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Unique hyaluronan structure of expanded oocyte-cumulus extracellular matrix in ovarian follicles

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In preovulatory follicles, after the endogenous gonadotropin surge, the oocyte-cumulus complexes (OCCs) produce hyaluronan (HA) in a process called "cumulus expansion". During this process, the heavy chains (HCs) of the serum-derived inter-alpha-trypsin inhibitor (IαI) family bind covalently to synthesized HA and form a unique structure of the expanded cumulus HA-rich extracellular matrix. Understanding the biochemical mechanism of the covalent linkage between HA and the HCs of the IαI family is one of the most significant discoveries in reproductive biology, since it explains basis of the cumulus expansion process running in parallel with the oocyte maturation, both essential for ovulation. Two recent studies have supported the above-mentioned findings: in the first, seven components of the extracellular matrix were detected by proteomic, evolutionary, and experimental analyses, and in the second, the essential role of serum in the process of cumulus expansion in vitro was confirmed. We have previously demonstrated the formation of unique structure of the covalent linkage of HA to HCs of IαI in the expanded gonadotropin-stimulated OCC, as well as interactions with several proteins produced by the cumulus cells: tumor necrosis factor-alpha-induced protein 6, pentraxin 3, and versican. Importantly, deletion of these genes in the mice produces female infertility due to defects in the oocyte-cumulus structure.

Keywords: cumulus expansion, extracellular matrix, hyaluronan, inter-alpha-trypsin inhibitor

Mammalian ovulation occurs in female reproductive cycle and is triggered by a surge of pituitary luteinizing hormone (LH). In preovulatory follicles, oocytes are surrounded by numerous layers of cumulus cells forming the cumulus cell-oocyte complex (OCC). In 2005, Richards has proposed a schematic model explaining LH signaling cascades, the matrix components being induced and associated factors being essential components of the expanded cumulus extracellular matrix (ECM) that is required for the release of the OCC into the oviduct during ovulation (Richards 2005). However, to better understand ovulation, which represents the essential requisite for fertilization and subsequent embryonic development, we have to move almost 50 years backwards.

In 1976, Hillensjo et al. (1976) have described the basic morphological changes occurring in the maturing oocyte, such as the resumption of meiosis, germinal vesicle breakdown, and polar body formation. They have shown close functional relationship between the two cell types of the OCC as a response to stimulation with gonadotropins, and have described a principal cumulus structure created by expanded cumulus cells surrounding the oocyte embedded in a viscous matrix. Other authors have clarified how the timing of the cumulus expansion coincided with the oocyte maturation (Dekel et al. 1979; Schuetz and Swartz 1979) and have defined the general conditions for the gonadotropin-stimulated cumulus expansion *in vitro* in mice (Eppig

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1979a). In addition, Eppig (1979b) has demonstrated gonadotropin-stimulated hyaluronan (HA) synthesis by OCCs isolated from mouse preovulatory follicles and emphasized that the presence of the fetal bovine serum in the culture medium is required for retaining HA within the OCC, since in the absence of serum, HA was released into the medium. Thus, the essential role of serum in the process of the cumulus expansion *in vitro* was to promote the retention of HA within the OCC (Eppig 1980).

New evidence supporting the essential role of serum in the cumulus expansion process

In 2023, new evidence strongly supporting the essential role of serum in the cumulus expansion process *in vitro* has been published by Goetten et al. (2023). The authors have revealed different profiles of key genes involved in the mechanism of the double-stranded DNA (DSB) repair in bovine cumulus cells of OCCs matured in two different media, supplemented with either fetal calf serum or bovine serum albumin (BSA). These results showed for the first time that the expression of the key genes is involved in DSB repair mechanisms in cumulus cells. In fact, the higher mRNA expression of DNA repair-associated BRCA1 (BRCA1) and tumor suppressor P53-binding protein 1 (TP53BP1) and lower mRNA expression of tumor necrosis factor-alpha-induced protein 6 (TNFAIP6) demonstrated an increase in apoptosis rate and DNA damage in cumulus cells cultured in BSA-supplemented medium and explained the reduced developmental potential of the oocytes matured in the serum-free medium (Goetten et al. 2023).

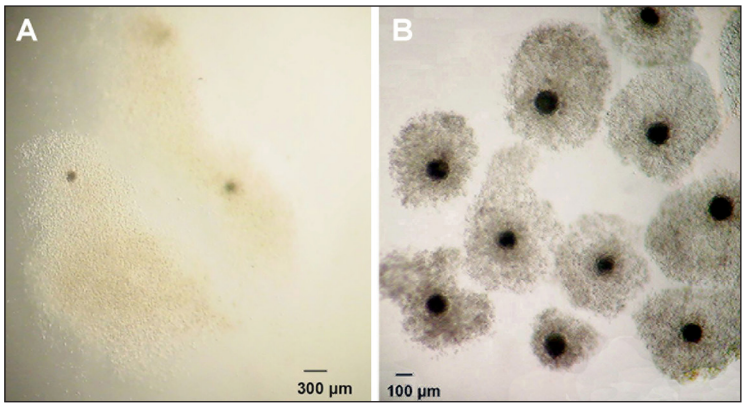

Figure 1. Cumulus expansion: **(A)** *in vivo* expanded (porcine) oocyte-cumulus complex (OCC) consisting of oocyte and granulosa/cumulus cells that form a large expanded viscous hyaluronan-rich structure of extracellular matrix and **(B)** *in vitro* expanded (porcine) OCC consisting of oocyte and cumulus cells that form the viscous hyaluronan-rich structure of the cumulus extracellular matrix.

How can the developmental competence of the oocyte be impaired?

The acquisition of the developmental competence of the oocyte involves the synthesis and storage of a wide range of molecules during the oocyte growth followed by the reprogramming and ordered utilization of these stored products during maturation, fertilization, and early embryogenesis. The regulatory signals for these molecular changes are produced by the follicular cells in response to circulating levels of gonadotropins, as suggested by Moor et al. (1998). These authors have reported that some ovarian stimulation protocols distort these regulatory signals, thereby leading to disruption of the molecular reprogramming of the oocyte and reduction of subsequent developmental competence. Indeed, the production of a viable egg depends on the synthesis and storage of mRNAs and proteins during oocyte growth along with the mobilization and reorganization of these molecules in the final few hours before releasing the oocyte from the follicle during ovulation. These authors have also stressed that the viability of the oocytes matured *in vitro* depends on a wide range of endocrine and paracrine signals being present not only at the appropriate time, but also at the sufficient concentrations, and the correct form. The failure to meet these demanding prerequisites is the reason for most of the developmental failures associated with the impaired maturation of oocytes under *in vitro* conditions (Moor et al. 1998).

The association of the developmental failure with infertility has been demonstrated in mice with deletions of the cumulus expansion-related genes, such as TNFAIP6 (also called TSG-6) (Fulop et al. 2003), pentraxin 3 (PTX3) (Varani et al. 2022), and bikunin (a light chain essential for the synthesis of inter-alpha-trypsin inhibitor (IαI) family, known as a serum-derived factor) (Zhuo and Kimata 2001; Suzuki et al. 2004), and also versican as well as HA synthase 2 (Toole et al. 2002). Since cumulus expansion and oocyte maturation are central processes in ovulation, and the newly organized structure of the cumulus ECM in the OCC (Figure 1) is essential for successful ovulation and fertilization (Russell and Salustri 2006; Yerushalmi et al. 2014; Nagyova 2018), we will further discuss the formation of the unique structure of covalent linkage of HA to heavy chains (HCs) of IαI in the expanded gonadotropin-stimulated OCC (Figure 2).

Biochemical basis of a newly formed structure: involvement of the covalent linkage between hyaluronan and heavy chains of inter-alpha-trypsin inhibitor family in the cumulus expansion process

To elucidate the structure and function of the covalent linkage between HA and HCs of the IαI family, Zhuo et al. (2004) have provided an excellent schematic model showing that the family of IαI molecules is synthesized in hepatocytes, where one or two HCs are linked to the chondroitin sulphate chain of bikunin in the Golgi apparatus. Upon an appropriate stimulus, the molecules are recruited to extravascular sites, where only the HCs are transferred into the locally synthesized HA to form the HCs-HA complexes (transesterification), which are involved in the formation of ECM by aggregating into a "cable-like structure" containing the complexes.

In 1992, Chen et al. (1992) have identified a serum factor (proteins of the IαI family) that was critical for organizing and stabilizing the expanding oocyte-cumulus matrix. Later, it has been shown that the direct interaction between proteins of the

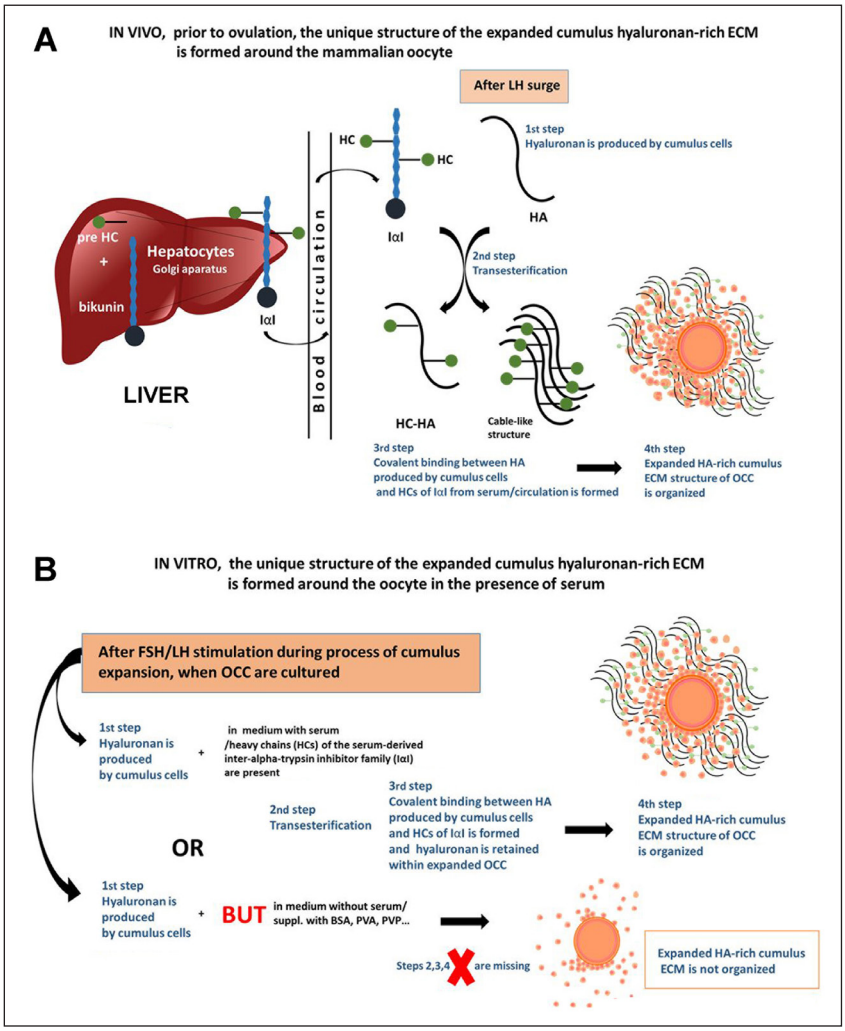

Figure 2. Modified schematic model explaining the process of cumulus expansion and formation of the unique structure of the expanded cumulus hyaluronan (HA)-rich extracellular matrix (ECM). **(A)** *In vivo*, in preovulatory follicles, after the endogenous gonadotropin surge, the oocyte-cumulus complex (OCC) synthesizes HA in a process called "cumulus expansion". During this process, the heavy chains (HCs) of the serum-derived inter-alpha-trypsin inhibitor family (IαI) bind covalently to HA and form the unique structure of the expanded cumulus HA-rich ECM. **(B)** *In vitro*, after gonadotropins stimulation, the OCC synthesizes HA during the cumulus expansion process. OCCs cultured in a medium supplemented with serum (in the presence of HCs of the serum-derived IαI family) form the unique structure of the expanded cumulus HA-rich ECM. In contrast, in the absence of serum, although the cumulus cells synthesize HA, it is not retained within the OCC and the cumulus ECM structure is missing.

IαI family and HA plays a major role in preventing the release of HA into the culture medium (Chen et al. 1994) and the majority of IαI within the ovulated cumulus ECM is covalently linked to HA (Chen et al. 1996). The IαI family members synthesized and assembled in the liver are secreted into the blood at a high concentration (0.5 mg/ml) (Mizon et al. 1996). The members of the IαI family are composed of a common light chain, bikunin, and one or two of the three genetically different HCs (HC1, HC2 and HC3) (Salier et al. 1996). These HCs are transferred onto the HA polysaccharide to form covalent HCs (of IαI)-HA complexes, thereby stabilizing the HA-rich cumulus ECM structure around the oocyte, as it has been detected in mice and porcine OCCs (Chen et al. 1994, 1996; Nagyova et al. 2004).

The new structure of the expanded cumulus ECM is formed after gonadotropin stimulation by the interaction of cumulus-produced HA with the serum-derived IαI (Nagyova et al. 2012) and also proteins produced by cumulus cells, such as TNFAIP6, PTX3 (Nagyova et al. 2004, 2008, 2016) and versican (McArthur et al. 2000; Russell et al. 2003; Rodgers and Irving-Rodgers 2010; Nagyova et al. 2020). Deletion of these corresponding genes in mice results in female infertility due to disorganization of the oocyte-cumulus ECM structure (Toole et al. 2002; Nagyova 2018). These findings show how HCs (of IαI)-HA binding contributes to the understanding of the cumulus expansion process in gonadotropin-stimulated OCC matured *in vivo* or *in vitro* (Nagyova et al. 2021), and how this binding forms the correct HA-rich expanded cumulus ECM structure and consequently regulates the developmental potential of oocytes (Nagyova 2015; Salustri et al. 2019).

Bikunin is essential for the biosynthesis of inter-alpha-trypsin inhibitor: its role in the reproductive physiology

The importance of bikunin in reproductive physiology has been demonstrated previously (Sato et al. 2001; Zhuo and Kimata 2001). Mice deficient in bikunin were infertile with abnormalities in ovulation due to the lack of expanded HA-rich cumulus ECM structure. The structure of cumulus ECM was not formed, since the linkage of HCs (of IαI)-HA during the process of cumulus expansion was abolished by the bikunin deletion. Bikunin is essential for the biosynthesis of IαI family proteins (Sato et al. 2001; Zhuo and Kimata 2001). To determine the consequences of bikunin deletion, Suzuki et al. (2004) have analyzed RNA isolated from ovaries of Bik–/– and wild-type female mice, and identified at least 34 bikunin target genes using cDNA microarray hybridization. These IαI-selective targets included genes involved in the regulation of signal transduction modifiers, cytokines, apoptosis, and ovulation-related, aging-associated, stress-related and neuroendocrine factors. We emphasize that Suzuki et al. (2004) have provided the full repertoire of endogenous IαI-regulated genes and showed how proteins of the IαI family regulate different events of reproductive physiology. The identification of these genes was essential not only for revealing the nature of all signaling pathways used by IαI, but also for the definition of the set of proteins that are induced or repressed by this glycosaminoglycan.

Extracellular matrix components detected by proteomic, evolutionary, and experimental analyses

The characterization of the new cumulus ECM structure is also supported by a recent study of Keeble et al. (2021). Through a combination of proteomic, evolutionary, and experimental analyses, the authors have detected a subset of seven OCC components: ITIH1, ITIH2, ITIH3, PTX3, TNFAIP6, versican (VCAN), and vitronectin (VTN) at high spectral counts. They have suggested that this subset of the OCC proteome might have evolved as a mechanism for females to modulate fertilization outcomes, since it is involved in the process of cumulus expansion and in the formation and stabilization of the expanded structure of HA-rich cumulus ECM. The results were obtained from the mouse OCCs through the proteomic experiments, which produced over 24,000 mass spectra and identified 711 proteins. Seven cumulus expansion-related components/proteins known to form the HA-rich expanded cumulus ECM structure were especially abundant. Through separate evolutionary analyses, they showed that three of these (ITIH1, ITIH3, and VTN) evolved rapidly as a classic signature of genes that influenced fertilization rate. In addition, they characterized the global OCC proteome through cell fractionation and identified a small set of highly abundant proteins involved in the ECM structure in the process of cumulus expansion, some of which, ITIH1, ITIH3, and VTN, exhibit signatures of recurrent adaptive evolution. IαI, HC1 (ITIH1) was the protein identified with the most spectra (identified with 1,368 spectra), with more than twice as many spectra as the second highest (ITIH3, identified with 683 spectra) (Keeble et al. 2021). This work provided two main insights. First, the above-mentioned seven

proteins are involved in the process of cumulus expansion by forming and stabilizing the structure of the HA-rich cumulus ECM and as a large portion of the cumulus ECM proteome acts to control access to eggs. Second, three of these proteins (ITIH1, ITIH3, and VTN) showed evidence of recurrent positive selection, a common signature among proteins that influence fertilization rates (Keeble et al. 2021). We and others have already shown that the products of ITIH genes; the HCs of the IαI family bind covalently to HA and form the expanding structure of cumulus HA-rich ECM during the process of oocyte maturation and cumulus expansion in mice and pigs (Chen et al. 1994, 1996; Nagyova et al. 2004).

Collectively, these studies have supported our proposal about the cumulus expansion, first in terms of the role of each domain of IαI, i.e. the HCs are directly related to the physiological function of IαI through transfer to HA, and their coupling to bikunin is crucial for the biosynthesis of IαI family proteins. Second, several different laboratories have reported key ECM proteins in expanded HA-rich oocyte-cumulus ECM structures: PTX3 (Salustri et al. 2004; Nagyova et al. 2016), TNFAIP6 (Mukhopadhyay et al. 2001; Fulop et al. 2003; Nagyova et al. 2008, 2009), and VCAN (Russell et al. 2003; Nagyova et al. 2020). These studies clearly demonstrated the essential cumulus ECM components that complete the process of cumulus expansion *in vivo* and *in vitro* essential for ovulation and fertilization (Russell and Salustri 2006; Nagyova 2018).

We emphasize that the size of the expanded area should not be considered the absolute proof of the proper cumulus expansion. Although it is a measurable parameter, it is not the exclusive indicator for the evaluation of cumulus expansion, since it fails to provide complete evidence about the quality of the newly formed expanded cumulus ECM structure. In addition to the area size, it is relatively simple to identify whether the OCC, either obtained *in vivo* or after the stimulation in the culture, is fully expanded. When using mechanical manipulation of the individual OCC (repetitive passing of the expanded OCC into and out of the pipette tip), the fully expanded OCC will not lose any of the cumulus cells, since these are embedded with the sticky viscous matrix structure resulting from the biochemical interactions described above.

Conclusions and future perspectives

In conclusion, we demonstrate that understanding the biochemical mechanism of the covalent linkage between HA and the HCs of the IαI family is one of the most significant discoveries in reproductive biology, since it explains the basis of the cumulus expansion process running in parallel with oocyte maturation, both essential for ovulation. The OCC formation of a new ECM structure is associated with the oocyte maturation after gonadotropin hormone stimulation and with not only morphological, but also marked biochemical and physiological changes in the cumulus cells preceding ovulation. The cumulus expansion process, characterized by the formation of the HA-rich expanded cumulus ECM structure, is strongly correlated in time with the induction of the meiotic maturation process (Nagyova 2015, 2018; Salustri et al. 2019). Strong support was provided by Hess et al. (1999), who have shown that the developmental potential and viability of oocytes were compromised during the process of fertilization and early cleavage when the oocyte was surrounded by a destabilized and morphologically abnormal cumulus mass. This confirms that the binding of IαI to HA in the cumulus ECM is required for optimal ovulation and development of oocytes (Hess et al. 1999).

According to Anthony Day, President of the International Society for Hyaluronan Sciences, understanding the biochemical mechanism of the covalent linkage between HA and HCs of IαI is the major finding in HA biochemistry (Briggs et al. 2020; ISHAS 2024).

From our point of view, it is also one of the most significant discoveries of the last thirty years in reproductive biology, since it explains the biochemical mechanisms of the cumulus expansion process running in parallel with the oocyte maturation, both essential for ovulation. Elucidation of the biochemical mechanism of the covalent linkage between HA and HCs of IαI as well as the significance of the cumulus ECM structure has been well established (at least in mouse and porcine OCC). However, still much more remains to be learned about the interactions and functions of HA, TNFAIP6, PTX3 and versican in the preovulatory follicles.

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