strands (Fig. 2), additional molecular interactions involving PTX3 are required for organizing HA in a stable gel-like ECM.

Cooperation between HCs and PTX3 in stabilizing cumulus matrix

PTX3 is a 45-kDa protein predominantly assembled in a multimeric complex of eight protomers by interchain disulfide bonds [53]. It consists of a C-terminal 203-amino-acid pentraxin domain, sharing homology with the classic short pentraxins, C-reactive protein and serum amyloid P component, coupled to an N-terminal portion of 174 amino acids that does not show any significant homology with any other known protein. The expression of PTX3 is up-regulated under inflammatory stimuli in several cell types [54]. In the ovary, PTX3 is specifically up-regulated in CCs following an ovulatory stimulus, in parallel with TSG-6 and HAS2, and localizes in the matrix [26,27]. In *Ptx3* deficient mice, as well as in *bikunin*- and *TSG-6* null mice, cumulus ECM formation is deeply altered and prevents oocyte fertilization leading to female sterility. In COCs ovulated from *Ptx3* null mice, CCs appear to form a uniform unstable mass rather than layers arranged around a central positioned oocyte. The viscoelastic ECM spontaneously dissolves in a short time, quickly leading to CCs and oocyte dispersion in the oviduct. The deletion of *Ptx3* does not alter HA synthesis by CCs, neither prevents or decreases the formation of HC-HA complexes [27]. *Ptx3* null COCs, stimulated to undergo expansion *in vitro*, do not retain HA even though IαI or serum is present in the medium, but a normal phenotype can be restored by adding recombinant human PTX3 to the culture medium. Recombinant N-terminal PTX3 domain can replace full length PTX3 indicating that ECM assembly activity is independent of the pentraxin domain and exclusively resides in this unique sequence of the protein, then assigning a specific role to PTX3 in HA matrix organization. Solid binding assays show that PTX3 does not bind to HA but interacts with IαI HCs and with TSG-6 [27,40]. Direct interaction between PTX3 and HCs in biological context has been documented by their co-localization in the cumulus ECM and co-precipitation from cumulus matrix extracts as well as by using purified molecules. The physiological relevance of such interaction is supported by the evidence that the HC binding site

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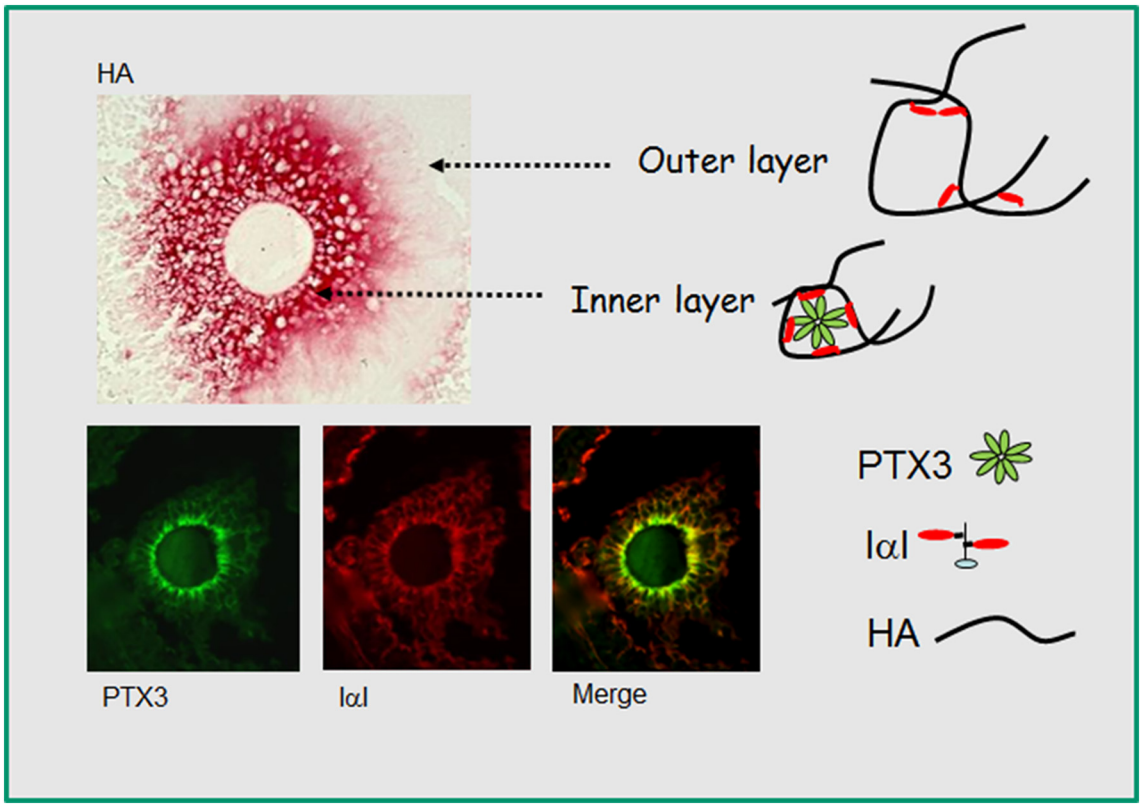

Fig. 2. Localization of HA, PTX3 and IαI in a COC and the proposed model of interaction. In this model HA strands are loosely organized at the periphery of the cumulus for the predominance of weak HC-HA or HC-HC interactions, while a greater aggregation occurs in the inner layers for the abundance of PTX3 and the formation of 'nodal' crosslinking generated by the HC-PTX3-HC. For HA detection, sections of ovaries collected at 10 h from an ovulatory dose of hCG were stained with biotinylated HA binding protein and successively incubated with peroxidase-conjugated streptavidin. Note the filamentous appearance of the HA staining extending from the outer cell layer into the follicular fluid. For PTX3 and IαI detection, sections of ovaries collected at 6 h from an ovulatory dose of hCG were first incubated with biotin-labeled rabbit antihuman PTX3 polyclonal antibody and with Alexa Fluor 488 streptavidin (green). After washing, the same sections were probed with rabbit anti-human IαI and with the secondary Cy3 goat anti-rabbit IgG (red). Note the more intense staining for PTX3 in the corona radiata cell layer. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

resides in the PTX3 N-terminal domain, which is required and sufficient to organize HA. Moreover, a monoclonal antibody generated against this portion of the molecule inhibits their interaction and neutralizes full length recombinant hPTX3 in restoring normal phenotype in *Ptx*3 deficient COCs [40]. The multimeric status of PTX3 plays a key role in defining its biological activity. Site direct mutagenesis of cysteines involved in the formation of disulphide bonds among the PTX3 protomers indicates that the tetrameric assembly of the full length and the N-terminal domain is the minimum oligomeric state required for supporting HA organization while the dimers retain the ability to bind HC [53,55]. This suggests that the octameric structure of PTX3 provides at least four binding sites for this ligand. Thus, it is likely that PTX3 might substantially strengthen and stabilize the HA network by binding several HCs covalently linked to distinct HA molecules, acting as a "node" (Fig. 1). Interestingly, TSG-6 also binds to a dimer of the N-terminal domain of PTX3 [55,56], implying that multiple TSG-6 can bind to the full length PTX3 and, in principle, participate in cross linking HA by its link domain. However, several lines of evidence are against a structural role of TSG-6. First, HA hexasaccharides, which compete with the binding of TSG-6 to HA, do not inhibit ECM assembly in COC [22,57]. Accordingly, mutants of TSG-6 link domain showing highly reduced binding to HA, but retaining the capacity to transfer HC to HA, do support ECM assembly of *Tsg-6* deficient COC *in vitro* [58]. Finally, TSG-6-HC complexes, but not free TSG-6 molecules, are found in the cumulus ECM up to few hours before ovulation, indicating that almost all the TSG-6 synthesized by CCs is engaged in transferring HCs to HA [59]. On these bases, it has been proposed that the binding of TSG-6 to PTX3 might lead to the integration of PTX3 into the ECM at the same time and in coordinate fashion to HCs [40]. This hypothesis found

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a strong support and further elucidation by a novel *in vitro* binding assay in which PTX3, IαI and TSG-6 can interact with well-defined film of HA in controlled sequence. It was demonstrated that PTX3 can be incorporated into the HA film only if it is pre-mixed with IαI and TSG-6 [56]. Thus, it seems that the HA organization in the cumulus ECM is a highly orchestrated process rather than a casual encounter of molecules with relative binding affinity. In this regard, we should reconsider earlier studies on the temporal effect of serum on HA retention in COC matrix. It was, in fact, shown that if serum is removed from the culture during COC expansion, HA synthesized before the removal is retained in the matrix, while that synthesized afterwards diffuses in the medium [22]. Therefore, it is time to speculate that HCs are associated to HA during the process of HA elongation, before its release from HAS2. Targeting of IαI and/or its ligands in the pericellular matrix could help in this matter. Interestingly, TSG-6 can bind to adhesive molecules of the pericellular matrix [60] and interaction with thrombospondin 1 seems even to enhance the formation of TSG-6-HC complexes [61].

Spatio-temporal regulation of versican synthesis and its cleavage in the preovulatory follicle

Versican belongs to the hyalectan family of proteoglycans, so called for the ability to specifically bind to HA through their N-terminal (G1) domain and to lectins present in the matrix or at the cell surface *via* their C-terminal (G3) domain [62]. Versican is produced by a variety of cell types in different tissues and localizes in the ECM as well as in the pericellular space. It is involved in regulating cell adhesion and migration during physiological and pathological conditions, including embryo development and tumor invasion [63]. The central domain of versican core protein carries up to 30 chondroitin sulfate chains conferring to the molecule large molecular size, negative charge and hydrodynamic characteristics. This domain comprises two subdomains, designated glycosaminoglycan α and β (GAGα and GAGβ), which can be both present (V0) or alternatively spliced to generate three main variants containing the G1 domain and the GAGβ domain, (V1), or the GAGα domain (V2) or neither GAG domain (V3). The V0 and V1 represent the isoforms most ubiquitously expressed and extensively studied. During matrix remodeling, versican is a substrate for proteases belonging to ADAMTS family, *i.e.* ADAMTS1, ADAMTS4 and ADAMTS5 [64]. The expression, localization and cleavage of versican show a tight spatial and temporal regulation in the preovulatory follicle. Early studies showed that a large dermatan sulfate proteoglycan, later recognized as versican, is synthesized by granulosa cells and mostly released into the medium, suggesting that granulosa cells are the principle producers of versican accumulating in the follicular fluid during the formation of the antrum [65–67]. In the follicular fluid, the concentration of versican is high, ranging between 0,8–1 mg/ml [65,68], and substantially contributes to the osmotic pressure and water entrapment in the follicular cavity, preventing the collapse of follicle and providing an appropriate spatial and physical environment for granulosa cells and COC [36]. Before ovulation, a spike in the expression of both versican and ADAMATS1/ADAMTS4 proteases occurs [23,69,70]. These proteases specifically cleave out the G1 HA binding domain from Versican1 and, accordingly, a fragment of about 70 kDa is detected in the medium of hormonally-stimulated mouse granulosa cells [23]. This is consistent with early observation that versican in follicular fluid is unable to bind to HA [65]. The reason for versican cleavage by granulosa cells is not clear. Likely, this is a mechanism for increasing the colloid osmotic pressure and decreasing the viscosity of follicular fluid in order to facilitate COC release at ovulation. A detectable increase of versican also occurs in COCs during expansion, but in this follicle subpopulation versican is retained in the ECM [69–71]. The link protein, a well-known HA binding protein which stabilizes the binding of hyalectins to HA and confers them resistance to enzyme degradation, is also produced by CCs [71,72]. The aggregation of versican to HA might contribute to the swelling of the cumulus ECM, as suggested for other soft matrices [63]. Co-immunolocalization studies have shown an intense immuno-staining for both versican and HA at the surface of CCs suggesting that they form a thick pericellular coat [23], which in other cell types has been shown to favor cell detachment and migration, processes that recent studies indicate essential for successful expansion (see next section). ADAMTS1 and ADAMTS4 are expressed by CCs and are activated at the cell surface few hours before ovulation. Accordingly, the 70 kDa G1 fragment accumulates in the cumulus ECM with a temporal pattern that matches that of these enzymes [23,69]. Thus, the versican proteolytic cleavage begins at the end of cumulus matrix formation and increases in the ovulated COC, with ECM disassembly and cumulus cell apoptosis [21]. The relevance of versican degradation in cumulus ECM dynamic cannot be studied in *versican* null mice, since they die at early stages of embryogenesis. However, involvement of versican in COC expansion might be suggested by studies performed in *ADAMTS1* null mice. Versican cleavage is markedly reduced in COCs ovulated by *ADAMTS1* deficient mice, and COCs are resistant to disaggregation, suggesting an altered ECM remodeling [73]. This scenario resembles the events occurring during heart development, where the spatio-temporal expression of versican, HA and ADAMTS allows the formation and dissolution of the cardiac jelly required for heart wall remodeling [74–76]. Cumulus cells also upregulate the expression of urokinase

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plasminogen activator [8], which, through plasmin activation, could act in concert with ADAMTS to degrade versican, as well as other structural ECM components. Certainly, the cleavage of the G1 HA domain from G3 lectican domain of versican breaks the bridge between HA and adhesive proteins and could substantially contribute to HA network destabilization. Interestingly, the G1 domain fragment has been reported to create a permissive environment for apoptosis during limb bud development [77] and it could have similar function in the postovulatory COCs [21].

Cytoskeleton reorganization is required for cumulus expansion

The deposition of cumulus ECM is accompanied by an extensive rearrangement of actin microfilaments in CCs, which results in the formation of several cytoplasmic expansions, mainly blebs of various size and density, and a few microvilli [5,78–81]. Membrane blebbing is dynamic and is associated to migratory capacity in non-adhesive environment [82,83]. Early studies with microfilament disrupting agents suggested that cytoskeleton changes are essential in the cascade of events leading to COC expansion [84]. This hypothesis has been confirmed and extended by recent findings [81]. It has been shown that CCs stimulated to undergo expansion rapidly cleave and activate calpain 2 (or m-calpain) which is temporally associated to degradation of paxillin and talin, two integral components of focal adhesion complexes, allowing cell detachment and formation of bleb-like protrusions. Paxillin and calpain 2 co-localize at the cell membrane, specifically at the base of cell protrusions. Injection of a calpain inhibitor *in vivo* prevented the formation of blebs, and COC expansion failed to occur, even though HAS2 was highly expressed [81]. Moreover, inhibition of focal adhesion kinase (FAK) phosphorylation at Tyr397, dependent on integrin engagement, has been shown to stimulate the expression of HAS2 and cumulus expansion in the absence of any additional stimulus, indicating that alternative mechanotransduction pathway can control HAS2 expression [85].

The study of prostaglandin E_2 receptor subtype 2 (EP_2) null mice helped in understanding the intracellular pathway controlling cytoskeleton reorganization. The genes encoding for prostaglandin synthase-2 (*Ptgs2*), the rate-limiting enzyme of PG biosynthesis, and its EP_2 receptor (*Ptger2*) are highly expressed by CCs before ovulation [86,87]. Cumuli of *Ptger2* null mice are less expanded, and show resistance to hyaluronidase-induced disaggregation and sperm penetration, albeit they contain normal amount of HA [88,89]. The CCs show enhanced cortical actin polymerization, which results in rapid recruitment and clustering of $\alpha_1\beta_v$ integrin on cell membrane and an evident increased assembly of fibronectin fibrils in the pericellular matrix. This effect is due to enhanced activation of Ras homolog gene family member A (RhoA) and following activation of Rho-associated protein kinase (ROCK)/myosinII pathway likely mediated by over-production of chemokines [90]. Therefore, in physiological conditions, PGE_2 generated cAMP dependent signaling negatively regulates RhoA activation to maintain an appropriate pericellular matrix to allow full expansion. In addition, maintenance of high intracellular cAMP level inhibits the release of HA from fully expanded COCs, stabilizing them for days. Although the mechanisms have yet to be clarified, it seems that RhoA/ROCK inhibition is involved also in this cAMP action [21].

Altogether, these findings show that the formation of the mucoelastic ECM in the COC is achieved by the coordination between HA deposition and cytoskeleton-mediated rearrangement of adhesive molecules in the pericellular matrix, and cumulus cell detachment and motility. Interestingly, an interplay between HA and fibronectin fibrillar matrix has been recently reported and showed to induce the myofibroblastic phenotype in inflammation, possibly by altering ECM swelling pressure, stiffness or viscosity [91].

Tenascin C (TNC), a hexameric matrix glycoprotein which modulates cellular functions during tissue remodeling, is also strongly upregulated in the mouse COC approaching ovulation, and localizes to specific regions of the cumulus cell surface [92]. The function of this protein in the context of cumulus ECM organization has not been investigated yet, although it could have a relevant role. In the human cumulus, TNC and fibronectin are differently expressed, with the former mostly present in the innermost layer of cells and the latter associated to the cells scattered in the cumulus mass [80,93]. TNC is well known to inhibit fibronectin-mediated adhesion by binding to fibronectin and preventing its binding to the proteoglycan syndecan 4 [94,95]. Noteworthy, the expression of syndecan 4 by CCs parallels that of TNC [20].

Ultrastructural analysis of ovulated COCs suggests that the HA-protein network is attached to cytoplasmic projections of CCs [5]. However, the relevance of direct binding of HA to specific receptors is still unclear. The major HA receptors CD44 and RHAMM (Receptor of HA Mediated Mobility) are expressed by cumulus cells [92,96–98] and could elicit multiple intracellular signals leading to anchorage-independent cell growth [99,100]. Nevertheless, CD44 deficient mice have normal fertility [101,102] and deletion of the RHAMM gene produces hypofertility [103], which, however, has been recently associated to altered proliferation of granulosa cells rather than cumulus ECM alteration and oocyte fertilization failure [104].

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Impact of ECM organization on cumulus function

Several lines of evidence indicate that molecules synthesized by the CCs and/or the oocyte attract the sperm [105,106]. Therefore, it is intuitive that the central position of the oocyte and the higher cumulus cell density around the germ cell are essential prerequisites for creating a chemoattractant gradient. Regional difference in COC HA organization seems to play an important role in this cell spatial distribution. HA staining of ovarian sections with a specific probe clearly shows that HA is present throughout the COC matrix, but it forms filaments at the periphery, extending into the follicular fluid, while, it shows a more compact structure between the inner layers [19]. Differences in relative concentrations of PTX3, HCs and TSG-6 throughout the cumulus could account, at least in part, for such peculiar HA organization. It has been shown that PTX3 deposition is spatiotemporal regulated in the COC with the most precocious and abundant accumulation in the matrix among corona radiata cells, while the HCs appeared more uniformly distributed throughout the cumulus mass [40] (Fig. 1). Likely, this depends by the fact that PTX3 is exclusively produced by the CCs under oocyte influence [26,27], while TSG-6, which catalyzes the HC transfer from IαI to HA, is expressed by cumulus as well as mural granulosa cells [26,39,46], and the latter release TSG-6 in the follicular fluid [39,107,108] Thus, it is reasonable to hypothesize that at the periphery of the cumulus, HA strands are loosely organized for the predominance of weak HC-HA or HC-HC interactions while a greater aggregation occurs in the inner layers for the abundance of PTX3 and the formation of "nodal" crosslinking generated by the HC-PTX3-HC. In agreement, in TSG-6 and bikunin null mice, that cannot form either types of interactions (HC-HC and HC-PTX3-HC), CCs mostly disaggregate within the follicle deeply impairing ovulation [24]. Conversely, in PTX3 null mice ovulation occurs normally because a matrix is formed, although loose and unstable, and corona radiata cells are disorganized and the oocytes randomly located in the cumulus mass, a condition sufficient for preventing fertilization by the sperm [27].

Mechanical analyses by colloidal-probe atomic force microscopy confirmed that the cumulus ECM is effectively a hydrogel stably cross-linked throughout [109]. It also revealed that its mechanical properties are heterogeneous, in agreement with the hypothesized regional difference in the HA cross-linking. The most outer layer displayed a unique combination of elasticity and extreme softness below 1 Pa, which is 2–3 order of magnitude smaller than in the inner layer, as indicated by a quantitative stretch analyses of COC in rabbit [110]. The estimated mesh size formed by the HA network is in the order of a few hundred of nanometers suggesting that physical constraints are probably of minor importance for the distribution of signaling molecules through the cumulus ECM [109]. Therefore, heterogeneities in matrix composition in different regions of the COC might also serve to establish an immobilized gradient of factors affecting sperm maturation and mobility during its progression toward the egg.

In the oviduct, only vigorously moving sperm can enter the cumulus mass. The surface of the cumulus appeared to be quite resistant to spermatozoa entry and then penetration is slowly completed [111]. Once inside, the sperm continues to move, alternating between turning and swimming straight ahead, a progression typical of chemoattractant response. Electron microscopy analyses show an intimate association of the ECM with the head and tail plasma membrane [5]. No obvious degradation of the ECM is observed and fertility of male mice deficient for Sperm Adhesion Molecule 1 and hyaluronidase 5, two hyaluronidases acting at neutral pH, questioned the relevance of HA degradation in sperm penetration of the cumulus [112]. However, recent findings suggest that a limited and localized digestion of HA may occur during sperm swimming through the action of acid-active hyaluronidase 2 and its coactivator CD44 present on the sperm surface [113], similarly to what reported for tumor cells [114]. Indeed, it has been shown that low molecular weight HA can activate the Toll-like receptor 2 and 4 on CCs and induce the production of chemokines that increase sperm motility and fertilization [115]. In addition, sperm is able to respond to rheological changes of the environment, for example sensing and swimming against the oviductal fluid flow for reaching the ampulla [116,117]. Therefore, mechanically gated channels activated by forces imposed on the plasma membrane by local viscoelasticity of the COC matrix could contribute to control the flagellar bending and consequently the swimming mode.

In conclusion, the combined action of multiple factors, including local HA degradation, chemoattractant gradients and mechanosensory modulation, may contribute to determine the behavior of the sperm in the cumulus ECM in order to achieve oocyte fertilization.

New vision and perspectives

The studies conducted in the last 20 years have made great progress in understanding the biochemical and rheological characteristics of the cumulus matrix. However, information on its molecular composition is still fragmentary and a systematic study of the expression of matrix molecules is essential to fully understand its function. It is now well established that spermatozoa cannot find the egg in the oviduct without the help of the cumulus oophorus.

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However, for doing this, they must have acquired all the molecular machinery necessary to respond to biochemical and mechanical signals that allow them efficiently cross the matrix and reach the oocyte. The evidence that the essential components so far identified in the mouse cumulus ECM, such as PTX3 and IαI-HCs, are also present in the human cumulus ECM makes these studies relevant for clinical purposes. Male infertility is increasing in the population and any efforts have to be made to develop more efficient technique to select gold sperm in programs of assisted reproduction technology. The multiple interactions reported in this review between the spermatozoa and the cumulus suggest the possibility that biomimetic HA gel functionalized with structural and biochemical components of the cumulus matrix could constitute an innovative method for sperm selection, mimicking the physiological conditions.

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Abbreviations used:

ADAMTS, disintegrin and metalloproteinase with thrombospondin motifs; BM, basement membrane; COC, cumulus cell-oocyte complex; ECM, extracellular matrix; EGF, epidermal growth factor; EP_2, prostaglandin E_2 receptor subtype 2; FSH, follicular stimulating hormone; HA, hyaluronan; HC, heavy chain; HAS2, hyaluronan synthase 2; HS, heparan sulfate; hCG, human chorionic gonadotropin; HSPG, heparan sulfate proteoglycan; IαI, inter-α-trypsin inhibitor; LH, luteinizing hormone; PGE_2, prostaglandin E_2; PTX3, pentraxin 3; RhoA, Ras homolog gene family, member A; TSG-6, tumor necrosis factor alpha-induced protein 6; TNC, tenascin C.

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