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REVIEW ARTICLE

Oocyte growth in vitro: potential model for studies of oocyte–granulosa cell interactions

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Abstract Various factors such as gonadotrophins, growth factors, and steroid hormones play important roles in the regulation of oocyte/follicular growth in mammalian ovaries. In addition to these factors, there is a bidirectional interaction between oocytes and granulosa cells that is essential for achieving optimal oocyte developmental competence. Oocytes play a key role in this interaction by secreting paracrine factors that alter the activities of neighboring cumulus cells, such as the expression of a specific amino acid transporter, cholesterol biosynthesis, and levels of glycolysis in the cumulus cells. Among the known oocyte-derived factors, growth differentiation factor 9 (GDF9) is the dominant factor mediating the regulation by oocytes leading to cumulus expansion and granulosa cell proliferation. GDF9 frequently interacts with other oocyte-derived factors in a synergistic manner. It seems reasonable to speculate that oocytes growing in vitro require interactions similar to those in vivo. Some of the oocyte-mediated regulations have been confirmed in vitro, providing evidence of the usefulness of culture systems as a strong tool for such studies. This review discusses in vitro culture of growing oocytes in terms of oocyte–granulosa cell interactions.

Keywords BMP15 · GDF9 · In vitro growth · Oocyte–granulosa cell interaction · Ovarian follicle

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Introduction

In the mammalian ovary, numerous oocytes degenerate either before growth or at various stages of growth [1]. Those redundant but "potential" oocytes can be rescued if they are provided with suitable culture conditions that allow them to escape degeneration and continue to grow. It is desirable for oocytes' health and growth to be maintained in culture systems, and granulosa cells around the oocyte ought to proliferate to prevent spontaneous oocyte denudation. On that basis, both oocytes and granulosa cells should be functional. In addition to this, recent studies have indicated that a bidirectional interaction between oocytes and granulosa cells is essential for normal oocyte development. It seems reasonable to speculate that oocytes growing in vitro require similar interactions. In this review, the oocyte–granulosa cell interactions are considered in terms of culture conditions, which are presumed to be crucial for supporting optimum oocyte growth in vitro.

Fertility of oocytes grown in vitro

We already have evidence that oocytes can grow and mature into "normal" ova in vitro. Eppig and Schroeder [2] reported the production of the first mice derived from oocytes cultured for the latter half of their growth period. Several other studies have also reported viable mouse offspring produced from a similar growth stage of oocytes [3–6]. A combination of preantral follicle culture following an 8-day organ culture of newborn mouse ovaries produced the first live offspring, named Eggbert, from an oocyte grown in vitro for the entire growth period [7]. The culture system has since been remarkably improved [8]. Besides the mouse, the cow is the only mammal the offspring of which

have been born from oocytes grown in vitro from the stage where they were approximately 75–80% of the maximum oocyte diameter after a 14-day culture period [9, 10].

On the other hand, rat oocytes grown in vitro were able to complete meiosis, but did not undergo preimplantation development [11]. Similarly, pig oocytes cultured for 16 days from preantral follicles were able to mature and undergo fertilization, but were unable to develop the male pronucleus [12].

Basic culture conditions

Before discussing the oocyte–granulosa cell interactions, it is useful to summarize the culture systems developed to date and the basic conditions necessary to support the survival of oocytes and granulosa cells. This is because if culture conditions are suboptimal for either oocytes or granulosa cells, no further bidirectional communication would be expected.

Two major types of culture systems

Several different culture systems have been developed [13]. These can be divided into two types according to the structure of the follicles or oocyte–granulosa cell complexes [13] depending on whether follicles/complexes spread on a substratum (the substratum-adhering type, Fig. 1a–d) or maintain their spherical shape (the sphere type, Fig. 1e, f). In the former type, preantral follicles or oocyte–granulosa cell complexes adhere to the substratum and proliferate outward, creating a gentle swelling around the oocyte [4, 5, 7, 14]. The simplicity of this type of system gives it an advantage in terms of narrowing down the basic conditions for oocyte growth regulation [15]. Alternatively, follicular cells on the substratum proliferate to form a dome-like structure, as has been reported in the mouse [16–18], rat [19], cow [10], and pig [20]. In the sphere type of system, each preantral follicle or oocyte–granulosa cell complex maintains or grows into a spherical shape, developing an antral cavity if subjected to proper stimulation, as has been reported in the mouse [3, 21–24], cow [25–28], pig [12, 29], sheep [30, 31], goat [32–34], and human [35, 36]. To achieve a 3-D culture, follicles/complexes are often embedded in collagen- [37] or alginate-based matrices [36, 38, 39].

Medium supplements affecting follicle/oocyte viability

Studies on the mitogenic effect of follicle-stimulating hormone (FSH) and its second messenger cyclic adenosine monophosphate (cAMP) have been conducted. While FSH

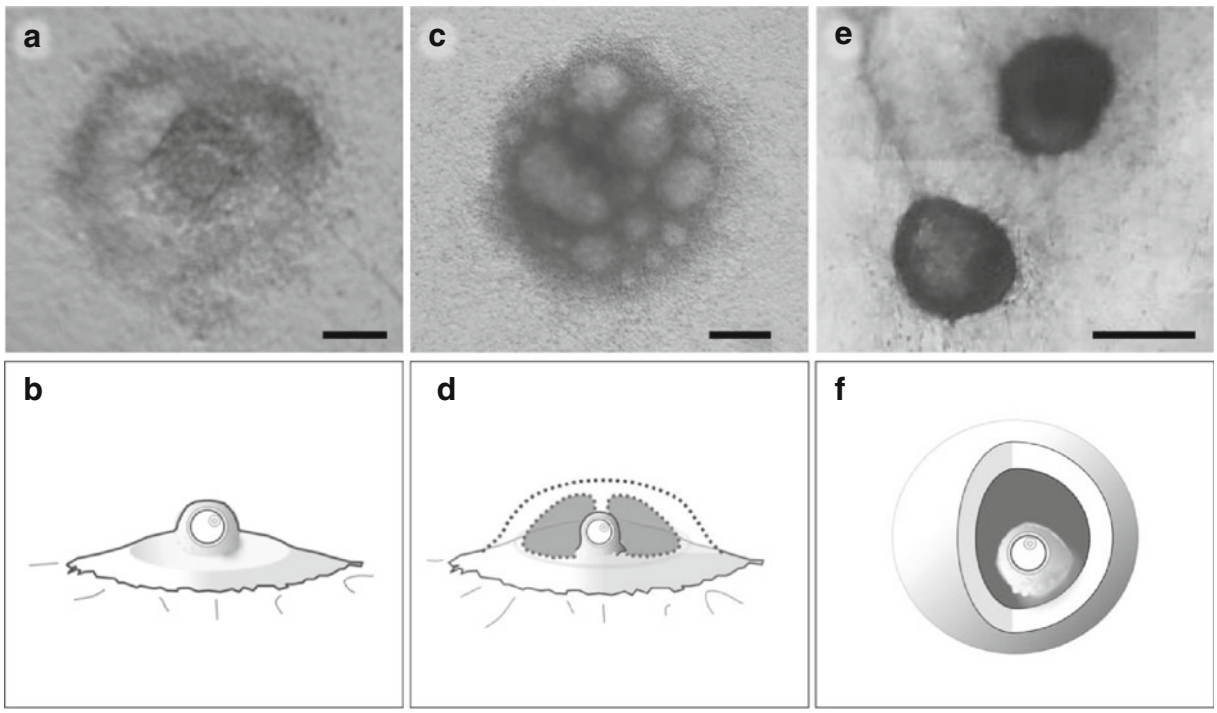

Fig. 1 Typical morphologies of oocyte–granulosa cell complexes developed in vitro. **a** Mouse complexes after a 10-day culture period on collagen-coated substratum, and **b** a simplified illustration. **c** Bovine complexes after a 14-day culture period on collagen-coated substratum, and **d** a simplified illustration. **e** Bovine complexes after a 16-day culture period within the collagen matrices, and **f** a simplified illustration. *Scale bars* 100 μm (**a**), 200 μm (**c**), and 500 μm (**e**)

is not indispensable to mouse oocyte growth [2], intact preantral follicles (composed of the oocyte, granulosa cells, theca cells, and the basement membrane) survive and grow better in FSH-supplemented medium than in a control medium [40, 41]. Similarly, porcine oocyte–granulosa cell complexes survived better in a medium containing FSH than in one without [42]. In contrast, a luteinizing hormone (LH)-supplemented medium diminished the viability of follicles [43].

Cyclic AMP is known to be involved in various aspects of ovarian regulation [44]. Dibutyryl cyclic AMP (dbcAMP), a cell-permeable analogue of cAMP, has frequently been used to test its effect in culture. Mouse follicle growth in vitro was promoted with dbcAMP [22]. Similarly, 4 mM hypoxanthine, a natural inhibitor of phosphodiesterase, which degrades intracellular cAMP [45], was beneficial to the survival of bovine oocyte–granulosa cell complexes [25, 27]. The concentration of hypoxanthine in mouse follicular fluid was estimated to be 2–4 mM [46].

The effects of other factors on the viability of follicles and oocytes have been also examined. Epidermal growth factor (EGF)-supplemented medium was twice as effective as the control medium in promoting the survival of mouse follicles [7]. A synergistic effect of EGF and insulin-like growth factor I (IGF-I) on oocyte survival has been observed in the goat [32] and pig [29]. In the pig, however, the same combination increased apoptosis in a serum-free medium [29].

Medium supplements affecting oocyte growth

Mouse oocytes grown in a medium containing fetal bovine serum are more competent in embryogenesis than those grown in a serum-free medium [47]. The addition of dbcAMP [22] or hypoxanthine [25, 27, 42] to the medium is also beneficial to the growth and acquisition of meiotic competence of oocytes. Similarly, a combination of FSH and LH can promote the acquisition of oocyte meiotic competence [43] and developmental competence [48]. However, under culture conditions optimized without the use of FSH, supplementary FSH can reduce oocyte developmental competence [49].

Epidermal growth factor [7] and IGF-I [50] used in mouse oocyte growth cultures have improved the developmental competence of oocytes. In addition, the growth of caprine preantral follicles is improved by a synergistic effect of IGF-I and EGF [32]. Activin A is also beneficial to bovine oocyte growth [28], but follicle survival was impaired after activin A-treatment in a mouse study [51].

Androgens are other biological factors that promote the acquisition of meiotic competence in vitro [52, 53]. Conversely, oocytes grown in a medium containing androgenic compounds are not capable of maturation [18]. Therefore, it appears to be beneficial to add androgen to the medium for oocyte growth, particularly when theca cells are removed before the culture. On the other hand, exogenous estradiol may be unnecessary for the production of oocytes that are capable of maturation [41, 54]. Rather, excess exposure to estrogen during oocyte growth appears to decrease the probability of successful fertilization [48]. However, a recent mouse study has uncovered a role for estrogen and oocyte-derived factors together in promoting the ability of cumulus cells to undergo expansion [55].

It is well established that c-Kit and c-Kit ligand (KL) are involved in oocyte–granulosa cell interactions [56]. The addition of the KL to the culture medium promotes the growth of mouse oocytes within the cultured follicles [57, 58] and even in those without associated granulosa cells [59]. Within the follicles, however, appropriately supplemented FSH is needed in the modulation by KL to promote oocyte growth [60].

Besides biological factors present in ovaries, high concentrations of polyvinylpyrrolidone (PVP; molecular weight: 360,000) improve the survival and growth of bovine oocyte–granulosa cell complexes [10]. A calf was produced from an oocyte grown in medium supplemented with 4% PVP [10]. Furthermore, a combined supplement of PVP and fibroblast growth factor-7 improved the growth of bovine oocytes [61].

Medium supplements affecting granulosa cells

Carroll et al. [22] have reported a remarkable improvement in the growth of mouse preantral follicles after the addition of dbcAMP to the medium. Similarly, hypoxanthine promotes the survival of intact bovine early antral follicles [27] and helps to maintain the association between oocytes and the surrounding granulosa cells [62]. In another study, a specific phosphodiesterase present in granulosa cells was targeted with two phosphodiesterase type 3-inhibitors (PDE3-Is), org9935 and cilostamide, resulting in the promotion of growth, differentiation, and survival of mouse preantral follicles [63].

Intact mouse preantral follicles cultured in medium without FSH show reduced survival during long-term culture [64]. In addition, FSH plays an important role in promoting granulosa cell differentiation in cells from preantral follicles, so that these cells respond to LH stimulation [65]. However, granulosa cells exposed to excess FSH during follicle growth in vitro, show unusual expression of LH receptors, which may impair the developmental competence of oocytes [49].

Besides the factors described above, estradiol [66] and androstenedione [67, 68] are potent stimulators of follicular growth in vitro. IGF-I also enhances granulosa cell

proliferation, maintenance of follicular integrity, and the survival of oocytes in vitro [29, 32].

Medium supplements affecting antrum formation

Intact mouse preantral follicles develop into morphologically normal antral follicles in FSH-supplemented medium and retain their spherical shape [23]. On the other hand, in a substratum-adhering system, the spherical shape collapses, instead transforming into a dome-like structure [16, 19, 21, 40, 69]. Bovine and porcine oocyte–granulosa cell complexes develop a dome-like structure in media containing high concentrations of PVP [10, 20]. However, FSH was not added to the culture medium in these studies. It is interesting that the "mural" granulosa cell masses grow in a rim pattern remotely from the oocyte at the center, even in the absence of the dome formation [14].

Besides FSH, antral formation of mouse preantral follicles is promoted by the addition of dbcAMP [69] and androstenedione [67, 68] to the medium. Activin A has also been shown to promote follicular antrum formation in the rat [70] and humans [35].

Oocyte–granulosa cell interactions

The discovery and molecular characterization of oocyte-derived factors in the 1990s showed that oocytes secrete paracrine factors, thereby generating bidirectional interactions between oocytes and granulosa cells, which are essential for achieving optimal oocyte developmental competence [71, 72]. Until then, many lines of experimental evidence drew attention to the passive activity of the oocyte during growth, for example, a large part of the nutrition of growing oocytes is delivered by the associated granulosa cells [73]. However, a recent study still identified a role for granulosa cells in the regulation of intraoocyte pH via gap junctions [74]. Therefore, oocytes and granulosa cells are both important in the elaborate mechanisms controlling oogenesis and folliculogenesis.

Dominant roles for oocytes

In the bidirectional oocyte–granulosa cell interactions, oocytes play a key role [75]. Using the expression of LH receptor mRNA as a marker of the mural granulosa cell phenotype in the mouse, Eppig et al. [76] clearly demonstrated that paracrine factor(s) secreted by oocytes play a dominant role in the establishment of granulosa cell phenotypic heterogeneity. Later, by using cumulus marker mRNA transcripts such as *Slc38a3* and *Amh*, Diaz et al. [77] demonstrated that oocytes induce cumulus cell differentiation through the SMAD2/3 signaling pathway. Bovine oocytes are capable of determining phenotypic differences between cumulus cells and mural granulosa cells [78].

Mouse oocytes alter the metabolic activity of neighboring cumulus cells to counterbalance oocyte-specific metabolic deficits [79–81]. The paracrine signals secreted by oocytes promote the expression of a sodium-coupled neutral amino acid transporter in cumulus cells, which then increases the oocytes' uptake of amino acids via the cumulus cells [79]. Oocytes apply the same strategy to cholesterol biosynthesis [80] and the glycolytic enzymes of cumulus cells to increase metabolism cooperatively [81]. Furthermore, bone morphogenetic protein-15 (BMP15) and fibroblast growth factors (FGFs) secreted by oocytes cooperate to promote glycolysis in cumulus cells [82].

Growth differentiation factor 9 and BMP15

Growth differentiation factor 9 and BMP15 represent two major oocyte-derived factors essential for regulating folliculogenesis [83–85]. GDF9 and BMP15 are known to regulate the function of cumulus cells in a synergistic manner [86–88]. Both of these factors are expressed in mouse oocytes growing in organ-cultured ovaries [89]. The characteristics of GDF9-null mice are arrested folliculogenesis

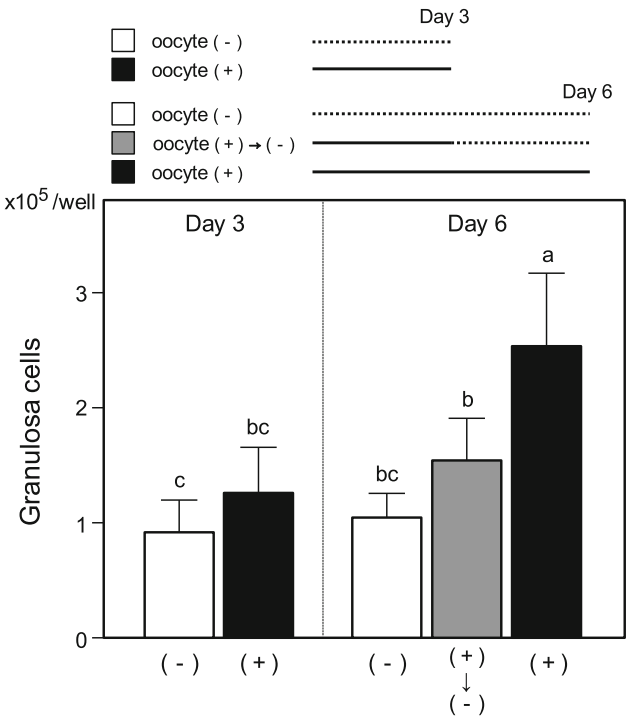

Fig. 2 Number of granulosa cells cultured for 3 or 6 days as an oocyte–granulosa cell complex (+) or as a granulosa cell mass after the removal of the oocyte (−) in the wells of 96-well culture plates. [a–c]Values with different superscripts are significantly different ($p < 0.05$, Tukey test)

at the primary stage and aberrant expressions of various genes are involved in follicular functions [90].

One of most well-known activities of oocyte-derived factors is the promotion of granulosa cell proliferation [91–93]. In the culture of bovine oocyte–granulosa cell complexes isolated from early antral follicles, granulosa cell proliferation ceases after removing the oocyte (Fig. 2). GDF9 is the major contributor to growth-promoting activity [94]. In fact, GDF9-supplemented medium enhances the growth of preantral follicles [95–97]. However, GDF9 does not account for the entire mitogenic activity originating from oocytes [98]. For example, BMP15 can promote the growth of granulosa cells [99].

A rat study suggested an anti-apoptotic effect of GDF9 on the cultured preantral follicles, and also the involvement of PI3/Akt pathway in the activity [100]. Using bovine cumulus–oocyte complexes, however, Hussein et al. [101] found that bone morphogenic proteins prevent cumulus cell apoptosis but GDF9 does not.

Cumulus expansion-enabling activity

Cumulus expansion-enabling factors (CEEFs) were the first oocyte-derived factors experimentally ascertained to play a regulatory role in cumulus cell differentiation [102, 103]. Later, studies utilizing GDF9 null mice [104] and an RNA interference approach [105] confirmed that GDF9 is a mediator in oocyte regulation of cumulus expansion. A recent study found that the SMAD 2/3 signaling pathway was involved in the oocytes' cumulus expansion-enabling process [106]. In fact, activins as well as GDF9, both SMAD 2/3 signaling pathway activators, can act as CEEFs [106].

Granulosa cells in mouse preantral follicles do not expand in response to hormonal induction. The reason for this has been identified as a lack of CEEFs from the growing oocytes and insufficient expression of necessary transcripts such as *Tnfaip6* mRNA [107].

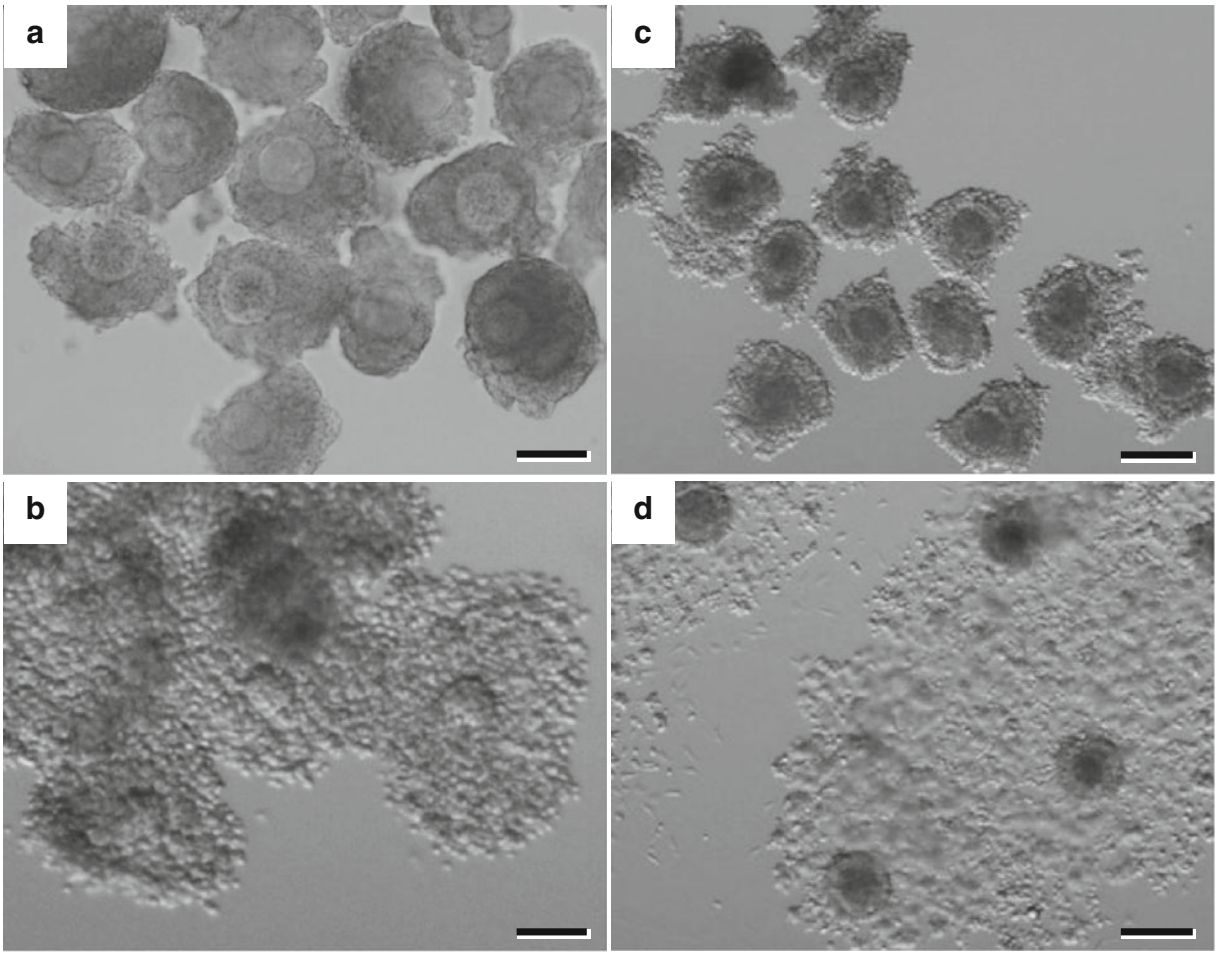

Fig. 3 Expansion of mouse and bovine cumulus cells observed around maturing oocytes after growth in vitro. **a** Mouse complexes after a 10-day culture period and **b** after maturation in vitro. **c** Bovine complexes after a 14-day culture period and **d** after maturation in vitro. *Scale bars* 100 μm (**a**, **b**) and 200 μm (**c**, **d**)

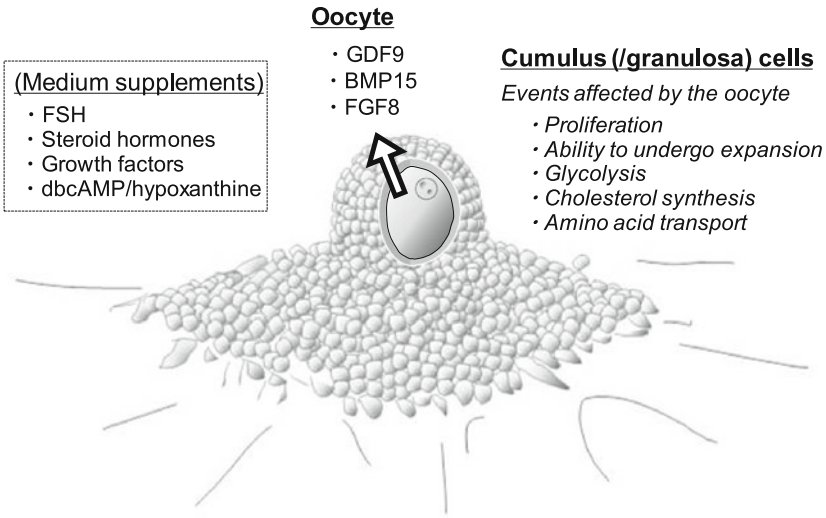

Fig. 4 Oocyte-derived paracrine factors and hormonal factors known to be involved, or presumably involved, in the regulation of the oocyte and neighboring granulosa cells during oocyte/follicle growth in vitro

Oocyte–granulosa cell interactions reproduced in vitro

In the author's experiments with bovine oocyte–granulosa cell complexes, granulosa cells proliferate better in the presence of oocytes (Fig. 2), and, after a 14-day culture period, cumulus cells acquire the competence to undergo expansion (Fig. 3). These observations suggest possible interactions between oocytes and granulosa cells as discussed above.

In mouse studies, some of the oocyte-mediated regulations have been realized specifically in vitro [18, 55, 89, 108]. Therefore, culture systems for oocyte growth can provide a strong tool for studies of the oocyte-mediated regulation of granulosa cell differentiation, such as differentially expressed androgen receptor protein in mural granulosa cells and cumulus cells in follicles [18], the acquisition of the ability of cumulus cells to undergo expansion in response to EGF [108], and the coordinating activity of GDF9 and BMP15 [55, 89].

Conclusions

Some of the culture systems discussed in this review have already been used to address important questions with regard to the oocyte–granulosa cell interactions (summarized in Fig. 4). Although many other oocyte factors presumably mediate granulosa cell differentiation in vitro, the in vitro oocyte growth systems will provide a strong platform for the analysis of the activities of these factors. In the meantime, it is clear that further improvements to existing culture systems are necessary, because oocyte competence does not yet match its in vivo counterparts. A greater understanding of the oocyte–granulosa cell interactions will benefit us in our search for the optimal conditions to culture growing oocytes.

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