

Review

**Control of oocyte growth and maturation by follicular cells and molecules present in follicular fluid.
A review**

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Abstract – The aim of this review is to summarize the interactions between the oocyte and its surrounding granulosa cells which are involved in the control of oocyte growth or apoptosis as well as those playing a key role in the ability of the oocyte to undergo nuclear (resumption as meiosis to reach the MII stage) or cytoplasmic maturation (ability to fertilize and develop to the blastocyst stage). The respective roles of the oocyte and of the granulosa cells in controlling the initiation of growth are poorly understood. During the preantral follicular stage when most oocyte growth is achieved, a local regulation appears to be in operation involving growth factors such as fibroblast growth factor (FGF) or epidermal growth factor/transforming growth factor α (EGF/TGF α), together with two proteins (c-kit present on the oocyte's membrane and its ligand KL produced by granulosa cells). In-situ techniques used to detect apoptosis demonstrate apoptotic oocytes in the reserves of primordial follicles but seldom within preantral follicles (because it is too fast?). Proteins involved in cell death (bax) or cell survival (bcl2) are present in oocytes as well as compounds (TNF α , Fas) involved in the initiation of apoptosis. However, the molecular and cellular mechanisms triggering oocyte apoptosis are not fully clarified. Three approaches have been used to identify compounds which are relevant to the oocyte's nuclear or cytoplasmic maturation. a) Correlation between amounts of specific compounds in follicular fluid or within follicle cells and the oocyte's ability to mature. b) Analysis of the consequences of pharmacological disruption of mechanisms such as steroidogenesis on oocyte maturation. c) Analysis of the consequences of addition of graded amounts of specific compounds on oocyte maturation in defined media. Factors playing a key role in stimulating nuclear maturation appear to be epidermal growth factor (EGF) and the inhibin (cattle)/activin (rodents) family, while testosterone has an inhibitory effect. Cytoplasmic maturation of the oocyte appears to be stimulated by oestradiol, EGF and inhibin. © Inra/Elsevier, Paris

oocyte / granulosa / maturation / apoptosis / growth / growth factors

Résumé – Interactions entre l'ovocyte et les cellules folliculaires. Conséquences pour la croissance, l'apoptose et la maturation ovocytaire. L'objectif de cette revue est d'analyser l'impact des

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interactions cellulaires somatiques–cellules germinales dans la croissance ou l'apoptose ovocytaire puis dans l'acquisition par l'ovocyte de l'aptitude à réaliser séquentiellement sa maturation nucléaire (reprise de la méiose) et sa maturation cytoplasmique (permettant la fécondation et le développement embryonnaire précoce). L'importance respective de l'ovocyte et des cellules de granulosa pour l'initiation de la croissance est mal connue. En revanche, au cours de la période préantrale où l'ovocyte effectue la plus grande partie de son accroissement en taille, des régulations locales impliquant des facteurs de croissance (FGF : *fibroblast growth factor*, EGF/TGF α : *epidermal-transforming growth factor* α) et un couple de protéines (c-kit présent sur l'ovocyte et son ligand KL produit par la granulosa) paraissent jouer un rôle clé. L'apoptose ovocytaire est principalement visualisée par les techniques in situ dans les réserves de follicules primordiaux. Elle est rare dans les follicules préantraux (parce qu'elle est rapide ?). Les protéines (bax, bcl2) impliquées dans la survie ou la mort cellulaire sont présentes dans les ovocytes, tout comme des composés (TNF α , Fas) impliqués dans l'initiation de l'apoptose. Les mécanismes cellulaires et moléculaires contrôlant l'apoptose ovocytaire sont encore inconnus. Trois types d'approches ont été employés pour identifier les composés impliqués dans l'acquisition par l'ovocyte de son aptitude à faire maturation nucléaire puis maturation cytoplasmique. a) L'étude des corrélations entre cette aptitude et les quantités de composés spécifiques présents dans le follicule, le cumulus ou le liquide folliculaire. b) L'étude des conséquences du blocage de certaines fonctions (stéroïdogénèse en particulier) par des approches pharmacologiques sur la maturation ovocytaire. c) L'étude de l'effet de la supplémentation de milieux définis utilisés lors de la maturation in vitro sur la maturation ovocytaire. Les composés affectant la maturation nucléaire paraissent être l'*epidermal growth factor* (EGF), la famille inhibine (bovin)/activine (rongeurs) qui stimulent la maturation nucléaire alors que la testostérone a un rôle inhibiteur. Les principaux composés affectant la maturation cytoplasmique sont l'œstradiol, l'EGF et l'inhibine qui la stimulent. © Inra/Elsevier, Paris

ovocyte / granulosa / maturation / apoptose / croissance

1. INTRODUCTION

Folliculogenesis in mammalian species is a highly selective process. Only a very small proportion of the follicles ($\cong 0.1\%$) survive apoptosis, which takes place in the stores of primordial follicles (rodents: Byskov [9]; sheep: Driancourt et al. [29]; human: Gougeon et al. [42]) as well as in preantral follicles (sheep: Driancourt et al. [29]; cattle: Schotanus et al. [86]). At these two stages, the oocyte is the first cell within the follicle to be affected by apoptosis. Whether oocyte apoptosis is related to oocyte defects or to an improper dialogue between the oocyte and its surrounding granulosa cells remains unclear. If the oocyte succeeds in evading apoptosis, it will grow actively while the follicle is at the preantral stage, since when antrum forms, the oocyte has reached about 85% of its final size. During the initial section of this review, the local interactions between the oocyte and its surrounding cells involved in the control of growth and apoptosis will be summarized.

Once antrum formation occurs within the follicle (at about 0.2 mm in diameter), changes in oocyte size are limited. However, functional changes which play a key role in oocyte maturation (nuclear and cytoplasmic) do occur. During nuclear maturation, meiosis resumes so that the oocyte, when ovulated, has reached the metaphase II stage. During cytoplasmic maturation, complex changes occur in the oocyte which are not fully understood. Changes in cortical granule distribution, alterations in the repartition of cytoplasmic organelles, accumulation or destruction of specific mRNAs or proteins have been observed. All these changes are of key importance in generating good quality oocytes (i.e. oocytes capable of producing a live young following fertilization and development).

In all mammalian species, there are strong links between follicular growth and oocyte maturation. For example, in cattle only 1.4% of the oocytes originating from follicles smaller than 1 mm in diameter have the ability to reach metaphase II [38]. This

Table I. mRNA and proteins detected amongst oocytes or follicle cells at specific stages of follicular growth (preantral: top panel, antral: bottom panel). At the antral stage, only the new molecules are indicated.

Molecules	Preantral		Antral	
	Follicle cells (granulosa)		Follicle cells (granulosa)	
	Proteins	mRNA	Proteins	mRNA
KL	Manova et al. [65]	Tisdall et al. [99]		
TGF α	Singh and Armstrong [87]	Singh and Armstrong [87]		
TGF β	Chegini and Flanders [15]			
MIS	Bézar et al. [4]			
	Ueno et al. [101]			
TGF β RII	Roy and Kole [84]		Roy and Kole [84]	
EGF receptor	Wandji et al. [109]			
FGF receptor	Wandji et al. [109]			
Follistatin				
c-kit		Braw Tal [7]		
TNF α		Tisdall et al. [98]		
TNF α receptor			Manova et al. [64]	Manova et al. [64]
TGF β 1			Marcinkiewicz et al. [66]	Chen et al. [16]
FGF				Naz et al. [71]
			Chegini and Flanders [15]	
			Van Vezel et al. [105]	
Molecules	Preantral		Antral	
	Follicle cells (granulosa)		Follicle cells (granulosa)	
	Proteins	mRNA	Proteins	mRNA
Inhibin				
Activin A		Tisdall et al. [98]		
IGF1 receptor		Braw Tal [7]		
TGF β 2				
			Wandji et al. [110]	
			Chegini and Flanders [15]	

proportion is at least 10 times lower than the level reached when oocytes are obtained from follicles 1 to 3 mm in diameter. These data were extended by Fair et al. [34] who showed that follicles 2.5 mm in diameter (and an oocyte diameter of 110–120 μm) represent a threshold at which the oocyte becomes capable of completing meiosis to metaphase II. It is only when the oocyte has acquired the ability to resume meiosis that it begins to be capable of undergoing cytoplasmic maturation. For example, in cattle Pavlok et al. [77] showed that following in vitro maturation (IVM) and fertilization (IVF), oocytes obtained from follicles smaller than 2 mm in diameter could not reach the blastocyst stage. The proportion of oocytes developing to the blastocyst stage increased from 21 to 28 % when the oocytes were obtained from follicles 2–4 mm or 4–8 mm in diameter. The superiority in terms of ability to develop to the blastocyst stage of oocytes originating from large follicles was confirmed by Lonergan et al. [61]. Furthermore, the observation that zygotes recovered after in-vivo maturation and fertilization (i.e. ovulated from follicles 12–16 mm in diameter) develop better in culture than those produced totally in vitro [68] also strongly supports the hypothesis that functional interactions between the maturing follicle and the oocyte take place.

Good evidence exists suggesting that the mechanisms involved in the control of oocyte growth and those involved in nuclear or cytoplasmic maturation are distinct. Support for this claim was obtained by Canipari et al. [11] who demonstrated that small oocytes at different stages of growth cultured on a feeder layer of fibroblasts do not grow but have the ability to resume meiosis and reach metaphase II at a time which corresponds strictly to the in-vivo situation. Furthermore Eppig and O'Brien [33], in an elegant study, demonstrated that 22-day-old oocytes obtained from 6-, 8- and 12-day-old mice (and cultured in vitro for 16, 14 and 10 days, respectively), despite having the same size, had vastly different abilities

to cleave to the blastocyst stage. Hence, in the final part of this review, the specific compounds produced by follicle cells which are relevant to nuclear and cytoplasmic maturation will be identified and their role discussed.

2. REGULATION OF FOLLICLE FUNCTION CHANGES THROUGHOUT FOLLICULAR GROWTH

Little information is available on the control of follicle function in follicles which grow from the reserves of primordial follicles and in preantral follicles. Data obtained in models where gonadotrophin concentrations are minimal (hypophysectomy: Dufour et al. [32], in vitro culture in the absence of gonadotrophins: Wandji et al. [111], knock out of the FSH β gene: Kumar et al. [56]) suggest that local (autocrine and paracrine) regulation is of key importance at this stage. Proteins, growth factors and their receptors which are expressed and/or present in the oocyte or in granulosa cells at the preantral stage are summarized in *table 1*. Paracrine signalling between the oocyte and granulosa cells could occur for FGF (fibroblast growth factor) and EGF (epidermal growth factor) which are expressed in oocytes and whose receptors are present in granulosa cells. This conclusion is also valid for c-kit which is present on the oocyte, while its ligand (KL) is produced by granulosa cells.

Additional regulatory factors which may become functional when follicles form an antrum are insulin-like growth factors (IGF1/IGF2) owing to the appearance of IGF receptors on granulosa cells at this stage. Their importance is shown by the observation that in IGF1 null mice, there are no antral follicles [3]. In addition, inhibin and activin become detectable at this stage (sheep: Tisdall et al. [98]; primates: Gougeon [41]). This is summarized in *table 1*.

The main processes involved in the growth and differentiation of the ovulatory follicle throughout the gonadotrophin dependent phase (starting at 2 mm in sheep, human and 3 mm in cattle) are summarized in *figure 1*. Detailed information can be found in other reviews [28, 41]. During the recruitment to selection phase (from 2 to 4 mm and 2 to 8 mm in sheep and human, respectively), the key gonadotrophin is FSH which induces aromatase activity in granulosa cells. This FSH action is potentialized by an autocrine action of activin produced by granulosa cells. During the dominance phase (from 4 and 8 mm to ovulatory size in sheep and human, respectively), LH becomes of key importance in stimulation of thecal steroidogenesis. This LH action is further increased by a paracrine action of the high amounts of inhibin produced by granulosa cells as well as by the high bio-availability

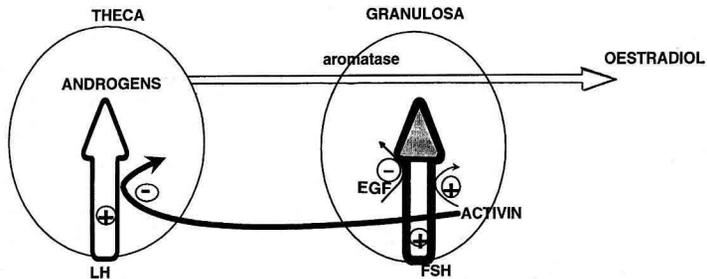
of IGFs (owing to the reduced amounts of its binding proteins). The high androgen output by theca cells together with the high aromatase activity in the granulosa cells differentiated earlier during the follicular phase, results in a high oestradiol output by the preovulatory follicle.

3. THE CLOSEST FOLLICULAR ENVIRONMENT FOR THE OOCYTE: THE CUMULUS CELLS

Cumulus cells have features which are different from the granulosa cells which are further away from the oocyte. The main differences are:

- the absence of expression of the mRNA coding for the LH receptor [79]. As a consequence, the number of LH receptors per cumulus cell is markedly reduced compared to that observed on granulosa cells [14];

Recruited Stage



Dominant Stage

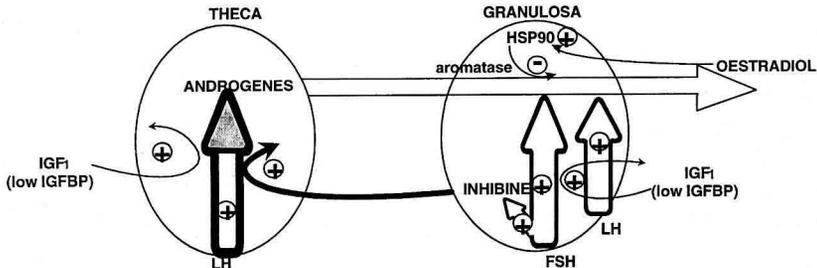


Figure 1. Regulation of follicular maturation by both endocrine and local mechanisms. The top of the figure describes the follicle at the recruited stage while the bottom describes it during its dominant stage. During the recruited stage, FSH is the main gonadotrophin and activin the main locally acting compound. During the dominant stage, LH is the main gonadotrophin and IGF1 together with inhibin are the main locally acting compounds.

– a markedly reduced ability to produce steroids; cumulus cells contain reduced amounts of 3β hydroxysteroid dehydrogenase (involved in progesterone production) [120] and very low amounts of aromatase [96, 119];

– interestingly, higher amounts of the mRNA coding for most of the members of the inhibin family (α inhibin, follistatin, MIS) have been reported in cumulus cells [7, 102]. Whether the amounts of these proteins are also higher within cumulus cells has not been established. Activin, in contrast, does not appear to be preferentially located to cumulus cells at least in sheep;

– a more active proliferation of the cells [47]. This increased ability of cumulus cells to divide may be a consequence of their more limited differentiation;

– a lower sensitivity to atresia. In atretic follicles, cumulus cells are always the last ones to enter atresia. Pycnotic bodies amongst cumulus cells start to appear when atresia is widespread in the rest of the follicle [30].

It is not yet clear whether this altered environment is important for the proper development of the oocyte (which is linked to cumulus cells by numerous gap junctions) or if it is the consequence of the presence of the oocyte [106, 107].

4. THE OOCYTE CUMULUS COMPLEX FLOATS IN FOLLICULAR FLUID

Follicular fluid is a complex mixture of serum proteins and proteins secreted by follicle cells. Transit of proteins through the basal membrane appears to be related to molecular weight and charge and possibly protein conformation. Following two dimensional electrophoresis of newly synthesized proteins (S35 incorporated) by intact follicles, 100–150 spots are observed of which 95 % are present in follicular fluid and are transferred through the basal membrane

towards incubation medium [31]. One protein (52 kDa) is stored in follicular fluid and not secreted outside the follicle and three proteins (30, 31 and 90 kDa) are more abundant in follicular fluid than outside the follicle. However, none of these newly synthesized proteins can be visualized on silver stained gels of follicular fluid. This is because most of its proteins originate from serum. Hence compounds originating in serum as well as ovary specific compounds are candidates for affecting oocyte maturation.

5. THE OOCYTE IS SENSITIVE TO FOLLICULAR SECRETIONS

Follicular cells (granulosa and theca) produce large amounts of steroids and growth factors or proteins [41]. Most of them are at least partly stored in follicular fluid.

As regards steroids, Wu et al. [115] have demonstrated that the human oocyte expresses mRNA for the oestrogen receptor (α type). This suggests that oestradiol may directly act on the oocyte. This finding has not, however, been confirmed in farm animals either by RT-PCR or immunohistochemistry. There is no information, at present, on expression or presence of the β type oestradiol receptor or of the androgen receptor on oocytes of mammalian species.

With respect to growth factors, the oocyte appears to bear receptors for EGF/TGF α [17, 81], for IGF1 and IGF2 [95] and PDGF [113]. There has been no demonstration that receptors for other growth factors are present (TGF β , MIS, FGF) despite observations that these compounds (TGF β , MIS) can affect function of denuded oocytes [35, 93].

Finally data demonstrating the presence of activin receptors on the oocyte have been presented in rats [10] and cattle [50]. This is in good agreement with the accumulation of ^{125}I activin along the zona pellucida after *in vivo* injection [114].

6. AUTOCRINE AND PARACRINE REGULATION BETWEEN OOCYTE AND GRANULOSA CELLS INVOLVED IN THE CONTROL OF OOCYTE GROWTH

Three sequential phases of growth can be visualized when oocyte diameter is plotted against follicle diameter: a) initiation of oocyte enlargement; b) rapid oocyte growth up to antrum formation in the follicle; c) slow growth between this stage and ovulation. Possible regulatory mechanisms for each specific phase are detailed below by addressing three specific questions.

1) What triggers initiation of oocyte growth?

Whether initiation of oocyte growth is caused by compound(s) originating from the oocyte or from the surrounding granulosa cells has not yet been clarified. This issue is complicated by the existence, at least in rodents, of different reserves of primordial follicles, each containing oocytes of different diameters and surrounded by variable numbers of granulosa cells [67]. When follicles present 10–15 granulosa cells in the largest cross section, oocyte nucleolar RNA-polymerase increases [60] suggesting that oocyte growth is initiated. However, the relationships between granulosa cell proliferation and activation of nucleolar RNA polymerase in the oocyte have not been clarified. On the one hand, granulosa cell proliferation may increase the amounts of compounds (such as kit ligand, KL) stimulating oocyte growth [76] while on the other, oocyte growth may affect granulosa cell proliferation [106].

Key compounds for growth initiation are c-kit, a receptor present on the oocyte membrane at the primordial stage and its ligand (KL) produced by granulosa cells. Strong evidence supporting this claim has been obtained from two lines of mice (St^t and St^{pan}) which harbour mutations in the KL gene. In these lines few follicles initiate growth despite numerous primordial fol-

licles being present in the ovaries [49, 57]. The role (if any) of other compounds present in the oocyte (myc oncoprotein, retinoblastoma protein; Li et al. [59]; Bukowsky et al. [8]) or in granulosa cells of resting follicles (WT1 [48]) remains to be clarified. New genes involved in growth initiation may be identified from two lines of sheep (Cambridge, Inverdale) where major genes are segregating [22, 43] and where part of the ewes are sterile owing to the lack of synchrony between oocyte growth (which occurs) and granulosa cell proliferation (which is absent) in Cambridge ewes or owing to absence of initiation of follicular and oocyte growth in Inverdale ewes [23].

2) Which compounds are responsible for the rapid growth of the oocyte during the preantral stage?

There is solid evidence for involvement of three main factors (c-kit + KL, EGF and FGF) in oocyte enlargement, during the rapid growth phase. The key mechanism again appears to be the interactions between c-kit and KL. In the elegant study of Packer et al. [76], a stimulatory effect of graded doses of KL was observed on oocyte growth. Furthermore, incubation of oocytes on monolayers of granulosa cells increased the amounts of mRNA coding for KL [76]. This loop whereby the oocyte stimulates the production of KL and where KL stimulates oocyte growth is of utmost importance at this stage. This is further supported by the observation [117] that blocking the c-kit-KL interaction *in vivo* completely abolishes preantral oocyte and follicular growth.

Fibroblast growth factor (FGF) which is present in oocytes of preantral follicles [105], for which receptors are present on preantral granulosa cells [109] and which is mitogenic for granulosa cells [72] may also be operating to promote growth at this stage by a paracrine signalling between the oocyte and the granulosa cells.

The same reasoning could apply to epidermal growth factor (EGF)/transforming growth factor α (TGF α) which is present

in oocytes [87], for which receptors are present in granulosa cells [109] and which also stimulates granulosa cell replication [40]. The FGF and EGF stimulated increase in the number of granulosa cells will increase the amounts of KL produced by this layer, which will in turn stimulate further oocyte growth. The cAMP produced after binding of FSH to its receptors which are present on granulosa cells at the preantral stage may also operate in a similar way [76]. Interestingly, a novel regulator of this phase was identified [24] when it was shown that follicular growth was blocked during the preantral phase in GDF9 (a member of the TGF β superfamily) knock out mice.

3) Why does oocyte growth ceases when antrum forms?

This growth arrest could be explained either by the appearance of compounds which inhibit oocyte growth (for compounds appearing at the antral stage, see *figure 1b*) or by the disappearance of growth promoting compounds. While there is no data supporting an effect of insulin-like growth factors (IGF1/IGF2), TGF β 2, inhibin or activin A on oocyte growth, there is good evidence [70] that the cessation of oocyte growth at the antral stage is associated with the cessation of KL expression in the cumulus cells surrounding the oocyte. This block in the c-kit-KL stimulatory loop may explain the slow pace of growth noted in oocytes throughout the antral phase.

7. AUTOCRINE AND PARACRINE REGULATIONS BETWEEN THE OOCYTE AND GRANULOSA CELLS INVOLVED IN THE CONTROL OF OOCYTE APOPTOSIS

Signals that lead to the induction of apoptosis include oxydative stress, the withdrawal of extracellular signals (specific growth factors) as well as the stimulation of some cell surface molecules such as the TNF α receptor or the Fas/Apo1 molecule. A num-

ber of excellent reviews are available on the general mechanisms involved [18, 108]. Briefly, induction of apoptosis is followed by ceramide generation, which will ultimately activate caspases (CPP 32) which are proteases able to induce nuclear DNA fragmentation, plasma membrane (blebbing) and mitochondrial changes (alterations in mitochondrial membrane potential). Cell death (Bax, Bcl \times short) or cell survival (Bcl2, Bcl \times long) proteins modulate caspase activation.

All the apoptotic machinery is present in oocytes. TNF α receptor is present on the surface of oocytes [71] as is Fas/Apo1 [112]. Bcl2 and Bax can be identified by immunohistochemistry within oocytes (K. Reynaud and M.A. Driancourt, unpublished data). That both compounds are involved in oocyte apoptosis is shown by the observations that genetic ablation of Bcl2 and Bax expression [82, 97] markedly alter the number of primordial follicles present in post-natal ovaries. A similar conclusion also stands for caspases since caspase 2 knockout mice exhibit an increased number of primordial follicles in post-natal ovaries [97]. While the pathways used for the development of the apoptotic process are relatively clear, the way in which oocytes and granulosa cells interact to escape or promote apoptosis is poorly understood. In fact, it is not even known if the normal fate of oocytes is apoptosis (survival signals are then of utmost importance to permit growth initiation) or growth (death signals then determine the numbers of follicles initiating growth). The answer to that question may vary with the follicle type. Within the reserves of primordial follicles, the high expression of proteins such as TNF α [16], TNF α receptor [71], myc [59], retinoblastoma protein [8], Fas/Apo1 [112] may indicate that, for primordial follicles, the normal fate is cell death. However, it is puzzling that cell death within the primordial follicle population appears to be spread throughout reproductive life, particularly in primates. Survival factors have not yet been identi-

fied. Within preantral follicles, the opposite answer may be valid since the extent of apoptosis is far more limited than in primordial follicles. With the development of *in vitro* cultures of preantral follicles in rodents, significant information regarding compounds modulating apoptosis was obtained. A pro-apoptotic (TGF β at 10 ng/mL) and two anti-apoptotic (FGF and EGF at 50 ng/mL) growth factors for preantral follicles were identified [33, 111]. Whether or not they work by acting directly on the oocyte has not been established. While demonstrating their mechanism of action may be difficult because oocytes isolated from surrounding somatic cells survive and grow poorly, it should be remembered that, in male gonocytes, TGF β 1 and β 2 have been shown to be pro-apoptotic [74] while FGF appears to be anti-apoptotic [104]. Interestingly, when preantral follicles are cultured *in vitro* individually, very few of them become apoptotic [19]. In contrast, when two such follicles are cultured in contact in the same culture well, one of them displays a reduced growth rate, ultimately leading to death [89]. The compounds involved in these between follicle interactions remain to be identified.

8. AUTOCRINE AND PARACRINE REGULATIONS BETWEEN THE OOCYTE AND GRANULOSA CELLS INVOLVED IN THE DEVELOPMENT OF THE OOCYTE'S ABILITY TO RESUME MEIOSIS

Regarding the links between the amounts of steroids in follicular fluid and the ability of the oocyte to resume meiosis to reach the metaphase II stage, all available data have been obtained in humans undergoing ovarian stimulation and following hCG administration. Owing to the pharmacological amounts of hormones used and to the heterogeneity of the follicular cohort generated, they should therefore be considered with caution. Most reports agree that follicular fluids which

contain immature (i.e. not having undergone germinal vesicle breakdown – GVBD) oocytes usually contain lower amounts of oestradiol than those containing metaphase II oocytes [2, 6, 54]. There are no identical data available in other models such as farm animals. In one of the above studies, the follicular fluid concentrations of EGF and IGF1 were also compared between follicles yielding mature and immature oocytes [2], demonstrating similar amounts of these growth factors in the two types of follicular fluids. This claim should be viewed with caution because assessment of EGF concentrations in follicular fluid is hampered by the presence of EGF binding compounds and also because IGF binding proteins, which are the key factor affecting IGF1 bioactivity, were not measured in the above study.

The role of steroids on nuclear maturation was also explored by using experimental alterations of steroidogenesis (administration of inhibitors of steroidogenesis) or by studying patients genetically deficient in specific steroidogenic enzymes. In primates [118], administration of an aromatase inhibitor during the mid follicular phase produced a drop in oestradiol and a rise in testosterone outputs by the maturing follicle. This altered steroidal environment markedly reduced the proportion of oocytes reaching the metaphase II stage. This observation is supported by the findings obtained in sheep [75] where follicles were cultured for 24 h in the presence of an inhibitor of 17 α hydroxylase. Oocytes contained in such follicles could undergo GVBD but were blocked before or at the metaphase I stage. The limitation of such approaches is, however, that they do not identify the steroids which are of key importance (low oestradiol, varying amounts of testosterone or their ratio). Interestingly, a recent study using an inhibitor of progesterone synthesis, epostane [91] did not demonstrate any effect of withdrawing progesterone on oocyte maturation in primates. Overall, these pharmacological approaches may suggest that the ratio be-

tween specific steroids may be more important than the actual amounts of these steroids. In any case, steroids do not appear to be a strict prerequisite for nuclear maturation as there is evidence that patients genetically deficient in 17α hydroxylase [80] or $17-20$ desmolase [78] can successfully produce mature oocytes after administration of exogenous gonadotrophins despite very low amounts of androgens + oestrogens ($17-20$ desmolase) or oestrogens (17α hydroxylase) in follicular fluid. Along the same line, the observation of a patient with Laron syndrome [25] who had minimal IGF1 levels and managed to develop mature oocytes following administration of exogenous gonadotrophins also demonstrates that IGF1 is not a prerequisite for successful maturation of the oocyte.

Most of the solid information on the interactions between follicular products and nuclear maturation has been obtained *in vitro* by supplementation of IVM culture media with specific steroids, growth factors or proteins. For example, a very clear inhibitory effect of testosterone on the nuclear status of the oocyte after 18 h of culture has been reported in mice [1]. This effect appeared to be dose dependant and could also be demonstrated on denuded oocytes. Although such an effect appears clear cut, no confirmatory studies have been conducted in other species including domestic animals. Epidermal growth factor (EGF/TGF α) is the growth factor for which a major effect on nuclear maturation of most species has been demonstrated. In mice [21, 26], cattle [44, 51, 62, 63], pig [20, 83] and primates [39], EGF/TGF α appears to have stimulatory effects on GVBD and/or on the percentage of oocytes reaching the metaphase II stage. Because the information on the amounts of EGF present in follicular fluid of these species is not solid, it is impossible to state whether the amounts used are actually physiological. It is also unclear if EGF/TGF α has the same potency on denuded oocytes since contradictory reports exist in mice [21, 26]. There is a large consensus on the lack of

effects of IGF1 on resumption of meiosis (mice: Downs [27]; cattle: Lorenzo et al. [63]; pig: Reed et al. [83]). Interestingly, as regards TGF β , effects ranging from stimulatory [35] or null [27, 100] have been described in rodents, while inhibitory effects have been reported in swine [20]. Finally, the protein family which appears to have large effects on oocyte's nuclear maturation is the inhibin/activin/follistatin/MIS family of proteins. Stimulatory effects of inhibin (10 ng/mL) on nuclear maturation of cattle oocytes were reported recently [90]. This positive effect of inhibin is also suggested by data obtained in pigs by Miller et al. [69] who demonstrated higher amounts of immunoreactive follicular fluid α inhibin concentrations in follicles which contained GVBD oocytes compared to immature ones. Hence, it is puzzling that O et al. [73] failed to demonstrate such a positive effect in rats. In contrast, in the latter species, activin appears to have the ability to dose dependently induce GVBD [53, 85] although it is unclear whether it is also effective on denuded oocytes [53, 73]. By comparison, oocytes of large animals do not appear to be sensitive to activin (pig: Coskin and Liu [20]; cattle: Van Tol et al. [103]; Stock et al. [90]). As MIS appears to be preferentially produced by cumulus cells [4], it may play a role in resumption of meiosis since Takahashi et al. [93] and Ueno et al. [101] demonstrated in rats that MIS could dose dependently inhibit GVBD and that this inhibition was independent of cumulus cells.

Another pair of proteins (c-kit and its ligand KL) also appear to be relevant for the control of oocyte maturation since a recent report [52] claimed that c-kit expression when decreased was associated with an increased ability to resume meiosis. Furthermore, addition of KL to the culture medium of oocytes could also delay resumption of meiosis.

An attempt to summarize the effects of all these molecules on resumption of meiosis is presented in *figure 2*.

9. AUTOCRINE AND PARACRINE REGULATIONS INVOLVED IN THE CYTOPLASMIC MATURATION OF THE OOCYTE

Three approaches have been used to study the relationships between follicular products and oocyte cytoplasmic maturation. In the first one, the concentration of specific compounds in follicular fluid was related to the oocyte's ability to develop, mostly in human. Oocytes and follicular fluid were collected following ovarian stimulation and hCG administration. Regarding the relationships between specific steroids and quality of the oocyte, the numerous studies performed have generally found that oocytes enclosed by oestradiol-rich follicular fluid were of better quality [6, 13, 36] although some other studies failed to confirm this finding [92, 94, 116]. In contrast, there is a general agreement between all studies for the lack of correlation between progesterone concentrations in follicular fluid and oocyte quality [6, 92, 94, 116]. The interpretation of these findings is further complicated by the observation that

steroid binding proteins are present in follicular fluid (SHBG and CBP) and that the amounts of steroid binding proteins appear to be also related to the ability of the oocyte to develop following IVF [116]. Interestingly, the two studies which have related the amounts of gonadotrophins (FSH, LH) in follicular fluid to oocyte quality have shown that high concentrations of FSH [92] or hCG [58] are associated with an improved ability of the oocyte to develop following IVF. This may suggest that follicular vascularization (which regulates gonadotrophin delivery to follicles) may play a role in oocyte quality, a finding confirmed by the study of Itskovitz et al. [54] showing that prorenin concentration in follicular fluid can be related to the success of IVF. Only one study has related the amounts of growth factors in follicular fluid and oocyte quality. Artini et al. [2] could not relate the ability of the oocyte to fertilize with the follicular fluid concentrations of either IGF1 or EGF. Franchimont et al. [37] demonstrated a link between high follicular fluid inhibin and low aromatase inhibitor and an oocyte of good quality.

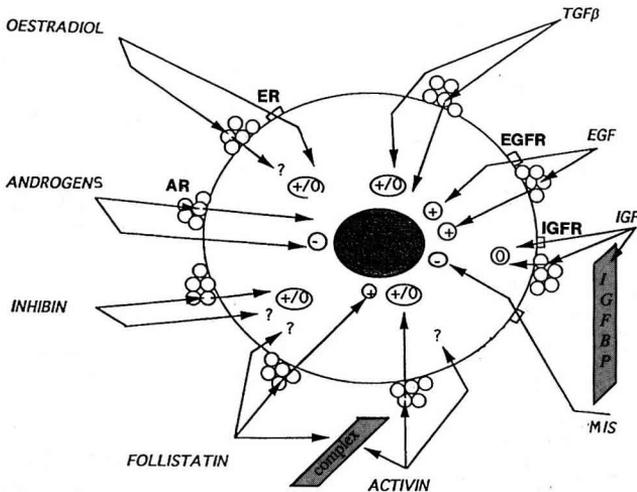


Figure 2. Molecules modulating oocyte nuclear maturation either directly (effects obtained on denuded oocytes) or via the surrounding cumulus cells (○). Positive effects are indicated by (+) while inhibitory effects are indicated by (-). (0) is used when no clear effects were obtained. Receptors are indicated on the oocyte surface (AR: androgen receptor; ER: oestradiol receptor).

However, because all this information was obtained at stages of maturation markedly different from that in domestic animal IVF, caution should be exercised before extrapolating these conclusions to cattle. In this species, only one study [45] has attempted to correlate blastocyst development with features of follicular fluid following analysis of individual oocyte/follicles. They showed that high progesterone was detrimental to oocyte quality while there was no relationship between amounts of oestradiol and IGF1 in follicular fluid and oocyte quality. We (Driancourt et al., unpublished results) have recently completed a study combining IVM/IVF/IVD of individual oocytes with the study of numerous follicular markers to extend these findings. Oocytes ($n = 93$) were grouped in three categories following IVD. Class A were oocytes which developed to the blastocyst stage. Classes B were oocytes which cleaved but did not reach the blastocyst stage. Class C were oocytes which did not cleave following IVF. The respective proportions of classes A, B and C were 40, 35 and 25%. The main conclusions of this study were that: 1) there was no difference between class A, class B and class C oocytes in the amounts of steroids (oestradiol, testosterone, progesterone) of their follicles of origin; 2) healthy follicles (as evidenced by high aromatase activity) contained more class A oocytes than atretic follicles; 3) high amounts of specific α inhibin forms could be found in follicular fluid containing class A oocytes. In contrast, amounts of β inhibin were identical in follicular fluid containing class A, B or C oocytes; and 4) numerous compounds (LH receptors, FSH receptors, EGF receptors, progesterone receptors, amounts of matrix metalloproteinases and of TIMP's in follicular fluid, etc.) could not be related to oocyte quality.

In the second one, pharmacological models in which follicular steroidogenesis is altered have been used to assess whether steroids may be involved in cytoplasmic maturation of the oocyte. Administration of an aromatase inhibitor [118] or of an inhi-

bitor of 3β hydroxysteroid deshydrogenase to primates during the mid follicular phase [91] did not alter follicle development but markedly reduced the ability of the oocyte to develop following fertilization. Both studies provide solid evidence that a proper steroid environment within the follicle is important for oocyte quality. The limit of these approaches is, however, that it is impossible to identify which steroids are important, because prevention of synthesis of a specific steroid results in accumulation of its precursors.

In the third one, *in vitro* approaches have been undertaken to test the physiological effects on oocyte quality of adding specific molecules to the maturation medium. This approach proved very fruitful to identify compounds which were stimulatory (EGF/TGF α , inhibin/activin), inhibitory (androgens) or had no clear activity (IGF1/IGF2) to cytoplasmic maturation. The only steroid for which a clear effect has been observed is testosterone for which Andriesz and Trounson [1] demonstrated a dose-dependant inhibitory effect on cytoplasmic maturation. Regarding growth factors, several studies have demonstrated a positive role of EGF on cytoplasmic maturation. Beneficial effects of addition of EGF/TGF α to the maturation medium were observed on cytoplasmic maturation of mice [21] or cattle [55, 61] oocytes. There is no general agreement on the effects of IGF1/IGF2 on cytoplasmic maturation. On the one hand Herrler et al. [46] found a beneficial effect while Carolan et al. [12] found no effect at all. Finally, neither TGF β or FGF appear to have the ability to affect cytoplasmic maturation [55]. Interestingly, a recent report described a positive effect of inhibin or activin on cytoplasmic maturation [90]. Both compounds were active and there was no synergy between inhibin and activin. This finding is, however, difficult to reconcile with the observations that follicular fluid (inhibin/activin rich medium) has the same effect on cytoplasmic maturation as serum (inhibin/activin poor medium) [61, 88]. A summary of these effects is presented in *figure 3*.

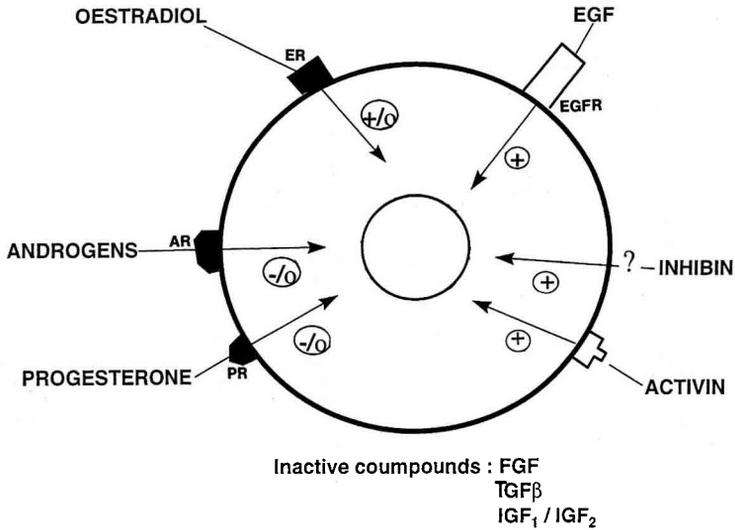


Figure 3. Molecules modulating cytoplasmic maturation of the oocyte. Positive effects and inhibitory effects are noted (+) and (-), respectively.

10. CONCLUSIONS

Despite the large amount of literature searched in writing this review, our understanding of most of the key phases of oocyte development is far from complete. Significant progress requires that several questions are addressed.

1) Are all oocytes similar? Heterogeneity within the oocyte population is obvious. What causes this heterogeneity and whether it tends to change with ageing also needs further investigation. Is the time of oocyte formation and of primordial follicle formation important for the fate (growth or apoptosis) of the follicle?

2) Amongst the compounds already shown to affect oocyte quality, which are of critical importance and which are only of limited importance?

3) How does follicular atresia affect oocyte quality? Are oocytes in atretic follicles better [5] or worse (Driancourt et al., unpublished results) than those originating from healthy follicles?

4) What are the mRNAs or proteins produced in the oocytes at the stage of follicular growth when the oocyte gains the ability to undergo nuclear or cytoplasmic maturation? Differential display techniques will be helpful to address this problem.

5) What are the mRNAs still actively produced in the oocytes on the day before ovulation (in vivo maturation) or during IVM (in vitro maturation)? How do they differ between the in vivo and in vitro groups? Again in vitro transcription techniques or differential display could be useful.

Major improvements in the IVM/IVF/IVD systems would surely benefit from clear answers to the above questions.

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