

# Paracrine actions of oocytes in the mouse pre-ovulatory follicle

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**ABSTRACT** In mammals, ovulation requires a tight control of extracellular matrix modifications, within both the follicle wall and the inner mass of granulosa cells surrounding the oocyte, namely the cumulus cells. During the pre-ovulatory period, mural granulosa cells promote selective degradation of perifollicular matrix, resulting in the formation of a follicle rupture site. Conversely, cumulus cells synthesize a large amount of a muco-elastic matrix that plays an essential role in the extrusion of the oocyte from the follicle and in the subsequent fertilization process. Formation of such matrix by cumulus cells in the pre-ovulatory follicle appears to be controlled by a paracrine influence by the oocyte. We have shown that mouse oocytes modulate the response of cumulus cells to an ovulatory gonadotropin stimulus by promoting the synthesis and preventing the degradation of cumulus matrix. Therefore, although gonadotropins are essential for triggering the complex events involved in ovulation, the oocyte appears to have an active role in this process. In the present review current data and hypotheses concerning molecular mechanisms involved in the organization and synthesis of cumulus matrix are discussed.

**KEY WORDS:** *oocyte, cumulus cells, gonadotropins, ovulation, extracellular matrix.*

## Introduction

In mammalian antral follicles, granulosa cells surrounding the oocyte, namely cumulus cells, and granulosa cells lining the follicle wall, referred to as mural granulosa cells, activate divergent functions in response to gonadotropin stimuli. Under follicle stimulating hormone (FSH) action, mural granulosa cells synthesize increasing amounts of luteinizing hormone (LH) receptors and steroid hormones, while cumulus cells show a limited increase of such functions (Zoller and Weisz, 1979; Lawrence *et al.*, 1980; Channing *et al.*, 1981; Oxberry and Greenwald, 1985; Zlotkin *et al.*, 1986; Ishimura *et al.*, 1989). These two cell populations also differ in growth factor expression: higher mRNA levels of insulin-like growth factor (Oliver *et al.*, 1989), vascular endothelial growth factor (Dissen *et al.*, 1994), inhibin beta A (Braw-Tal, 1994) and mullerian inhibiting substance (Ueno *et al.*, 1989) are reached in cumulus cells in comparison to mural granulosa cells, while the opposite occurs for the c-kit ligand (Manova *et al.*, 1993; Motro and Bernstein 1993). Following an endogenous LH-FSH surge or administration of an ovulatory dose of hCG, mural granulosa cells promote selective degradation of peri-follicular matrix bringing to the formation of a rupture site in the follicle wall. Conversely, cumulus cells synthesize an abundant muco-elastic extracellular matrix promoting detachment of cumulus cell-oocyte complex (COC) from the follicle wall and a 20-40 fold increase in the volume of the cumulus mass, a process termed cumulus expansion or mucification (Tsafirri and

Reich, 1999). At ovulation, the expanded COC leaves the follicle through the ruptured wall, while mural granulosa cells remain in the follicle anchored to the basement membrane.

Cumulus cells provide the proper environment for successful oocyte maturation and fertilization. Soluble factors, synthesized and secreted by cumulus cells, improve meiotic progression, fertilizability of oocytes and embryo development (Vanderhyden and Armstrong, 1989; Goud *et al.*, 1998; De La Fuente and Eppig, 1999). The muco-elastic extracellular matrix appears to be essential for oocyte extrusion from the follicle and for pick-up by the fimbria, since when its synthesis is prevented or altered, a significant lower number of oocytes are released from follicles and in addition, they are not transferred into the oviduct (Chen *et al.*, 1993; Hess *et al.*, 1999; Hizaki *et al.*, 1999). In addition, a function of selective barrier for sperm has been also advanced for this matrix (Carrell *et al.*, 1993; Bedford and Kim, 1993).

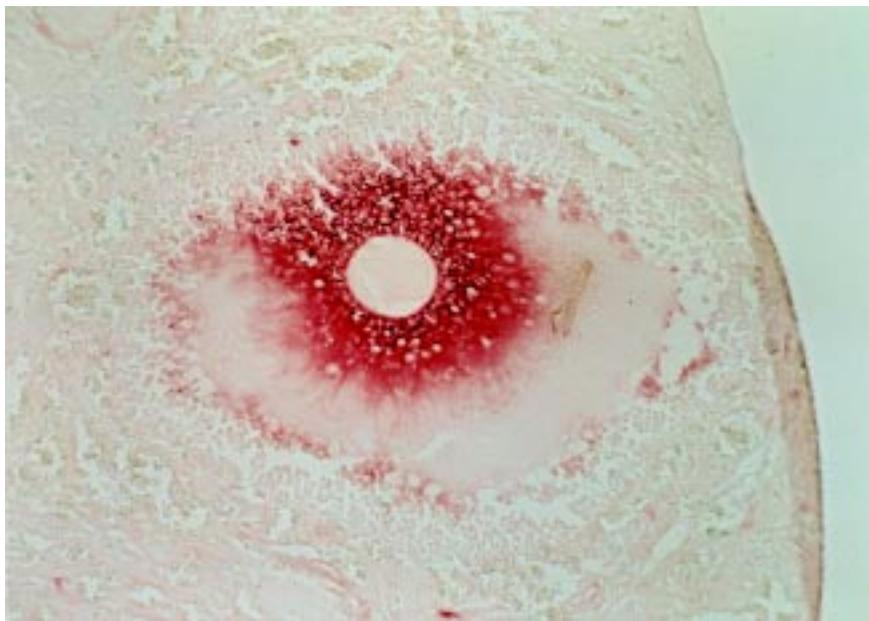
Mural granulosa cells luteinize to form mature corpus luteum,

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*Abbreviations used in this paper:* COC, cumulus cell-oocyte complex; CS-PG, chondroitin sulfate proteoglycan; dbcAMP, dibutyryl cAMP; FCS, fetal calf serum; FSH, follicle stimulating hormone; hCG, human chorionic gonadotropin; GDF-9, growth differentiation factor-9; HAS2, hyaluronan synthase 2; LH, luteinizing hormone;  $\alpha$ I, inter-alpha-trypsin inhibitor; P $\alpha$ I, pre-alpha-trypsin inhibitor; PMSG, pregnant mare serum gonadotropin; TGF beta1, transforming growth factor beta 1; TSG-6, tumor necrosis factor stimulated gene-6; uPA, urokinase plasminogen activator.

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**Fig. 1. Localization of hyaluronan in cumulus matrix.** The image shows a peri-ovulatory follicle from a hamster ovarian section stained with biotinylated hyaluronan binding proteins followed by avidin-peroxidase (Salustri *et al.*, 1992).

producing progesterone required for successful implantation of the embryo and maintenance of the pregnancy. Thus, the acquisition of divergent metabolic activities by the two follicle sub-populations is functional to their different role in follicle development, essentially steroidogenic for mural granulosa cells, and oocyte nursing for cumulus cells. Any alteration in the differentiation process of one or the other cell type may compromise fertility. Relative position of cumulus cells and mural granulosa cells within the follicle is essential in determining their response to gonadotropins. Evidence has been accumulating that contact with the basement membrane and soluble products secreted by theca cells, such as androgens and growth factors, co-operate with gonadotropins to promote mural granulosa cell phenotype (Adashi, 1994). In addition, the identification of some components of the expanded extracellular matrix and the study of the regulation of their synthesis in mouse COC has highlighted the essential role of oocytes in modulating cumulus cells' response to gonadotropins and in determining their functional pathway (Salustri *et al.*, 1990b; Buccione *et al.*, 1990). This research field is rapidly evolving and the list of cumulus cell activities affected by the oocyte is lengthening. Here we will focus on the progress of knowledge about the regulation of cumulus expansion which has allowed us to propose a model of the molecular mechanisms involved in the organization and synthesis of cumulus matrix during the pre-ovulatory period.

### Structure and molecular components of expanded cumulus matrix

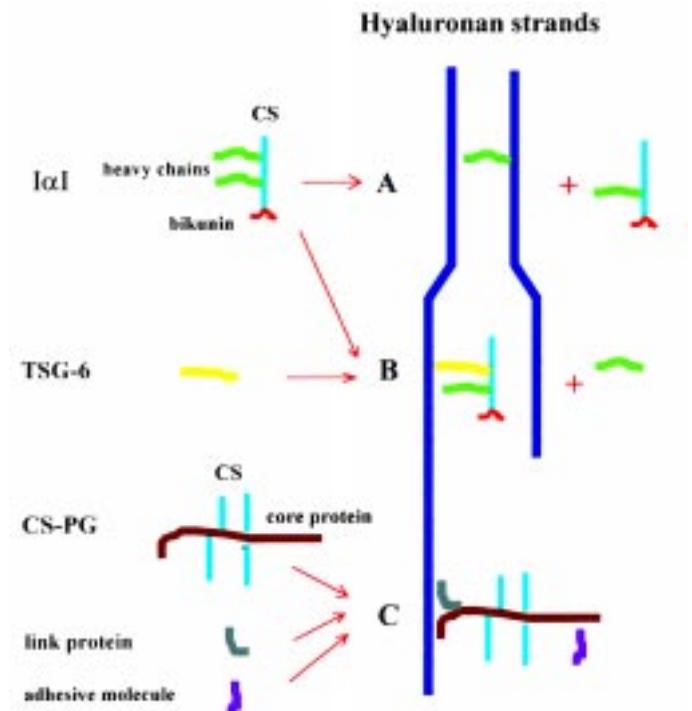
Cumulus expansion depends on synthesis and secretion of hyaluronan by cumulus cells (Fig. 1). Hyaluronan is a glycosaminoglycan with exceptionally high molecular weight (up to a few million Dalton) and large hydrodynamic domains which is synthesized at the plasma membrane, with the elongating chain being extruded into the extracellular space. Its ability to attract

water is most likely responsible for the hydration of COC extracellular matrix and the increase of intercellular space among cumulus cells. When this matrix is analyzed by electron microscopy, the hyaluronan strands appear to be organized into twisted or coiled fibrils which interconnect to each other to form a homogeneous mesh-like network (Yudin *et al.*, 1988). Protease treatment causes dissociation of the fibrils into individual hyaluronan filaments and produces loss of COC integrity (Cherr *et al.*, 1990). Therefore, proteins are required to organize and maintain hyaluronan in such a highly structured gel, conferring on the COC matrix unique physical properties. For instance, expanded COCs are only temporarily deformed when subjected to shearing forces. Such elasticity and stability of the matrix may play an essential role in ovulation by promoting the protrusion of the COC and protecting the oocyte from mechanical stress. The hyaluronan-protein fibrillar network is attached on cumulus cell surface and penetrates into the zona pellucida surrounding the oocyte (Yudin *et al.*, 1988). Most likely these interactions allow cumulus cells and oocyte to remain firmly bound within the matrix, so that they are not dispersed during extrusion.

Evidence obtained *in vivo* and *in vitro* suggests that proteins both derived from the serum and synthesized by cumulus cells participate in organizing hyaluronan in the matrix. Mouse COCs *in vitro* treated with FSH in the presence of fetal calf serum (FCS) undergo expansion (Eppig, 1979). However, when COCs are stimulated with FSH in the absence of serum the elastic-expanded matrix is not formed and cumulus cells lose contact from each other and settle individually on the bottom of the culture dish. In such culture condition, cumulus cells synthesize hyaluronan but they fail to organize it in the intercellular space so that most of hyaluronan diffuses into the culture medium (Eppig, 1980a). The absence of serum does not affect the size of the synthesized hyaluronan suggesting that the observed phenomenon is not due to changes in the physiological properties of this polymer (Salustri *et al.*, 1989). If serum is added at the beginning of culture and removed while COC expansion is ongoing, hyaluronan synthesized before the removal is retained in the matrix while that synthesized afterwards is released into the medium (Camaioni *et al.*, 1993). This finding indicates that serum factors must be present while hyaluronan is being synthesized in order to organize it into a matrix. Factors with such functional ability have been isolated from the serum and identified as inter-alpha-trypsin inhibitor ( $\text{I}\alpha\text{I}$ ) and a related protein pre-alpha-trypsin inhibitor ( $\text{P}\alpha\text{I}$ ). Each of these molecules can substitute for serum in retaining hyaluronan among cumulus cells, whereas serum depleted of them is inactive (Chen *et al.*, 1992). Co-localization *in vivo* of  $\text{I}\alpha\text{I}$ -related molecules and hyaluronan in the pre-ovulatory follicle suggests their physiological role. It has been proposed that these serum macromolecules can rapidly enter the follicle after a gonadotropin ovulatory stimulus increases the permeability of the selective blood-follicle barrier (Chen *et al.*, 1992).

How  $\text{I}\alpha\text{I}$  and  $\text{P}\alpha\text{I}$  retain hyaluronan in the cumulus matrix is still unclear. These molecules are proteoglycans containing a single chondroitin sulfate chain covalently attached to a core protein of

about 16 kDa molecular weight (MW), named bikunin or light chain, and to one (P $\alpha$ 1) or two (I $\alpha$ 1) additional peptides of about 80 kDa MW, referred to as heavy chains (Salier *et al.*, 1996). The two heavy chains of I $\alpha$ 1 and the single heavy chain of P $\alpha$ 1 are encoded by three different genes sharing a high degree of homology. These peptides show the ability both to bind to hyaluronan through stretches rich in positively charged amino acid residues and to covalently link to hyaluronan *via* displacement of the chondroitin sulfate chain (Chen *et al.*, 1994, 1996). Thus, these interactions can lead to cross bridges between adjacent hyaluronan strands thereby stabilizing cumulus matrix (Chen *et al.*, 1996) (Fig. 2A). Alternatively or in addition, I $\alpha$ 1 could act as an anti-protease within cumulus matrix. The bikunin peptide of I $\alpha$ 1 and P $\alpha$ 1 consists of two tandemly arranged domains, each with proteinase inhibitor activity (Wachter and Hochstrasser, 1979). Its anti-proteolytic activity is increased by tumor necrosis factor-stimulated gene-6 (TSG-6), a protein which can form a stable complex with I $\alpha$ 1 by displacing a heavy chain (Wisniewski *et al.*, 1994, 1996) (Fig. 2B). The expression of TSG-6 gene is up regulated in cumulus cells during COC expansion and high levels of this messenger are maintained throughout the pre-ovulatory period (Fulop *et al.*, 1997a). Interestingly, TSG-6 can also bind to hyaluronan through a highly specific binding site (Lee *et al.*, 1992). Therefore, TSG-6 could activate and specifically localize the anti-protease bikunin on hyaluronan strands



**Fig. 2. Models for interaction between hyaluronan, I $\alpha$ 1, TSG-6 and versican-like CS-PG.** (A) The heavy chain of I $\alpha$ 1 can be covalently linked to a hyaluronan through a reaction in which hyaluronan displaces the CS chain of I $\alpha$ 1. This polypeptide, tightly linked to hyaluronan can interact with another hyaluronan strand by positive charge residues leading to the formation of a cross-bridge. (B) TSG-6 displaces a heavy chain of I $\alpha$ 1 forming a stable complex with I $\alpha$ 1. TSG-6 increases the anti-proteolytic activity of I $\alpha$ 1 and it binds to hyaluronan through a specific binding site. (C) CS-PG binds to hyaluronan, an interaction that is stabilized by the link protein. This proteoglycan binds adhesive molecules through a C-terminal lectin-binding domain.

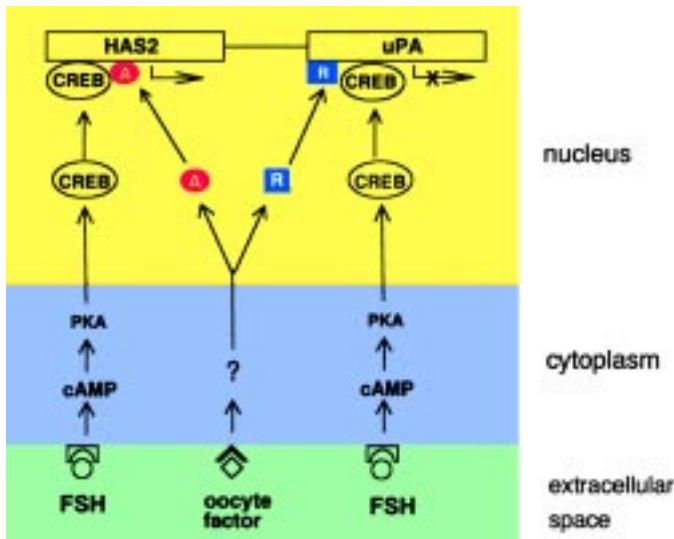
elongating at the cumulus cell membrane, thereby tightly protecting from degradation hyaluronan-associated proteins involved in matrix organization. Indeed, proteolytic activity increases in the follicular fluid during the pre-ovulatory period due to the increased production of proteases by mural granulosa cells (Tsafiri and Reich, 1999).

Cumulus cells synthesize two additional hyaluronan binding molecules which are retained within the matrix: a chondroitin sulfate proteoglycan (CS-PG) with a molecular weight larger than  $10^6$  Dalton (Camaioni *et al.*, 1996), and the link protein, a glycoprotein able to interact with both hyaluronan and a proteoglycan, thus stabilizing their linkage (Kobayashi *et al.*, 1999) (Fig. 2C). Inhibition of chondroitin sulfate chain elongation by b-xyloside treatment apparently does not affect cumulus expansion, implying that the negatively charged glycosaminoglycan chain of large CS-PG do not significantly contribute to the swelling of the matrix (Salustri *et al.* 1989). The large CS-PG in the COC matrix is more likely involved in matrix organization. For its structural characteristics and immunological affinity it can be considered a member of proteoglycan versican family (Camaioni *et al.*, 1996; Eriksen *et al.*, 1999). The core protein of this type of proteoglycan has a functional N-terminal hyaluronan-binding domain and a C-terminal lectin-binding domain, through which it can bind adhesive molecules (Aspberg *et al.*, 1995). Tenascin C and other adhesive proteins have been found in the extracellular matrix of COC (Familiari *et al.*, 1996) and versican-like PG may serve as cross-linking agent between such molecules and the hyaluronan scaffold (Fig. 2C).

### Role of gonadotropins and oocyte in cumulus expansion

When large antral follicles are punctured under a dissecting microscope before gonadotropin surge, cumulus cells remain closely associated with the oocyte, and intact cumulus cell-oocyte complexes (COCs) can be isolated and induced to expand *in vitro*. In all examined species, gonadotropins are potent inducers of COC expansion *in vitro*, although cumulus cells are more sensitive to follicle stimulating hormone (FSH) than to luteinizing hormone (LH) or human chorionic gonadotropin (hCG) (Magnusson *et al.*, 1982; Ball *et al.*, 1982; Singh *et al.*, 1993; Armstrong *et al.*, 1996). In fact, highly purified LH and hCG fail to induce expansion of isolated mouse COCs (Eppig, 1979). These findings are consistent with the low or undetectable levels of LH receptors in the cumulus cells in the pre-ovulatory follicle.

The study of hyaluronan synthesis by mouse COCs stimulated to expand *in vitro* by FSH has allowed molecular mechanisms involved in cumulus expansion to be studied in detail. Hyaluronan synthesis is first detected 2-3 hours after FSH stimulation, increases to a maximum rate sustained between 4-10 hours, and then declines and ceases by 12-18 hours, when maximum expansion has been reached (Salustri *et al.*, 1989). Several lines of evidence suggest that FSH exerts its influence on hyaluronan synthesis via cAMP. Follicle stimulating hormone stimulates cAMP production by cumulus cells, and dibutyryl cAMP (dbcAMP), a membrane-permeable cAMP analog, shows a potency and temporal pattern of hyaluronan synthesis induction similar to that observed with FSH (Tirone *et al.*, 1997). Interestingly, a level of 14 fmol of cAMP/COC has to be reached to obtain maximum hyaluronan synthesis by *in vitro* FSH-stimulated COCs, and a similar cAMP level is achieved by COCs *in vivo* 2-4 h after hCG injection into



**Fig. 3. A model for the regulation of HAS2 and uPA gene expression by FSH and the oocyte factor.** A, activator; CREB, cAMP responsive element binding protein; PKA, cAMP dependent protein kinase A; R, repressor.

PMSG-primed mice, a time at which hyaluronan synthesis is initiated. Since, as discussed above, mouse cumulus cells do not appear to have LH/hCG receptors, the hCG-dependent cAMP elevation observed in cumulus cells *in vivo* is likely mediated by mural granulosa either by direct transfer of cAMP via gap junctions or by the synthesis or activation of a molecule that can activate adenylate cyclase in cumulus cells (Schultz *et al.*, 1983; Eppig, 1980b, 1981; Downs and Longo, 1982; Salustri *et al.*, 1985). Based on *in vitro* results, it is reasonable to hypothesize a primary role for cAMP in regulating hyaluronan synthesis and in promoting cumulus expansion *in vivo*.

Regulation of hyaluronan synthesis in cumulus cells is primarily controlled at the level of hyaluronan synthase-2 (HAS2) gene expression (Fulop *et al.*, 1997b). Hyaluronan synthase-2 mRNA is at very low levels in the compact COCs isolated from PMSG-primed mice, but reaches high levels 2-3 hours after hCG injection into the animals. By the time hyaluronan synthesis ceases, HAS2 mRNA returns to basal levels. Therefore, it is likely that a cAMP dependent protein kinase promotes the activation of a cAMP responsive element binding protein acting as a transcription factor for HAS2 gene.

Although the oocyte does not synthesize hyaluronan, it has an essential role in cumulus expansion in the mouse. When the oocyte is mechanically removed from the COC, isolated cumulus cells exposed to FSH synthesize low levels of hyaluronan, and do not form an expanded matrix (Salustri *et al.*, 1990b; Buccione *et al.*, 1990). A high level of hyaluronan synthesis is restored in FSH-stimulated cumulus cells by adding back either oocytes or oocyte-conditioned medium to the cultures. Therefore, a soluble factor secreted by the oocyte is required for FSH to exert its action on cumulus cells. We have investigated at which intracellular level the oocyte factor synergizes with FSH to induce hyaluronan synthesis. An oocyte influence on FSH binding to its receptor and/or on the generation of an intracellular signal has been excluded by the evidence that equal amounts of cAMP are generated by hormone-

stimulated cumulus cells cultured in the presence or in the absence of oocytes (Buccione *et al.*, 1990) and that dbcAMP fails to increase hyaluronan synthesis by cumulus cells in the absence of oocytes (Salustri *et al.*, 1990b). FSH and the oocyte factor differ in their temporal patterns of action on hyaluronan synthesis (Tirone *et al.*, 1997). Mouse COCs produce maximal hyaluronan synthesis if they are exposed to FSH for only 2 hours, indicating that hormone action is completed within this time. Conversely, the presence of the oocyte is not required during this initial inductive phase, because nearly identical results were obtained when the oocytes were either added with FSH at the beginning of culture or 2 h later. However, continuous presence of the oocyte is required from 2 hours onwards to promote and sustain maximal hyaluronan synthesis. Therefore, it is likely that the oocyte factor(s) does not act on FSH signal transduction. The level of HAS2 mRNA significantly increases in cumulus cells stimulated *in vitro* by FSH in the presence of oocytes while it only slightly increases in cumulus cells cultured with either oocytes or FSH alone, implying that the oocyte factor synergistically acts with FSH to induce HAS2 gene expression (Fulop, Salustri and Hascall, unpublished results). Noteworthy, if the oocytes are removed from FSH-stimulated COCs at 6 h of culture, when maximum HAS2 transcription and HA synthesis are achieved, hyaluronan synthesis ceases in 2-3 h (Tirone *et al.*, 1997). The same result is obtained if transcription inhibitor actinomycin D is added at 6 h to FSH-stimulated COC cultures. Therefore, it is likely that removal of the oocyte rapidly down regulates HAS2 transcription and that continuous presence of oocyte factor is required to sustain the expression of this gene.

As reported above, cumulus cells synthesize hyaluronan-binding proteins that maybe are required for matrix assembly and stability. At the present it is not known if the oocyte can influence the synthesis of such proteins by cumulus cells. However, the oocyte inhibits gonadotropin-induced protease synthesis by cumulus cells, thereby contributing to protect matrix protein components (Canipari *et al.*, 1995). Cumulus cells show significantly lower levels of urokinase plasminogen activator (uPA) mRNA than mural granulosa cells in mice injected with an ovulatory dose of gonadotropins. Such difference is not observed when isolated cumulus cells and mural granulosa cells are stimulated *in vitro* with FSH. However, when cumulus cells are cultured with oocytes or oocyte conditioned medium, significantly lower levels of uPA mRNA are achieved under FSH stimulus, mimicking what occurs *in vivo*. Thus, up-regulation of uPA expression by hormones is suppressed by a soluble oocyte factor and differences in uPA mRNA levels observed *in vivo* between cumulus and mural granulosa cells are due to the inhibitory influence of the oocyte.

Based on the similarities in physicochemical characteristics and in pattern of secretion during oogenesis between the oocyte factor regulating hyaluronan synthesis and that regulating uPA synthesis (Vanderhyden *et al.*, 1990; Eppig *et al.*, 1993a, 1993b; Canipari *et al.*, 1995), we have advanced the hypothesis that both activities could be controlled by a single factor produced by the oocyte (Canipari *et al.*, 1995). The results are consistent with the following model: FSH activates a cAMP-responsive element binding (CREB) protein which acts as a transcription factor for both HAS2 and uPA gene. The oocyte factor operates through a separate receptor and signaling pathways to activate other proteins which regulate the activity of the cAMP-dependent transcription factor, positively on HAS2 and negatively on uPA gene (Fig. 3).

## Mechanisms limiting oocyte factor action in the pre-ovulatory follicle

Mural granulosa cells isolated from pre-ovulatory follicles assume cumulus cell phenotype when cultured *in vitro* in the presence of oocytes. For instance, in this condition they increase hyaluronan (Salustri *et al.*, 1990a) and decrease uPA (Canipari *et al.*, 1995) synthesis, as cumulus cells do. In addition, it has been observed that the synthesis of LH receptor mRNA is suppressed in these cells as well as in cumulus cells by a factor secreted by oocytes (Eppig *et al.*, 1997). However, some differences can be observed, such as mural granulosa cells do not require the gonadotropin stimulus to respond to the oocyte in terms of hyaluronan synthesis (Salustri *et al.*, 1990a). This may depend on the ability of mural granulosa cells to synthesize molecules, such as prostaglandin E and/or epidermal growth factor, which can substitute for FSH and act in an autocrine way synergizing with the oocyte factor (Salustri *et al.*, 1985; Salustri *et al.*, 1990a). Whatever the mechanism, results show that even though mural granulosa cells show functional specificity, still they can be modulated in their function by the oocyte. So, how do mural granulosa cells escape from the oocyte control in the follicle? Most likely this goal is accomplished by limited diffusion of oocyte factors. This hypothesis is supported by the evidence that media conditioned by FSH-stimulated COCs, in which the oocyte is enclosed within the cumulus cell mass, have only 10-20% of the hyaluronan stimulatory activity of media conditioned by an equal number of isolated oocytes when tested in mural granulosa cell cultures. Furthermore, the activity of medium conditioned by isolated oocytes is reduced to ~30% by pre-incubation with isolated cumulus cells (Salustri *et al.*, 1992). These results suggest that cumulus cells can bind and inactivate the factor, a process that would create a decreasing concentration gradient with increasing distance from the oocyte. In agreement with this hypothesis, the innermost layers of granulosa cells lining the antrum, that are closest to the COC, show an intermediate cell phenotype between cumulus and mural granulosa cells (Salustri *et al.*, 1992) (Fig. 1). In conclusion, limited diffusion of oocyte factor may have an essential role in ensuring that an appropriate number of granulosa cells differentiate and remain within the follicle to form with theca cells a corpus luteum suitable to support embryo implantation.

## Identification of oocyte factor

Mural granulosa cells are more abundant and easier to isolate than cumulus cells. For this reason, mural granulosa cell cultures have been used to test a series of known growth factors for ability to stimulate hyaluronan synthesis, as the oocyte factor does. Of growth factors studied, only transforming growth factor (TGF) beta1 resulted to induce hyaluronan synthesis and expansion by FSH-stimulated cumulus cells (Salustri *et al.*, 1990a; 1993). However, neutralizing antibodies against TGF beta1 do not inhibit the oocyte factor activity, indicating that they are different (Salustri *et al.*, 1990a). On the other hand, their effects are additive at suboptimal doses, and they show identical temporal pattern of induction of hyaluronan synthesis (Tirone *et al.*, 1997). Thus, we have proposed that the oocyte factor could be a member of the TGF beta family, different from TGF beta1 but triggering similar intracellular signals. This hypothesis has been recently confirmed. Recombinant mouse growth differentiation factor-9 (GDF-9) pro-

tein, a member of TGF beta family exclusively expressed by oocytes in mouse ovary (McGrath *et al.*, 1995), is able to increase HAS2 mRNA by mural granulosa cells and to induce cumulus expansion (Elvin *et al.*, 1999). This growth factor also shows the ability to mimic the oocyte factor in decreasing uPA and LH receptor mRNA levels in mural granulosa cells. The receptor and signal transduction pathways utilized by GDF-9 have not been studied yet. However, similarity of action between oocyte conditioned medium and TGFbeta1 suggests that GDF-9 could bind to serine/threonine kinase receptors and transduce signals through SMAD proteins, as TGF beta family members do. Following TGF beta binding to receptor, SMADs are phosphorylated and translocated to the nucleus. These proteins can access target genes by synergistically binding to DNA with cofactors (such as cAMP responsive element binding protein) and can induce or inhibit gene expression by recruiting co-activators or co-repressors (Massague and Chen, 2000). If GDF-9 is signaling through this intracellular pathway in FSH-stimulated cumulus cells, interaction of SMADs with different molecules acting at HAS2, uPA and LH-receptor gene level could explain the opposite effects of this growth factor on the expression of these cAMP-dependent genes. GDF-9 expression has been also demonstrated in rat, ovine, bovine and human oocytes (Jaatinen *et al.*, 1999; Bodensteiner *et al.*, 1999; Aaltonen *et al.*, 1999). In agreement with these findings, it has been shown that porcine, rat and bovine oocytes can substitute for mouse oocytes in inducing cumulus expansion. However, cumulus cells isolated from these species, in contrast with those from the mouse, can be stimulated to expand *in vitro* by FSH treatment in the absence of oocytes (Prochazka *et al.*, 1991; Singh *et al.*, 1993; Vanderhyden *et al.*, 1993; Ralph *et al.*, 1995). It should be noted, however, that if rat cumulus cell are pre-cultured for 5-6 h in the absence of oocytes, they can be induced to expand thereafter only if oocytes are added to the culture together with FSH (Vanderhyden, 1993). Thus, it is likely that species differences between cumulus cells are related to the duration of GDF9 activity or the stability of cumulus cell response to this factor.

## Acknowledgements

I wish to express my sincere thanks to Prof. Gregorio Siracusa, Dr. Vincent C. Hascall and Dr. Antonella Camaioni for a fruitful collaboration over many years. This work was supported by MURST grant for National Project "Development and Differentiation of Germ Cells" and by CNR grant n.98.00512.CT04.

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