doi:10.4149/endo_2015_01_37

Organization of the expanded cumulus-extracellular matrix in preovulatory follicles: a role for inter-alpha-trypsin inhibitor

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It has been shown that following endogenous gonadotropin surge, oocyte-cumulus complexes (OCC) synthesize hyaluronan (HA) in a process called cumulus expansion. During this process, HA associates with proteins and proteoglycans to form the expanded HA-rich oocyte-cumulus extracellular matrix (ECM), where the heavy chains of the serum derived inter-α-trypsin inhibitor family (IαI) bind covalently to HA. No study has been performed on the occurrence and regulation of this process during oocyte maturation in species other than mouse and pig, although, the heavy chains (of IαI)-HA complex was purified from human amniotic membrane. The present review pointing out that: 1/ formation of expanded HA-rich oocyte-cumulus ECM is dependent on the presence of IαI molecules, 2/ the heavy chains of IαI molecules identified in the serum are covalently linked to HA during cumulus expansion in mouse and pig, 3/ the family of IαI molecules can freely cross the blood-follicle barrier, and the follicular fluid collected at any stage of folliculogenesis can be successfully used instead of serum to form expanded cumulus ECM in pig, and 4/ proteins of the IαI family can affect reproductive process by modulating the expression of a large number of cellular genes during a preovulatory period. Finally, this review provides clear evidence that IαI family members present in the serum or follicular fluid become responsible for cumulus expansion, as without these proteins, expanded cumulus HA-rich ECM is not formed and HA is released into medium.

Key words: cumulus expansion, cumulus-extracellular matrix, hyaluronan, inter-α-trypsin inhibitor

In most mammals, the surge of luteinizing hormone (LH) stimulates the ovulatory response in Graafian follicles. This includes the resumption of meiotic maturation, differentiation of the mural granulosa cells, reprograming of their proteins and steroidogenic activity, expansion of the cumulus cells surrounding oocytes, rupture of the follicle wall, and release of the fertilizable ovum (Tsafriri and Reich 1999). *In vivo*, following endogenous gonadotropin surge or administration of an ovulatory dose of hCG, cumulus cells synthesize HA-rich ECM, a process called cumulus expansion or mucification. It has been shown previously that serum has a critical role in mouse cumulus expansion by promoting the retention of HA synthesized de novo by cumulus cells in response to FSH (Eppig 1979, 1980). In porcine OCC, follicle-stimulating hormone (FSH) pretreatment strongly induces an epidermal growth factor (EGF) response within 3 h, as evidenced by an increase in HA production and cumulus expansion (Prochazka et al. 2003). In mouse, EGF-like factors induced by LH (Park et al. 2004; Conti et al. 2006) or FSH (Downs and Chen 2008) are sufficient to induce cumulus expansion and oocyte maturation. The role of serum and HA in mouse OCC has been described in details by Camaioni et al. (1993). In gonadotropin-stimulated porcine OCC cultured in medium supplemented with or without serum, the role of serum and HA incorporated into the expanded cumulus ECM was described by Nagyova et al. (1999, 2012). Treatment of FSH/LH-stimulated porcine OCC with proteasome inhibitor MG132 prevented

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cumulus expansion and covalent binding between HA and heavy chains of inter-α-trypsin (IαI). In addition, MG132 arrested >90 % of oocytes at germinal vesicle stage and blocked degradation of F-actin-rich transzonal projections interconnecting cumulus cells with oocyte (Yi et al. 2008; Nagyova et al. 2011). It suggests that proteasomal activity has multiple functions in cumulus expansion. In contrast, treatment of mouse expanded OCC with protease resulted in a loss of OCC integrity and cumulus dissociation into individual HA-filaments (Cherr et al. 1990). It suggests that some HA-binding proteins are required to organize HA in the matrix. In agreement with these findings, it has been shown that proteins, both derived from the serum (IαI) and synthesized by granulosa cells after gonadotropin stimulation, are involved in organizing of HA within the cumulus ECM in mouse and porcine preovulatory follicles (Camaioni et al. 1993; Chen et al. 1994; Nagyova et al. 2004). Despite the importance assigned to serum proteins of IαI family to HA, and although the heavy chains (of IαI)-HA complex was purified from human amniotic membrane (He et al. 2009), no study has been performed on the occurrence and regulation of this process during oocyte maturation in preovulatory follicles in species other than mouse (Chen et al. 1992; Zhuo et al. 2001) and pig (Nagyova et al. 2004). This review describes the essential role of the IαI family proteins in the process of HA incorporation into the cumulus ECM (mouse and porcine) emphasizing that cumulus expansion is the well-organized process, which is strongly dependent on a specific cascade of intracellular signals inducing gene expression and formation of HA-rich cumulus ECM.

I. Mouse model

Cumulus expansion after FSH stimulation in serum supplemented medium

Mouse OCC treated *in vitro* with FSH in the presence of fetal calf serum undergoes expansion (Eppig 1979). However, when FSH-stimulated OCC are cultured in the absence of serum, the mucified-expanded matrix is not formed and cumulus cells lose contact from each other, and finally they are attached on the bottom of the culture dish. Although cumulus cells synthesize HA in the absence of serum, most of the produced HA is released into the culture medium (Eppig 1980). Several studies confirmed that serum factors must be present while HA is being synthesized in order to organize it into the matrix (Eppig 1980; Salustri et al. 1989; Camaioni et al. 1993). The effects of fetal bovine serum (FBS), exogenous HA and HA-oligomers on the expansion process were systematically investigated by Camaioni et al. (1993). It has been demonstrated that when 1% FBS is continuously present during the first 18 h of mouse OCC culture, the maximum retention of HA in the matrix and complete OCC expansion occurs. These findings support the hypothesis that the serum factor identified as IαI protein is the essential structural component of the cumulus ECM (Chen et al. 1992).

Inter-α-trypsin inhibitor (IαI) in mouse serum and follicular fluid

The serum components belonging to IαI family proteins consist of a small protein, named bikunin or light chain, with a chondroitin sulfate moiety that contains one or two evolutionarily related proteins, named heavy chains (HC1, HC2, and HC3). Proteins of IαI family carry two heavy chains, HC1 and HC2, while pre-α-inhibitor (PαI) and inter-α-like inhibitor (IαLI) have one heavy chain, HC3 and HC2, respectively (Salier et al. 1996; Zhuo et al. 2004). These IαI-related molecules are assembled in the Golgi apparatus of hepatocytes circulating in the blood. It has been proposed that the covalent linkage of heavy chains to HA is critical for cross-linking HA strands and stabilizing cumulus ECM in mouse (Chen et al. 1996). Serum active fractions were determined by their ability to stabilize the cumulus ECM in a bioassay system (Chen et al. 1992). The data of Western blot analysis using anti-human IαI IgG have shown that the antibody reacts positively with the purified serum factor, and immunodepletion of FBS with anti-IαI antibody eliminated its ability to stabilize the ECM. This factor has been also found in mouse follicular fluid collected 6 h following hCG injection to stimulate ovulation, but not in unstimulated mice. Anti-IαI-positive epitopes were also localized within the cumulus ECM of mouse preovulatory follicles supporting the possibility that this molecule or molecules may diffuse into follicular fluid after an ovulatory stimulus to act as structural linkers that ensure normal cumulus expansion. In agreement with this finding, it has been suggested by Camaioni et al. (1993) that cooperative binding to HA of either the serum factor, an endogenously synthesized factor(s), or both is required to stabilize the fully expanded ECM. Moreover, Camaioni et al. (1993) demonstrated that fully expanded OCC

disassemble when cultured longer than 18 h, a process which occurs also *in vivo* and which correlates with a loss of ability of oocyte to be fertilized both *in vivo* and *in vitro*. This process involves release of macromolecular HA from the matrix into the medium, with loss of 50% of the HA in the first 8 h of incubation after full expansion. Chen et al. (1994) studied the mechanism of interaction of HA with the cumulus ECM stabilizing factor and found that this factor stabilizes the expanding cumulus ECM by direct binding with HA, indicating that it may serve as a structural protein to organize the formation of the cumulus ECM. In the next study (Chen et al. 1996), it was documented that despite similarities in the morphology of mouse OCC expanding *in vivo* and *in vitro*, the OCCs which expand within intact follicles are more elastic and resistant to shear stress than the OCC expanded and stabilized *in vitro*. The Western blot analysis of mouse OCC demonstrated that only the heavy chains of IαI are incorporated into the cumulus ECM and appear to be covalently linked to HA after stabilization *in vivo*. In *in vitro* stabilized OCC, intact IαI is bound to the HA-rich cumulus ECM by a non-covalent mechanism. Nevertheless, Chen et al. (1994) have shown that HA and purified pre-α-inhibitor can form covalent linkage in the presence of granulosa cells or with granulosa cell-conditioned medium. The authors proposed that a factor(s) secreted by granulosa cells within the follicle may catalyze a transesterification reaction resulting in an exchange of chondroitin sulfate with HA at the heavy chain/chondroitin sulfate junction followed by release of chondroitin sulfate-bikunin into the follicular fluid. In addition, it has been suggested that the covalent linkage of heavy chains to HA is critical for cross-linking HA strands and stabilizing cumulus matrix in mouse (Chen et al. 1996).

Tumor necrosis factor-alpha-induced protein 6 (Tnfaip6) in mouse preovulatory follicles

In 1997, Fulop et al. revealed that granulosa cells within the mouse follicle secret factor(s) tumor necrosis factor alpha-induced protein 6 (Tnfaip6), also called tumor stimulating gene 6 (Tsg6), since *Tnfaip6* mRNA is specifically expressed by expanded complexes after hCG stimulation *in vivo*. They suggested that this protein may have a structural role in the expanded cumulus ECM matrix since TNFAIP6 binds to HA and interacts with IαI. In addition, Fulop et al. (1997) reported the complete coding sequence for the mouse TNFAIP6 protein and found that this gene has been localized to the murine chromosome 2. Mukhopadhyay et al. (2001) reported that after the LH-surge, a HA-binding protein, the translated product of Tnfaip6, is specifically accumulated in the expanded viscoelastic cumulus ECM. The *Tnfaip6* mRNA expression is quickly up-regulated and as assessed by quantitative RT-PCR, peaks at approximately 1500 copies/cell 4 h after the ovulatory stimuli. The localization of the TNFAIP6 protein and HA around the cumulus and granulosa cells was confirmed by immunohistochemistry (Carrette et al. 2001). Finally, data of Western blot analysis demonstrated that the TNFAIP6 protein exists in two distinct populations in the cumulus ECM. One population is a monomer (~ 35 kDa) that is anchored to the matrix by a non-covalent interaction. The second population (~ 120 kDa) is a covalent complex with either of the heavy chains of IαI proteins and is bound to HA through a strong interaction that is resistant to denaturation.

Bikunin knockout female mice

The family of IαI proteins, beside heavy chains, consist of a small protein, named bikunin or light chain. It has been established (Zhuo et al. 2001; Suzuki et al. 2004) that the bikunin knockout female mice display a severe reduction in fertility and forming the cumulus HA-rich ECM. Zhuo et al. (2001) have proposed that the serum-derived HA-associated proteins complex is a dominant component of the *in vivo* HA-rich cumulus ECM. The authors abolished the formation of the serum-derived HA-associated proteins complex in mice by targeting the gene of bikunin, which is essential for their biosynthesis. As a consequence, the OCC had a defect in forming the ECM during oocyte maturation and expansion. The ovulated oocytes were completely devoid of matrix and females were infertile. However, intraperitoneal administration of IαI, accompanied by the formation of the serum-derived HA-associated protein complex, fully rescued these matrix defects. It has been concluded that: 1/ mice lacking intact IαI family members fail to form a stable cumulus matrix and the naked ovulated oocytes are not able to be fertilized *in vivo*; 2/ organization of HA-enriched cumulus ECM does not occur with IαI immunodepleted serum while it does in the presence of purified IαI molecules; 3/ finally, *in vitro* FSH-induced mouse OCC requires the presence of serum (or follicular fluid) to incorporate the newly synthesized HA within the cumulus ECM. Suzuki et al. (2004) conducted a cDNA microarray hybridization screening using mRNA from ovaries of wild-type

or bikunin (-/-) female mice for identifying the full repertoire of the IαI deficiency-related genes from the bikunin-knockout female mice. Their screening identified that 29 (0.7%) and 5 genes (0.1%) of the genes assayed were, up- and down-regulated two-fold or more, respectively. The identified genes include stress-related, apoptosis-related, proteases, signaling molecules, aging-related, cytokines, hyaluronan metabolism and signaling, reactive oxygen species-related, and retinoid metabolism. In conclusion, the authors suggested that proteins of the IαI family have additional global effects on reproductive biology by modulating expression of a large number of cellular genes during a preovulatory period (Suzuki et al. 2004).

II. Porcine model

Cumulus expansion after gonadotropin stimulation in serum- or follicular fluid-supplemented medium

To investigate the organization of cumulus ECM in preovulatory follicles in other species, porcine OCC were cultured in serum- or follicular fluid-supplemented medium. In this supplemented medium, FSH- or FSH/LH-stimulated full expansion of intact OCC after 24 h of culture was observed (Nagyova et al. 2004; Yi et al. 2008; Nagyova et al. 2011). Hyaluronan is a glycosaminoglycan with high molecular weight and large hydrodynamic domains in the mouse cumulus ECM (Salustri et al. 2000). The specificity of incorporation of [3H]-glucosamine into the HA in porcine cumulus ECM was confirmed by the sensitivity to highly specific Streptomyces hyaluronidase (Nagyova et al. 1999). In the absence of serum, when PVP (3 mg/ml) was added to the culture medium, total HA accumulation was reduced by 35%. In addition, the retention of HA within the complexes decreased to less than 40% of the amount retained within the complexes cultured in the presence of serum. These experiments, in which serum was replaced with PVP, suggest that porcine cumulus cells are still capable of responding to gonadotropin and synthesizing HA, but the HA levels generated are reduced, both total and with respect to retention within the complexes (Nagyova et al. 1999). The data with porcine OCC are in agreement with earlier studies with mouse OCC, which showed that in the presence of serum, HA was retained within the complexes, but in the absence of serum it was released into the culture medium (Eppig 1980; Salustri et al. 1989; Camaioni et al. 1993).

Inter-α-trypsin inhibitor (IαI) in porcine serum and follicular fluid

As indicated previously, Chen et al. (1992) identified the factor that stabilizes the mouse cumulus ECM in FBS and in follicular fluid collected 6 h following hCG injection, but not in unstimulated mice. The family of IαI proteins was detected in porcine serum (Sigma-Aldrich, Prague, Czech Republic) and porcine follicular fluids collected at different stages of folliculogenesis using Western blot analysis (Nagyova et al. 2004). Our results have shown three major bands recognized by the IαI antibody in follicular fluid aspirated from medium-sized follicles that migrate at the relative position of IαI, PαI, and IαLI present in porcine serum. The identification of these proteins as IαI family members is supported by the evidence that they disappear in follicular fluid digested with Chondroitinase ABC and give rise to proteins migrating at 95 kDa and 75-85 kDa, corresponding to the molecular weight of free heavy chains: -HC3 and -HC1/HC2, respectively. Digestion of follicular fluid with Streptomyces hyaluronidase had no effect on the immunoreactive profile. The levels of IαI molecules in porcine follicular fluid changed neither in eCG-primed follicles nor in 8 h hCG stimulated follicles, while a detectable increase of concentration was observed at 24 h post-hCG injection. The results indicate that in pig, there is no apparent barrier to the transfer of IαI family molecules from the blood to the follicle and that LH/hCG only facilitates their diffusion (Nagyova et al. 2004). This observation is in agreement with the ability of porcine follicular fluid from medium-sized antral follicles to support full expansion of *in vitro* gonadotropin-stimulated OCC in the absence of serum (Kimura et al. 2002). Nevertheless, it is apparently in contrast with studies, which reported that serum IαI-related molecules are excluded from mouse antral follicles until an ovulatory dose of LH or hCG changes the permeability of the blood follicle barrier (Powers et al. 1995; Hess et al. 1998), implying that hormonal control of blood-follicle barrier might be critical for successful cumulus expansion.

Heavy chains of IαI molecules are covalently linked to HA in OCC expanded *in vivo*

Chen et al. (1994) demonstrated that heavy chains of IαI are incorporated into the cumulus ECM and are covalently linked to HA after stabilization *in vivo*. Zhuo et al. (2001) demonstrated that serum-derived

hyaluronan-associated protein complex is the dominant component of the HA-rich ECM of the mouse OCC. To determine whether in porcine complexes HCs of serum-derived IαI-related molecules are covalently linked to HA, OCC were isolated from antral follicles of gilts treated with eCG (unexpanded complexes) or eCG followed by hCG for 24 h (expanded complexes) (Nagyova et al. 2004, 2014; Yi et al. 2008). The results showed that unexpanded porcine OCC did not contain any protein reacting with IαI antibody. However, expanded OCC contained positive bands of about 220, 130, and 120 kDa, which were detected also in porcine serum, likely corresponding to IαI (bikunin plus HC1 and HC2), PαI (bikunin plus HC3), and IαLI (bikunin plus HC2), respectively (Rouet et al. 1992; Carrette et al. 1997). After digestion with hyaluronidase, two additional immunopositive bands of about 75-85 kDa and 95 kDa were detected in the extract of OCC, the former likely corresponding to the relative molecular mass of single HC1 and HC2, and the latter to that of a single HC3 (Salier et al. 1996; Flahaut et al. 1998). The obtained results clearly indicate that heavy chains from each IαI-related molecule identified in the porcine serum were transferred and covalently linked to HA during *in vivo* cumulus expansion. Analysis of the matrix and cell extracts confirmed that the immunoreactivity was exclusively associated with matrix proteins (Nagyova et al. 2004; 2014; Yi et al. 2008).

Heavy chains of IαI molecules are covalently linked to HA in OCC expanded *in vitro*

To find out whether heavy chains of IαI molecules are covalently transferred to HA during *in vitro* induced expansion, FSH-stimulated (Nagyova et al. 2004) or FSH/LH-stimulated (Yi et al. 2008; Nagyova et al. 2014) OCC were cultured in the presence of porcine serum or follicular fluid. Using IαI antibody, Western blot analysis of protein extracts of OCC cultured under both culture conditions revealed a positive 75-85 kDa material, corresponding to free heavy chains, that was fully associated with the matrix and released after hyaluronidase digestion, as in OCC expanded *in vivo*. In conclusion, our study provide clear evidence that heavy chains of IαI are covalently linked to HA in porcine OCC expanded *in vivo* and *in vitro*, thereby directly participating to the formation of cumulus HA rich-ECM (Nagyova et al. 2004; Yi et al. 2008; Nagyova et al. 2014). We also showed that IαI molecules can freely cross the blood-follicle barrier and that follicular fluid collected at any stage of folliculogenesis can be successfully used in formation of expanded cumulus ECM (Nagyova et al. 2004).

Tumor necrosis factor-alpha-induced protein 6 (TNFAIP6) in porcine preovulatory follicles

Fulop et al. (1997) suggested that Tnfaip6 (or Tsg6) may have a structural role in the expanded mouse cumulus ECM since TNFAIP6 binds to HA and interacts with IαI. Additionally, it has been shown that TNFAIP6 is produced by granulosa cells after an ovulatory stimulus in different species, in rat (Yoshioka et al. 2000), mouse (Carrette et al. 2001; Mukhopadyay et al. 2001), equine (Sayasith et al. 2007), bovine (Sayasith et al. 2008), and pig (Nagyova et al. 2008, 2009). Moreover, in OCC of Tnfaip6-null mice, covalent transfer of heavy chains of IαI to HA do not occur indicating that Tnfaip6 is critically involved in this process (Fulop et al. 2003). To find whether TNFAIP6 is involved in organization of porcine cumulus ECM, the expression of TNFAIP6 protein was investigated by Western blot analysis (Nagyova et al. 2008). Naturally expanding OCC from preovulatory follicles of cycling gilts or OCC expanded after *in vitro* culture (24 or 42 h) in medium supplemented with FSH and porcine serum were collected. The complexes were treated with Streptomyces hyaluronidase or Chondroitinase ABC. Matrix, cell pellet and total extracts were analyzed by Western blot. A band of about 35 kDa and a complex of about 120 kDa, corresponding to the molecular weights of the native and heavy chain-linked form of TNFAIP6, respectively, were detected by a rabbit anti-human TNFAIP6 polyclonal antibody in matrix extracts of expanded cumuli (Nagyova et al. 2008). For the first time, the presence of TNFAIP6 protein in porcine expanding OCC has been detected. It provided evidence that TNFAIP6 catalyzes the transfer of heavy chains to HA in porcine cumulus ECM since: 1/ a ~ 120 kDa TNFAIP6 species was revealed by Western blot in porcine (*in vivo* or *in vitro*) expanded cumulus ECM, which was also immunoreactive with anti-IαI antibodies; 2/ porcine follicular fluid collected after hCG injection promoted the HCs transfer to HA in a cell free system, where this activity was abolished by the anti-human TNFAIP6 monoclonal antibody A38. The direct evidence for a role of TNFAIP6 in porcine OCC was provided by stimulating porcine OCC with FSH in the presence of the A38 antibody. Under these culture conditions, neither the TNFAIP6-heavy chains (IαI) species nor heavy chains of IαI were present in the matrix extract, indicating that A38 antibody successfully

blocked TNFAIP6/IαI interactions and consequently heavy chains linkage to HA (Nagyova et al. 2008). Nevertheless, this antibody did not prevent cumulus expansion nor HA accumulation in the matrix of porcine OCC stimulated with FSH. It suggests a possibility of redundant mechanism in porcine OCC.

Expression of TNFAIP6 transcripts in porcine preovulatory follicles

To confirm the role of TNFAIP6 in porcine follicles, we studied expression of *TNFAIP6* in porcine preovulatory follicles (Nagyova et al. 2009; 2011). Mural granulosa cells (MGC) obtained from follicles on day 12 (D12) and day 15 (D15) of the estrous cycle, eCG-stimulated follicles, follicles at 4-32 h after hCG stimulation, and MGC and OCC obtained from immature gilts and cultured for 0-44 h *in vitro* with gonadotropins were used for extraction of total RNA and assessment of the relative abundance of *TNFAIP6* mRNA by RT-PCR. The levels of TNFAIP6 mRNA were low in the follicles on D12 and D15 of the estrous cycle and at 66 h after eCG stimulation, but were significantly increased at 4 h after hCG. The high level of *TNFAIP6* expression was maintained until 16 h after hCG stimulation and gradually decreased at 24 and 32 h after hCG. During *in vitro* culture, FSH/LH-induced *TNFAIP6* mRNA was expressed in both OCC and MGC in a similar temporal pattern as seen *in vivo*. We have shown that *TNFAIP6 is* expressed in porcine preovulatory follicles soon after following the LH/hCG surge and its expression decreases beyond 24 h. The OCC and MGC display the similar pattern of *TNFAIP6* expression that is also comparable under *in vivo* and *in vitro* conditions (Nagyova et al.

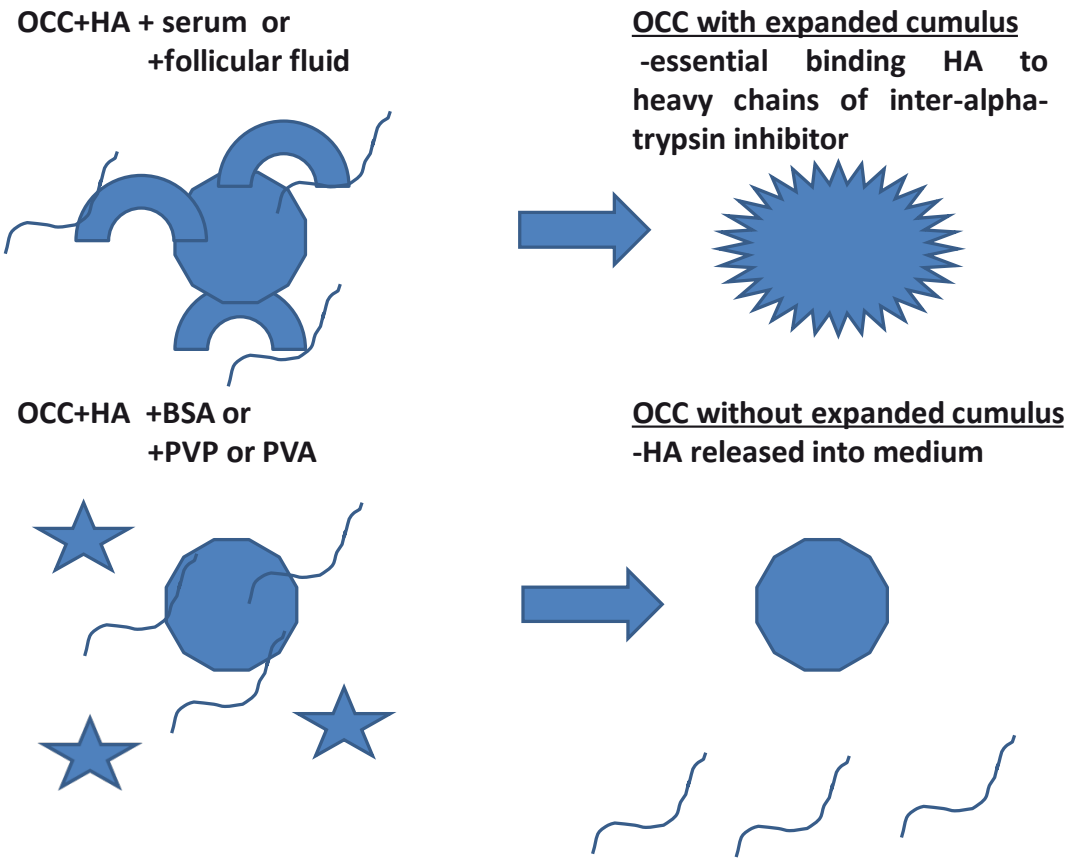

Fig. 1. Culture of FSH/LH-stimulated oocyte-cumulus complex (OCC) in the presence of serum or follicular fluid provide clear evidence that heavy chains of inter- alpha-trypsin inhibitor are covalently linked to hyaluronan (HA) in the expanded and elastic cumulus extracellular matrix. In contrast, FSH/LH-stimulated OCC cultured in the presence of either BSA or PVP or PVA are without the expanded and elastic cumulus, since HA is released into the culture medium.

2009, 2011). These results are consistent with the idea that TNFAIP6 acts in the preovulatory ovarian follicles in a coordinated way with HAS2 expression. Similar pattern of *Tnfaip6* mRNA expression was observed in mouse (Mukhopadhyay et al. 2001) and rat (Yoshioka et al. 200) preovulatory follicles. Sayasith et al. (2007) investigated the regulation of *TNFAIP6* mRNA in equine follicles isolated during estrus between 0 and 39 h post-hCG. Their study demonstrated the gonadotropin-dependent regulation of follicular *TNFAIP6* during the ovulation. Besides that the biphasic induction of *TNFAIP6* in equine theca and granulosa cells differs from the pattern observed in rodents suggesting a distinct control of gene expression in this monoovulatory species. Moreover, Sayasith et al. (2008) demonstrated that the ovulatory process in cow is accompanied with gonadotropin-dependent induction of *TNFAIP6* in granulosa cells and theca cells of preovulatory follicles. Similar results were observed by Tesfaye et al. (2009), they investigated the changes in gene expression profile of cumulus cells derived from bovine OCC with different developmental potential. The authors have shown an overexpression of *TNFAIP6* induced before ovulation in cumulus cells derived from *in vivo* OCC. The expression of *TNFAIP6* was without any changes after bovine OCC were stimulated with EGF. This is not surprising, since Sayasith et al. (2008) demonstrated gonadotropin-dependent induction of *TNFAIP6* in bovine ovarian follicles and provided the evidence that PKA activation plays an important role in the regulation of *TNFAIP6* in bovine granulosa cells.

Concluding remarks

In mouse, it has been shown that the heavy chains of serum-derived IαI molecules become covalently linked to HA during *in vivo* cumulus expansion and significantly contribute to cumulus ECM organization. Moreover, experiments with mice suggested that the incorporation of such proteins in cumulus ECM appears to be rather complex, involving LH/hCG-induced changes in blood-follicle barrier and functional cooperation between cumulus cells, granulosa, and oocyte within the follicle. In pig, we have demonstrated that heavy chains (IαI)-HA covalent complexes are formed during *in vivo* cumulus expansion as well. The similar amount of heavy chains were covalently transferred from IαI molecules to HA, when porcine OCC were stimulated *in vitro* with gonadotropins (FSH or FSH/LH) in the presence of porcine serum or follicular fluid from unstimulated or hCG-stimulated follicles. Furthermore, Western blot analysis with IαI antibody revealed that follicular fluids from medium-sized follicles and those from large follicles unstimulated with hCG contain high levels of all forms of IαI family members present in porcine serum. In conclusion, the results indicate that IαI molecules can freely cross the blood-barrier and that follicular fluid collected at any stage of folliculogenesis can be successfully used instead of serum in organizing of expanded HA-rich cumulus ECM. Thus, IαI family members present in porcine serum or follicular fluid become responsible for cumulus expansion, because without these members expanded cumulus HA-rich ECM is not formed and HA is released into medium (Chen et al. 1994; Nagyova et al.2004) (Fig. 1).

Since it has been shown that the proteins of the IαI family have additional global effects on reproductive biology in mouse by modulating the expression of a large number of cellular genes (including stress-related, apoptosis-related, proteases, signaling molecules, aging-related, cytokines, HA-metabolism), it is also important to verify these findings in other animal models.

Acknowledgements

I appreciate help of all people who have been involved in this research. I thank to Professors Salustri and Eppig for reading the manuscript and useful comments. This research was supported as follows: "Collaboration via a fellowship under the OECD Co-Operative Research Program (2013): Biological Resource Management for Sustainable Agricultural Systems" and by Grant 305/05/0960 from GA of the Czech Republic.

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