

# Oocyte control of ovarian follicular development and function in mammals

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A new perspective on ovarian follicular development has emerged over the last decade. Whereas the oocyte was previously considered only a passive recipient of developmental signals from oocyte-associated granulosa cells, it is now clear that communication between oocytes and granulosa cells is bidirectional. A complex interplay of regulatory factors governs the development of both types of cell. This interplay is essential not only for oocyte development but also for follicular development, beginning with the initial assembly of the primordial follicle and continuing throughout ovulation. The existence of an oocyte–granulosa cell regulatory loop, essential for normal follicular differentiation as well as for the production of an oocyte competent to undergo fertilization and embryogenesis, is proposed. Although gonadotrophins are essential for driving the differentiation of granulosa cell phenotypes, within its sphere of influence, the oocyte is probably the dominant factor determining the direction of differentiation and the function of the granulosa cells associated with it.

It has long been realized that follicular somatic cells support oocyte development. The close physical association of these two types of cell indicated this to early biologists, but physiological evidence was first presented by Pincus and Enzmann (1935), who found that fully grown oocytes removed from antral follicles underwent a spontaneous, gonadotrophin-independent resumption of meiosis in culture, and concluded that follicular somatic cells maintain oocytes in meiotic arrest. Subsequent studies demonstrated that follicular somatic cells promote the reinitiation of meiosis and its progression to metaphase II (nuclear maturation). Follicular somatic cells also promote oocyte competence to undergo fertilization and pre-implantation embryogenesis (cytoplasmic maturation) (Buccione *et al.*, 1990a) and granulosa cells participate in the global suppression of transcription in oocytes that occurs before nuclear maturation (Fig. 1) (De la Fuente and Eppig, 2001). In contrast to granulosa cell-to-oocyte communication, knowledge of oocyte-to-granulosa cell communication is relatively recent. The studies of Nalbandov and colleagues pioneered this field, observing a precocious luteinization of rabbit follicles *in vivo* after removal of the oocyte–cumulus complex (El-Fouly *et al.*, 1970). Similarly, granulosa cells from antral follicles cultured in the absence of oocytes were observed to resemble luteinized granulosa cells, whereas those cultured in the proximity of oocytes appeared to maintain a granulosa cell-like appearance (Nekola and Nalbandov,

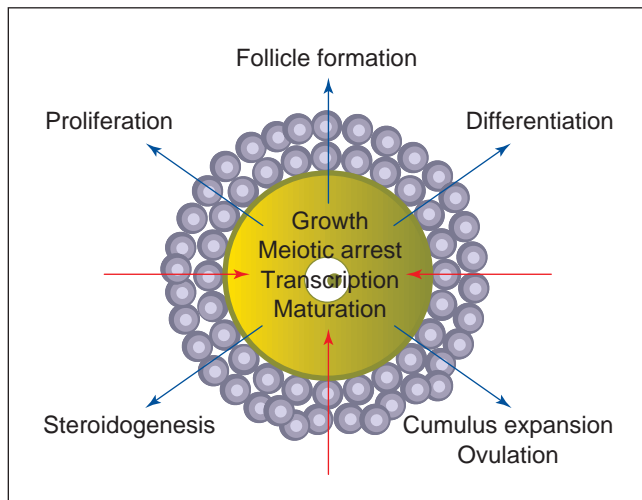
1971). These authors concluded that oocytes secrete an anti-luteinization factor. However, subsequent studies failed to support this hypothesis, as pig oocytes appeared to have no effect on progesterone production, a hallmark of luteinized cells, by granulosa cells *in vitro* (Channing and Tsafiriri, 1977). This last study had a discouraging effect on further research in this area for more than a decade. However, since 1990, data have emerged showing that oocytes can regulate progesterone production as well as play key roles in controlling granulosa cell development and function from the time of follicular organization and continuing throughout ovulation (Fig. 1).

## Co-ordination of oocyte and follicular development

Soon after oocytes enter a prolonged diplotene stage of meiosis, the dictyate stage, the precursors to the follicular somatic cells encompass the oocyte in a single squamous layer to form primordial follicles. The large population of non-growing primordial follicles serves as the source of developing follicles and oocytes until the end of a female's reproductive life. Until that time, there is a continuous recruitment of follicles from this pool, beginning with the formation of primary follicles. The oocyte in a primary follicle begins its extensive growth phase, and the surrounding follicular cells (now called granulosa cells) become cuboidal and proliferative. When the growing oocytes are surrounded by more than one layer of granulosa cells, the follicle is called a secondary follicle. Although primary and secondary follicular development can take place in the absence of gonadotrophins, these follicles are

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**Fig. 1.** Bidirectional communication between oocytes and companion somatic cells showing the influences of granulosa cells on oocyte development and the processes in granulosa cells controlled by oocytes.

responsive to gonadotrophins and therefore optimal development of preantral follicles may require these hormones (Fortune and Eppig, 1979; Cortvrindt *et al.*, 1997). The initial signals for the development of a follicular antrum are not well understood, but at some point, cavities form and fill with follicular fluid. Development beyond the early antral follicle stage is clearly dependent upon gonadotrophins; antral follicles are sometimes called tertiary follicles. Well-developed antral follicles are often referred to as Graafian follicles. However, after the preovulatory gonadotrophin surge, they are called preovulatory follicles. The formation of a follicular antrum divides the population of granulosa cells into two main groups: cumulus cells associated with the oocyte and mural granulosa cells lining the follicular wall. The mural granulosa cells nearest the antrum are called periantral granulosa cells.

About the time of transition from preantral to antral follicles, a critical developmental change also takes place in the oocytes. Before antrum formation, oocytes are unable to progress beyond the diplotene stage of meiosis I. These oocytes are therefore referred to as meiotically incompetent, a state that is attributable to an insufficiency in regulatory molecules necessary to drive meiotic progression. However, most oocytes in antral follicles are meiotically competent and will resume meiosis spontaneously if removed from the follicle and cultured in supportive medium (for a review, see Handel and Eppig, 1997). It was this observation that led Pincus and Enzmann (1935) to conclude that follicular somatic cells arrest the progression of meiosis in competent oocytes.

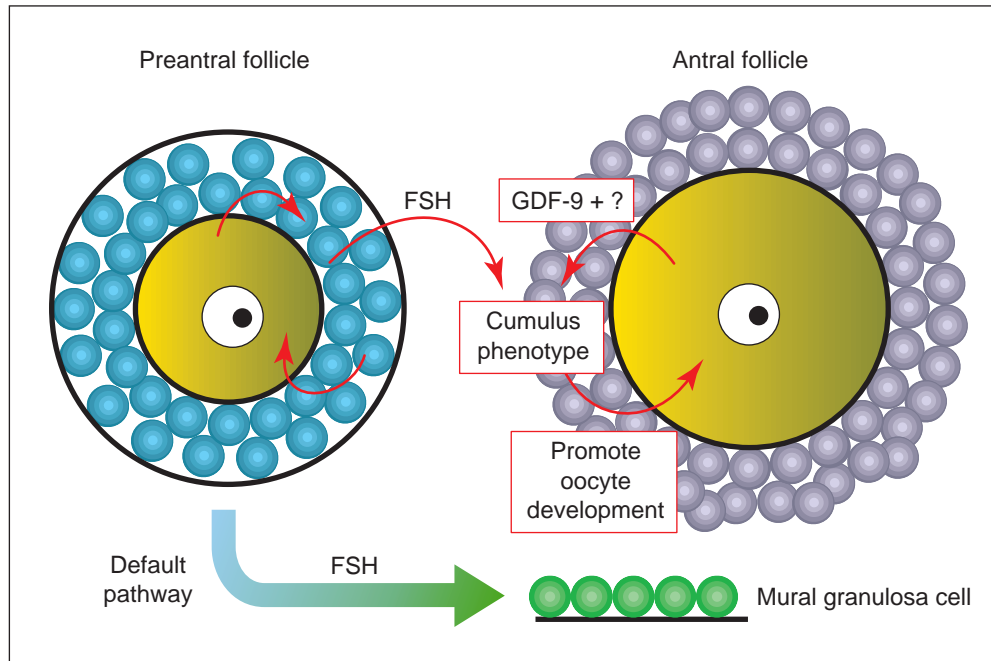
Although oocytes are generally thought to complete their growth phase near the transition from preantral to antral

follicles, this is not strictly true. The increase in oocyte diameter during antral follicular growth is relatively small, but the increase in oocyte volume, or mass, is significant. For example, the diameter of oocytes from medium size antral follicles of 18-day-old mice is about 73  $\mu\text{m}$ , whereas that of oocytes from larger antral follicles of 22-day-old mice is about 76  $\mu\text{m}$ , representing an increase in diameter of about 4%. However, at the same time, the volume increases by almost 13% from 203 770 to 229 940  $\mu\text{m}^3$  in oocytes from 18- and 22-day-old mice, respectively (Eppig and O'Brien, 1996). Nevertheless, the large oocytes isolated from gonadotrophin-primed 22–24-day-old mice will be referred to here as 'fully grown'.

Oocytes continue to grow after they acquire competence to resume meiosis. Nevertheless, oocytes competent to resume meiosis are not necessarily competent to undergo complete nuclear maturation and progress to metaphase II. Oocytes isolated from small antral follicles are competent to undergo germinal vesicle breakdown (GVBD) and progress to metaphase I, but the progression of meiosis in these oocytes usually becomes arrested at this stage. Therefore, further development of oocytes in antral follicles is required for oocytes to become competent to progress to metaphase II (Handel and Eppig, 1997).

The preovulatory surge of gonadotrophins induces marked changes in both the follicle and the oocyte–cumulus cell complex. Oocytes resume meiosis and progress to metaphase II before ovulation in most, but not all, mammals that have been studied. As oocytes mature in response to the preovulatory gonadotrophin surge, cumulus cells secrete hyaluronic acid, a non-sulphated glycosaminoglycan bound to the cumulus cells by linker proteins (Eppig, 1979; Salustri *et al.*, 1989; Chen *et al.*, 1996). When the hyaluronic acid becomes hydrated, spaces between cumulus cells become enlarged, and the cells become embedded in a sticky, mucified matrix. Hence, this process is called cumulus expansion or mucification. If cumulus expansion is suppressed, ovulation rate is greatly reduced (Chen *et al.*, 1993). Thus, cumulus expansion is one of several important processes that must occur in preovulatory follicles to enable ovulation. The mural granulosa cells remaining within the ovary undergo further differentiation and luteinization. These cells together with theca cells subsequently form the corpus luteum, which is essential for the maintenance of pregnancy.

It would be difficult to imagine that the highly coordinated development of the oocyte and somatic cell compartments of ovarian follicles could occur without constant intercommunication between the cell types. What follows is not only a summary of progress in the discovery of the role of oocytes in affecting the differentiation and function of granulosa cells, but also a presentation of the hypothesis that there is an oocyte–granulosa cell regulatory loop. Signals exchanged within this loop are thought to be essential for inducing and co-ordinating the differentiation of both the oocyte and somatic compartments from one developmental stage to the next (Fig. 2).



**Fig. 2.** Hypothesized oocyte–granulosa cell regulatory loop and its possible function during the transition from preantral to antral follicles. At this stage of follicular development, factors from granulosa cells, such as Kit Ligand, promote oocyte development, whereas factors from the oocyte influence granulosa cell development and function. These interactions, in concert with gonadotrophins and other factors, promote the transition to antral follicular development. In antral follicles, oocyte-derived factors, such as growth differentiation factor 9 (GDF-9), promote the development of the cumulus cell phenotype by suppressing expression of the mural granulosa cell phenotype. Without the influence of these factors from oocytes, mural granulosa cells differentiate as the default programme. The cumulus cell phenotype, promoted by GDF-9 and probably other factors as well, is essential for promoting the development of oocytes competent to undergo fertilization and embryogenesis.

## Role of the oocyte in follicular development

### Primordial follicle formation

Oocytes are essential for follicular formation; without oocytes, follicles are not assembled. This contrasts with the formation of the male homologue, the seminiferous tubule, which can form in the absence of spermatocytes. Although little is known regarding the mechanisms of follicular formation, at least one oocyte-expressed gene is required for this process. *Figla* (also known as *Fig $\alpha$* ) is a gene encoding a helix–loop–helix transcription factor that co-ordinates the expression of the structural genes for components of the zona pellucida. Expression of *Figla* mRNA begins as early as embryonic day 13 in mice, more than a week before zona pellucida genes are expressed in oocytes. Moreover, primordial follicles do not form after mutation of *Figla* by homologous recombination (Soyal *et al.*, 2000). Thus, *Figla* appears to regulate at least two important pathways in oocytes, although the relationship between these pathways is not obvious. One pathway co-ordinates the production of zona pellucida proteins, and the other regulates the production of one or more factors by

the oocyte that are essential for the initial organization of primordial follicles. This factor might be a secreted chemotactic factor that summons the prefollicular somatic cells to the oocyte, or a surface adhesion molecule that establishes and maintains contact with appropriate somatic cells. It remains a matter of speculation whether the follicular organization pathway remains functional during subsequent oocyte–follicular development when the zona pellucida is produced.

### Early follicular development

Although the mechanisms for selecting and activating primordial follicles for entry into the cohort of developing follicles are not known, the oocyte clearly plays a key role in development beyond the primary follicle stage. Growth differentiation factor 9 (GDF-9) is an oocyte-specific member of the TGF $\beta$  family produced by mouse, cow, sheep, rat and human oocytes (McGrath *et al.*, 1995; Laitinen *et al.*, 1998; Aaltonen *et al.*, 1999; Bodensteiner *et al.*, 1999). Thus, GDF-9 is probably a paracrine regulatory factor produced by all mammalian oocytes. It is expressed

by oocytes throughout ovulation. In mice, expression by oocytes begins in primary follicles, whereas in other species, it is also expressed by primordial oocytes. Mutation of the *Gdf9* gene by homologous recombination in mice does not prevent recruitment of primordial follicles to the primary stage. However, further development of the somatic cells beyond the primary follicle stage fails in *Gdf9*-null mice (Dong *et al.*, 1996). Nevertheless, the oocytes continue to grow, at an accelerated rate, and oocyte morphology eventually becomes abnormal (Carabatsos *et al.*, 1998). Granulosa cell expression of the *Kitl* gene, encoding Kit Ligand (KL) mRNA, appears to be increased in follicles of *Gdf9*-null mice (Elvin *et al.*, 1999b), and KL is thought to promote oocyte growth or development, or both (Packer *et al.*, 1994; Reynaud *et al.*, 2000). This finding indicates that GDF-9 suppresses *Kitl* expression in granulosa cells. Indeed, recombinant GDF-9 does suppress *Kitl* expression by granulosa cells isolated from both preantral and antral follicles (Joyce *et al.*, 2000). However, when these groups of granulosa cells were co-cultured with either medium-sized oocytes from preantral follicles or with fully grown oocytes from antral follicles, only the fully grown oocytes were able to suppress *Kitl* expression by either group of granulosa cells. The medium-sized oocytes either had no effect or a slightly stimulatory effect on *Kitl* expression by preantral granulosa cells *in vitro*. Removal of the fully grown oocyte (oocytectomy) from oocyte-cumulus cell complexes isolated from antral follicles resulted in increased *Kitl* expression, which was suppressed by co-culture with fully grown oocytes (Joyce *et al.*, 2000). Thus, the regulation of *Kitl* expression in the primary follicles of *Gdf9*-null mice seems more similar to the regulation of *Kitl* expression in the oocyte-cumulus cell complex of antral follicles than would be expected in normal preantral follicles. However, this issue is probably more complicated since the concentration of FSH in *Gdf9*-null mice is increased (Dong *et al.*, 1996) and FSH alone promotes *Kitl* expression by preantral granulosa cells but not by granulosa cells from antral follicles (Joyce *et al.*, 1999).

Assuming that GDF-9 from fully grown oocytes suppresses *Kitl* expression by cumulus cells, and that KL may promote oocyte growth, an oocyte-granulosa cell regulatory loop that affects oocyte growth can be postulated. In this model, KL produced by granulosa cells of preantral and early antral follicles would promote oocyte growth until a species-specific size is reached. At this point, GDF-9 secreted by oocytes would suppress *Kitl* expression in cumulus cells, which would slow or terminate oocyte growth. It is not known why GDF-9 from medium-sized oocytes does not suppress *Kitl* expression. Perhaps GDF-9 secretion is not constitutive by medium-sized oocytes, but instead is regulated by granulosa cells. Alternatively, GDF-9 may be secreted in an inactive form by medium-sized oocytes, or certain factors from these oocytes may inhibit its action. However, preantral follicular granulosa cells clearly respond to recombinant GDF-9 and to factors from fully grown oocytes in suppressing *Kitl* expression, although the

role of KL in promoting oocyte growth remains controversial. FSH markedly stimulates *Kitl* expression in preantral follicular granulosa cells, yet oocytes grow in the absence of FSH. Moreover, oocytes appear to grow in animals treated with neutralizing antibodies to KL despite clear effects of these antibodies on follicular development (Yoshida *et al.*, 1997). These effects are probably mediated by interstitial cells that, in addition to oocytes, also express KIT receptors. However, evidence indicates that KL plays some role in oocyte growth. Under some experimental conditions, KL treatment *in vitro* appears to accelerate oocyte growth (Packer *et al.*, 1994), and some mutant *Kitl* alleles, for example *Kitl<sup>SL-pan</sup>* (Huang *et al.*, 1993; Bedell *et al.*, 1995), cause both decreased oocyte growth and follicular development. However, the results of other studies *in vitro* do not support a role for KL in oocyte growth, but indicate that KL promotes oocyte survival and cytoplasmic maturation (Reynaud *et al.*, 2000). Nevertheless, expression of KL by granulosa cells, and its receptor by oocytes, strongly implies a functional relationship of some kind. This observation, together with the clear ability of oocytes to regulate *Kitl* expression, probably via GDF-9, reveals a complex oocyte-granulosa cell regulatory loop.

Another member of the TGF $\beta$  family, *Bmp15*, also known as GDF-9B, exhibits an expression pattern similar to that of *Gdf9* (Dube *et al.*, 1998; Jaatinen *et al.*, 1999; Elvin *et al.*, 2000b; Otsuka *et al.*, 2000). Homozygosity of a natural mutation in sheep (*FecX<sup>l</sup>/FecX<sup>l</sup>*) results in sterility due to failure to develop beyond the primary stage (Davis *et al.*, 1992; Galloway *et al.*, 2000), similar to that observed in *Gdf9*-null mice (Dong *et al.*, 1996). *FecX<sup>l</sup>* is homologous to *Bmp15* (Galloway *et al.*, 2000). Thus, BMP15 protein produced by oocytes is critically important for early follicular development in sheep. *FecX<sup>l</sup>/FecX<sup>+</sup>* heterozygotes exhibit increased ovulation rate with a high incidence of twin and triplet births (Davis *et al.*, 1992). The mechanisms involved in the augmentation of follicular development and ovulation due to the presumed 50% production of normal BMP15 protein are not known. One explanation is that feedback mechanisms between the ovary and pituitary are affected by the reduced expression of BMP15, resulting in increased gonadotrophin signalling and, consequently, augmented follicular development (Galloway *et al.*, 2000). If so, this would show that oocyte-derived factors have profound indirect effects on the dynamics of follicular recruitment via systemic mechanisms.

A null mutation of *Bmp15* induced in mice does not exhibit the same ovarian phenotype as that in *FecX<sup>l</sup>/FecX<sup>l</sup>* sheep (Yan *et al.*, 2001). Although fertility in *Bmp15*-null female mice is reduced, the morphology of follicular development appears essentially normal (Yan *et al.*, 2001); there is no arrest of early follicular development as seen in *FecX<sup>l</sup>/FecX<sup>l</sup>* sheep. Nevertheless, double mutant female mice homozygous for the *Bmp15*-null allele and heterozygous for the *Gdf9*-null allele exhibit more severe fertility defects than do the single mutant *Bmp15*-null females. Moreover, the percentage of fertilized oocytes found in the



oviducts after superovulation and mating was markedly reduced (Yan *et al.*, 2001). Thus, synergism between *Gdf9* and *Bmp15* is required for normal follicular and oocyte development in mice. It is possible that BMP-15 and GDF-9 proteins play similar functional roles in early follicular development, but BMP-15 may be more important in sheep than it is in mice.

## Oocyte regulation of granulosa cell development

### Proliferation

Fully grown oocytes probably promote the proliferation of granulosa cells via the secretion of one or more paracrine factors (Vanderhyden *et al.*, 1992; Lanuza *et al.*, 1998; Li *et al.*, 2000). This would account, at least in part, for the heterogeneous pattern of proliferation in which granulosa cells nearest the oocyte were observed to multiply more rapidly than distal granulosa cells (Hirshfield, 1986). Bovine oocytes rapidly lose mitogenic capability during oocyte maturation (Lanuza *et al.*, 1998), whereas medium-sized mouse oocytes appear to have not yet developed this capability (Gilchrist *et al.*, in press). Thus, the ability of oocytes to regulate granulosa cell proliferation appears to be developmentally regulated. Since recombinant GDF-9 and BMP-15 promote the proliferation of granulosa cells from small antral follicles (Hayashi *et al.*, 1999; Otsuka *et al.*, 2000; Vitt *et al.*, 2000), GDF-9 and BMP-15 are probably oocyte-derived mitogens. However, as described above, the mechanisms whereby oocytes regulate granulosa cell proliferation are complex, and involve multiple factors. Moreover, it seems likely that the regulation of granulosa cell proliferation is part of a larger mechanism of oocyte control over the development and function of the associated granulosa cell population.

### Differentiation

Formation of the follicular antrum effectively establishes the basis for a morphological definition of two granulosa cell populations: the mural granulosa cells associated with the follicular wall, and the cumulus cells associated with the oocyte. With advancing follicular development, which is driven by gonadotrophins and aided by various intrafollicular regulators, these populations can be distinguished in molecular as well as morphological terms. For example, cumulus cells express few, if any, LH receptors (LHR). In contrast, mural granulosa cells express these receptors and the highest expression is observed in cells that are in close apposition to the basal lamina (Amsterdam *et al.*, 1975). In fact, components of basal lamina augment gonadotrophin-induced expression of LHR (a product of the *Lhcgr* gene) (Furman *et al.*, 1986; Eppig *et al.*, 1997a). This pattern of gene expression is quite common and includes the expression of *Kitl* (Manova *et al.*, 1993; Motro and Bernstein, 1993; Joyce *et al.*, 1999). Oocytes play a key role in the establishment of this heterogeneous pattern of gene expression by granulosa cells. Microsurgical extirpation of

the oocyte from isolated oocyte–cumulus cell complexes promotes expression of both *Lhcgr* and *Kitl*. This increased expression is prevented by paracrine factors secreted by oocytes (Eppig *et al.*, 1997a; Joyce *et al.*, 1999). Thus, one or more factors secreted by the oocyte probably reduce the expression by cumulus cells of genes characteristic of the mural granulosa cell phenotype.

Oocytes also suppress expression of *Lhcgr* by mural granulosa cells *in vitro*. Fully grown oocytes prevent the FSH-induced increase in *Lhcgr* mRNA production by granulosa cells *in vitro*, after isolation from small antral follicles. Moreover, oocytes suppress *Lhcgr* mRNA in granulosa cells, expressing large amounts of *Lhcgr* mRNA when isolated from large antral follicles of gonadotrophin-stimulated females (Eppig *et al.*, 1997a). Thus, oocytes play a dominant role in the regulation of *Lhcgr* expression. Therefore, it appears that the default pathway of gonadotrophin-stimulated granulosa cell differentiation leads to the expression of a mural granulosa cell-like phenotype, at least insofar as *Lhcgr* expression is representative of this phenotype. Nevertheless, oocytes can abrogate this pathway of differentiation and promote a phenotype more typical of cumulus cells. Studies on *Kitl* expression produce similar results. However, oocytes are somewhat less able to suppress the expression of *Kitl*, stimulated by testosterone and FSH in mouse granulosa cells, than they are able to suppress expression of *Lhcgr* (Joyce *et al.*, 1999). GDF-9 mimics the actions of fully grown oocytes in suppressing the expression of *Lhcgr* and *Kitl* mRNA (Elvin *et al.*, 1999a; Joyce *et al.*, 2000). This finding supports the hypothesis that the actions of oocytes in regulating transcription of these genes are mediated by GDF-9. However, paradoxically, medium-sized oocytes have little or no ability to suppress *Lhcgr* or *Kitl* mRNA expression in granulosa cells despite expression of both GDF-9 mRNA and protein in these oocytes. More studies are needed to define the interactive roles of gonadotrophins, intrafollicular paracrine and autocrine factors, extracellular matrix components and oocytes in promoting the complex patterns of gene activity that completely define the cumulus and mural granulosa cell phenotypes.

In granulosa cells, luteinization is a terminal differentiative process that normally takes place during the periovulatory period. Nalbandov and colleagues suggested that oocytes produce an anti-luteinizing factor that prevents untimely terminal differentiation (El-Fouly *et al.*, 1970; Nekola and Nalbandov, 1971). Progesterone production is one of the hallmarks of luteinization. Although there are many other parameters of luteinization (Richards *et al.*, 1995), oocytes clearly affect the differentiation of granulosa cells in ways that suppress progesterone production before the LH surge, thus supporting the Nalbandov hypothesis. FSH stimulates progesterone production by oocyte-tomized complexes after isolation of oocyte–cumulus cell complexes from antral follicles. This progesterone production is suppressed by factors secreted by fully grown oocytes (Vanderhyden *et al.*, 1993; Coskun *et al.*, 1995; Vanderhyden and Tonary, 1995; Li *et al.*, 2000). Coincident with the

suppression of progesterone production, mouse oocytes promote oestrogen production. These two actions of oocytes on steroidogenesis appear independent of each other and occur downstream of FSH stimulation of increased cAMP (Vanderhyden and Tonary, 1995). The granulosa cells of preantral follicles also produce progesterone in response to FSH, and this steroid production is inhibited by medium-sized oocytes. Ovulated mature oocytes can also suppress progesterone production by cumulus cells, but only those cumulus cells isolated before the induction of ovulation. Cumulus cells from complexes recovered from oviducts appear refractory to the oocyte factors that suppress progesterone production. Thus, the progesterone-production-suppressing factor is produced by all stages of oocytes tested, from medium-sized through to mature ovulated oocytes. However, gonadotrophin-induced cumulus cell differentiation makes these cells refractory to the oocyte-derived factors (Vanderhyden and Macdonald, 1998). Therefore, although oocyte-derived factors can prevent precocious luteinization, once this process is initiated, the relevant oocyte factors apparently become superfluous. The identity of the oocyte-derived regulators of steroidogenesis are unknown but appear to be of low molecular weight and heat stable (Vanderhyden, 1996). Recombinant GDF-9 stimulates progesterone production by granulosa cells (Elvin *et al.*, 1999a) and, therefore, is probably not the oocyte-derived factor regulating steroidogenesis by cumulus cells before the preovulatory LH surge. Nevertheless, BMP-15 suppresses FSH-induced production of progesterone by granulosa cells (Otsuka *et al.*, 2000), although it is too large to be the same oocyte-derived factor described by Vanderhyden (1998).

### Role of the oocyte in ovulation

#### *Cumulus expansion*

The preovulatory surge of gonadotrophins induces a cascade of processes culminating in ovulation. One of the most marked of these processes is cumulus expansion, or mucification. Gonadotrophins promote cumulus cell production of hyaluronic acid, a non-sulphated glycosaminoglycan that binds to the cumulus cells and expands the spaces between the cells, embedding them in a mucinous matrix. This process is important for ovulation, as inhibiting hyaluronic acid synthesis or linking to cumulus cells *in vivo* reduces ovulation rates markedly (Chen *et al.*, 1993; Hess *et al.*, 1999). Oocytectomy of oocyte–cumulus cell complexes isolated from mouse follicles prevents FSH-induced hyaluronic acid synthesis by the cumulus cells, but co-culture of FSH-stimulated oocytectomized complexes with denuded fully grown oocytes enables expansion of the oocytectomized complex (Buccione *et al.*, 1990b). Similarly, denuded oocytes promote synthesis of hyaluronic acid by FSH-stimulated cultured mural granulosa cells (Salustri *et al.*, 1990). Thus, fully grown oocytes secrete a cumulus expansion-enabling factor (CEEF) that enables

granulosa cells to respond to FSH by producing hyaluronic acid. Medium-sized oocytes from preantral follicles do not secrete active CEEF; co-culture of these oocytes with FSH-stimulated oocytectomized complexes from antral follicles does not promote expansion. Although ovulated metaphase II oocytes secrete some active CEEF, this activity is low relative to fully grown GV-stage oocytes, and no CEEF activity is detected by the two-cell stage (Vanderhyden *et al.*, 1990). Thus, secretion of active CEEF by mouse oocytes is developmentally regulated.

Oocytectomy does not prevent expansion of rat, pig or cow cumulus cells (Prochazka *et al.*, 1991; Vanderhyden, 1993; Ralph *et al.*, 1995). However, fully grown oocytes from these species produce CEEF, even though it appears unnecessary to promote expansion, since co-culture of these oocytes with FSH-stimulated oocytectomized mouse complexes results in expansion (Singh *et al.*, 1993; Vanderhyden, 1993; Ralph *et al.*, 1995). Presumably, CEEF produced by rat, pig and cow oocytes is required to promote some function of granulosa cells other than hyaluronic acid synthesis. In fact, pig oocytes appear to secrete a factor that promotes binding of hyaluronic acid within the complex, even though the actual production of hyaluronic acid occurs independently of oocytes (Nagyova *et al.*, 2000). Moreover, pig cumulus cells apparently secrete a factor, similar in activity to oocyte-produced CEEF, which promotes expansion of mouse FSH-stimulated oocytectomized mouse complexes (Prochazka *et al.*, 1998). However, whether this factor is the same as the CEEF produced by the oocyte has yet to be determined.

Recombinant GDF-9 stimulates the expansion of oocytectomized mouse complexes and induces the expression of hyaluronan synthase 2 (*Has2*) by isolated granulosa cells without stimulation by FSH (Elvin *et al.*, 1999a). Therefore, it seems probable that GDF-9 and CEEF are identical. However, it is important to be mindful of an apparent paradox similar to that described above. If GDF-9 is the factor that suppresses expression of *Lhcgr* and *Kitl* by cumulus cells before the preovulatory LH surge, why does it not promote untimely expression of *Has2* and cumulus expansion before the surge? Perhaps other factors mediate granulosa cell responsiveness to GDF-9 before or after the surge. It is also possible that responses to GDF-9 are dependent upon the amount of stimulation, and that the amounts of GDF-9 released by oocytes are modulated by theca or granulosa cells *in vivo*. Thus, small amounts of GDF-9 may suppress *Lhcgr* and *Kitl* expression before the surge, whereas larger amounts promote expression of *Has2* and cumulus expansion after the surge. Fully grown GV-stage oocytes appear to secrete CEEF constitutively, but this may not be the mode of CEEF or GDF-9 secretion *in vivo*. Before the LH surge, granulosa cells may secrete only small amounts of GDF-9. However, in response to the ovulatory stimulus, the somatic cells may signal the oocytes to release a bolus of GDF-9, launching the preovulatory cascade of cumulus cell processes essential for ovulation. This mechanism would constitute an oocyte–somatic cell

regulatory loop in which the direction of function is altered by the imposition of ovulation-promoting gonadotrophic signals mediated by the somatic cells.

### *Ptgs2* expression

Targeted mutation of the prostaglandin–endoperoxide synthase 2 gene (*Ptgs2*, also known as *Cox2*) results in a greatly diminished rate of ovulation in mice (Lim *et al.*, 1997). Thus, production of prostaglandins is required for normal ovulation. The pattern of *Ptgs2* gene expression in mouse preovulatory follicles is complex. Within 2 h after hCG treatment, expression is observed in mouse mural granulosa cells but not in cumulus cells. By 4 h after hCG treatment, expression is seen in both cumulus and mural granulosa cells. However, expression is limited to the cumulus cells by 8 h after hCG treatment. Nevertheless, high expression is seen again in both types of cell by 12 h after hCG treatment. Thus, the pattern of expression in mural granulosa cells appears multiphasic: first an increase, then a decrease, and then another increase just before ovulation (Joyce *et al.*, 2001). Paracrine factors from oocytes augment expression of *Ptgs2* mRNA steady state concentrations in both cumulus and mural granulosa cells *in vitro*. However, the second increase in expression in the mural granulosa cells is not observed *in vitro* unless components of the follicular wall are co-cultured with the isolated mural granulosa cells (Joyce *et al.*, 2001). Thus, the complex pattern of *Ptgs2* mRNA expression during the pre-ovulatory period is probably orchestrated by non-oocyte factors, possibly derived from theca-interstitial cells, but the concentrations are augmented by oocytes. Therefore, oocytes probably affect granulosa cell functions in ways essential for ovulation by promoting prostaglandin production as well as cumulus expansion.

Recombinant GDF-9 stimulates expression of *Ptgs2* by granulosa cells *in vitro* and therefore participates in prostaglandin production (Elvin *et al.*, 1999a). GDF-9 also stimulates expression of *Ptgerep2*, the EP2 receptor of PGE<sub>2</sub> (Elvin *et al.*, 2000a). This pathway appears essential for promoting cumulus cell progesterone production during the periovulatory period (Elvin *et al.*, 2000a). Taken together, these findings indicate that GDF-9 is instrumental in inducing the expression of *Has2*, *Ptgs2* and *Ptgerep2* by cumulus cells after the LH surge and that, in addition to promoting normal ovulation, it is key to the expression of the cumulus cell phenotype during the periovulatory period.

### *Disassembly of the ovulated oocyte–cumulus cell complex*

The preovulatory gonadotrophin surge stimulates oocyte maturation and cumulus expansion. Before the preovulatory surge, oocytes suppress cumulus cell expression of urokinase plasminogen activator (*Plau* or uPA), a serine protease involved in tissue remodelling (Canipari *et al.*, 1995). However, cumulus cells in the oviduct express *Plau*

and the mature oocytes secrete tissue type plasminogen activator (tPA), a product of the *Plat* gene. Ovulated cumulus cells are insensitive to suppression of *Plau* expression by oocytes (D'Alessandris *et al.*, 2001). Thus, oocytes suppress what would be an inappropriate activation of uPA before the preovulatory LH surge, but participate in the degradation of the cumulus matrix after ovulation.

## Conclusions

A new perspective on ovarian follicular development has emerged over the last decade. As reviewed here, oocytes play an essential role in the development of ovarian follicles beginning with initial follicular formation and ending with the disassembly of the ovulated cumulus–oocyte complex. A complex interplay of regulatory factors governs the development of both the oocyte and the follicle, termed the oocyte–granulosa cell regulatory loop in this review. Although gonadotrophins most likely affect the function of this loop by driving the pathways of granulosa cell differentiation, within its sphere of influence, the oocyte is probably the dominant factor determining the direction of differentiation and the function of the granulosa cells associated with the oocyte. It is proposed that if the oocyte were hypothetically absent from the follicle, gonadotrophins and factors from the theca cells would drive the development of all granulosa cells toward the mural granulosa cell phenotype and ultimate luteinization. Thus, the mural granulosa cell phenotype is the 'default' pathway of granulosa cell development. However, oocytes can abrogate this default pathway and promote the development of the cumulus cell phenotype. In association with the oocyte, gonadotrophins probably also drive the development of the cumulus cell pathway, since oocyte-associated granulosa cells do not gain the ability to undergo cumulus expansion *in vitro* in the absence of FSH (Eppig, 1991).

Nourishment and signals from companion granulosa cells are essential to support oocyte development. Therefore, as proposed by early investigators, granulosa cells fulfil a role as nurse cells. It now appears that the signals from granulosa cells are complex, and it is possible that they must be presented to the oocyte in a way that is co-ordinated with both oocyte and granulosa cell differentiation. The central organizer that co-ordinates these signals may be the oocyte itself. Cultured granulosa cells from preantral follicles promote the growth of medium-sized oocytes also isolated from preantral follicles. However, fully grown oocytes appear to suppress the ability of preantral granulosa cells to promote oocyte growth. Moreover, granulosa cells of preantral follicles do not promote the further growth of oocytes from antral follicles (Cecconi and Rossi, 2001). Thus, there appears to be a complex regulatory loop controlling granulosa cell-dependent oocyte growth. When oocytes reach a size threshold, they suppress the ability of granulosa cells to promote oocyte growth. Although oocytes from antral follicles suppress the ability of preantral granulosa cells to promote oocyte growth, these fully grown



oocytes, but not growing oocytes, promote granulosa cell proliferation and therefore the growth of the follicle (Gilchrist *et al.*, in press). Thus, via the oocyte–granulosa cell regulatory loop, the oocyte orchestrates its own growth as well as that of the follicle.

Appropriate cumulus cell differentiation appears to be required for normal oocyte development. Inappropriate, or ambiguous, differentiation of oocyte-associated granulosa cells *in vitro* is correlated with the formation of oocytes lacking competence to complete preimplantation development (Eppig *et al.*, 1998; Latham *et al.*, 1999). Thus, there might be functions of mural granulosa cells antagonistic to normal oocyte development. Therefore, oocytes probably evolved to produce factors that both prevent the differentiation of granulosa cells expressing the mural granulosa cell phenotype and promote the cumulus cell phenotype. The specific attributes of either the phenotype of mural granulosa cells that are potentially antagonistic to oocyte development, or of the phenotype of cumulus cells that are beneficial to oocyte development, are unknown. However, the proposed oocyte–granulosa cell regulatory loop is probably essential for the progression of normal development in both the oocyte-associated granulosa cells and the oocyte. Therefore, this loop is key to successful follicular development.

Besides organizing and co-ordinating granulosa cell differentiation and function on its own behalf, the oocyte appears to play a role in its own delivery to the site of fertilization, the oviduct. Ovulation requires cumulus expansion and prostaglandin production, and factors secreted by oocytes participate in both of these processes, and possibly others as well. Moreover, the finding of increased fecundity in *FecX*<sup>-</sup>/*FecX*<sup>+</sup> heterozygous sheep indicates that oocytes have an indirect influence on follicular dynamics mediated by modulation of negative feedback mechanisms controlling gonadotrophin concentrations. Thus, the impact of the oocyte on reproductive processes extends beyond the boundaries of its own microenvironment.

Factors participating in the oocyte–granulosa cell regulatory loop, in addition to GDF-9, BMP-15 and KL, are being investigated. Although GDF-9 and BMP-15 are not the only important oocyte-derived factors, they appear to be critical for determining the preovulatory phenotype of cumulus cells. Metabolic labelling and detection of oocyte secretory proteins after separation by two-dimensional polyacrylamide gel electrophoresis reveals many individual proteins as well as proteins with complex post-translational modifications (Eppig *et al.*, 1997b). An important challenge for the future will be to characterize these proteins, in addition to non-proteinaceous oocyte-derived factors. This will not be a simple task, as these regulators are produced in minute quantities, and a variety of molecular technologies will be required for their detection and characterization. Moreover, discovery and identification are only the beginning. Resolution of function will require genetic modification, production of recombinant proteins, antibodies,

blocking agents, and implementation of imaginative cell culture techniques. Success in these tasks will generate exciting new knowledge of fundamental mechanisms that govern the development of both oocyte and somatic components of follicles, as well as a plethora of new targets for fertility control.

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