—Review—

Recent advances in understanding the regulation of oogenesis and its recapitulation *in vitro*: mouse and bovine models

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Abstract: The regulation of mammalian oogenesis *in vivo* is complicated because of numerous constantly changing events caused by ovarian cells interacting with or influencing each other. One of the most intractable questions for nearly the last 80 years has been the mechanism controlling the maintenance of meiotic arrest and the resumption of oocyte meiosis in a pre-ovulatory follicle. The question is now mostly resolved, as the regulatory mechanisms of cGMP, cAMP, and the NPPC/NPR2 system in the follicle, have recently been uncovered. Oocyte growth *in vitro* has also been the subject of extensive research utilizing growing oocytes at various stages in several species, including mice, cattle, pig, sheep, goat, and horse. Remarkably, the first reconstitution of the entire process of mammalian oogenesis *in vitro* from primordial germ cells (PGCs) was recently achieved in mice. Furthermore, even PGC-like cells, originally produced from mouse embryonic stem cells and induced pluripotent stem cells, can develop into functional oocytes *in vitro* with the help of gonadal somatic cells of female mouse fetuses. These updated findings and newly developed culture systems will assist in gaining a better understanding of the mechanisms of oogenesis and will also lead to the creation of new gamete resources for mammals.

Key words: Oocyte growth, Oocyte maturation, *in vitro*, Oocyte–granulosa cell complexes, Preantral follicles

Introduction

The regulation of mammalian oogenesis *in vivo* is so dynamic that examination of these events *in situ* is extremely difficult. The processes are complicated, not only because oocyte growth is a series of constantly changing events which take place over a long period of time, but also because a small proportion of primordial oocytes “periodically” enter the growth phase. Thus, the function of the ovary is also constantly changing. Various factors, such as gonadotrophins, growth factors, and steroid hormones, underlie the dynamic regulation of oocyte and follicular growth [1, 2]. In addition, there are also interactions between an oocyte and its associated granulosa cells [3]. One approach to simplify these processes is to isolate oocytes and their associated ovarian cells and culture them *in vitro*. In this review, recent findings regarding oocyte growth and maturation *in vitro* with a particular focus on the oocytes of mice and cattle are discussed.

Regulation of Meiotic Resumption

Prior to discussing oocyte-growth topics, it is appropriate to recount the history of the discoveries made regarding the mechanisms that trigger oocyte maturation in mammals.

Cyclic AMP in meiotic arrest

These mechanisms had been shrouded in mystery since the discovery of spontaneous maturation of rabbit oocytes in 1935 [4]. Subsequently, it was established that fully-grown oocytes, which are arrested at the prophase of the first meiosis, resume meiosis spontaneously after transfer into a medium, and that the spontaneous resumption of meiosis could be prevented by inducing a high level of intra-oocyte cyclic adenosine 3′, 5′-monophosphate (cAMP) [5]. On the other hand, a decrease in the cAMP level allows the resumption of meiosis [5]. Therefore, until an oocyte/follicle becomes fully grown and ready for meiotic resumption the level of oocyte

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cAMP must remain high; however, at the time when an oocyte needs to resume meiosis, the level of cAMP has to decline rapidly [6]. In oocytes, cAMP phosphodiesterase type 3A (PDE3A) is responsible for the timely hydrolysis of cAMP [7, 8] (Fig. 1A). However, the signal (s) causing the degradation of cAMP in response to the luteinizing hormone (LH) surge was not known.

Cyclic GMP in meiotic arrest

A crucial factor that had been known to be involved in oocyte maturation but essentially overlooked was cyclic guanosine 3′, 5′-monophosphate (cGMP), which also declines, as cAMP does, when oocytes resume meiosis [9, 10]. Although the decline of both cGMP and cAMP levels after the LH surge in rat ovaries was known from as early as the 1970s [11], the role of cGMP had not been examined extensively, in sharp contrast to the many studies performed to investigate the role of cAMP in oocyte maturation [12, 13]. Recently, it has been revealed that a high level of oocyte cGMP, which diffuses from cumulus cells to oocytes, inhibits PDE3A in oocytes, causing a high level of oocyte cAMP, thereby maintaining meiotic arrest [9] (Fig. 1A).

The NPPC/NPR2 system in meiotic arrest

In cumulus cells, the levels of cGMP are raised by the natriuretic peptide receptor 2 (NPR2), a guanylyl cyclase [14, 15], and cGMP diffuses into the oocyte through gap junctions [16]. The expression of *Npr2* in cumulus cells is promoted by oocyte-derived paracrine factors [3]. NPR2 is a receptor of the natriuretic peptide precursor type C (NPPC), and NPPC is expressed in mural granulosa cells in the follicle [14]. Thus, to maintain a high level of cGMP in an oocyte, not only the expression of NPR2, but also the expression of NPPC by mural granulosa cells is necessary (Fig. 1A). The existence of the NPPC/NPR2 system provides an explanation as to why cumulus cell-enclosed oocytes resume meiosis spontaneously after detachment from mural granulosa cells into a culture medium. The surge of LH reduces the activity of NPR2 guanylyl cyclase in mice [17] and rats [18] (Fig. 1B). Another important mechanism that may lower oocyte cGMP is the reduction of gap junction permeability after the LH signal [19]. Most recently, however, it has been suggested in a mouse study that the cause of the crucial decrease of oocyte cGMP, immediately after the LH surge, is due to diffusion of GMP from the oocyte to the granulosa cell mass, as a result of a decrease in the cGMP level in the peripheral granulosa cells, which can occur within 1 min of the LH application [20] (Fig. 1B). It was found that, after 20 min, the cGMP concentration in the follicle had become uniformly low enough to relieve the inhibition of meiotic cell cycle [20].

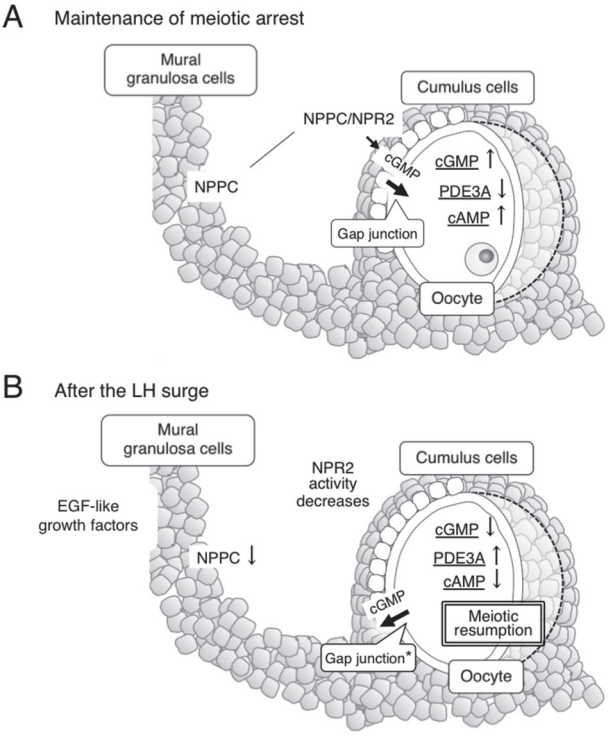

Fig. 1. A simplified illustration of the mechanisms involved in the maintenance of meiotic arrest (A) in the mouse oocyte and the meiotic resumption following the LH surge (B). NPPC, natriuretic peptide precursor type C. NPR2, natriuretic peptide receptor 2. PDE3A, phosphodiesterase type 3A. *Diffusion of cGMP out of the cumulus cells precedes the LH-induced decrease in gap junction permeability [20].

It has been observed that exogenous NPPC can delay meiotic resumption during *in vitro* maturation (IVM) of oocyte-cumulus cell complexes in pigs [21]. Similarly, the addition of NPPC into the medium prolonged the period of meiotic arrest in bovine oocytes to some extent [22]. A deeper understanding of the mechanisms of the NPPC/NPR2 system in species other than mice will be necessary to improve the efficiency of embryo production via enhancement of the quality of oocytes produced *in vitro*.

In Vitro Growth of Oocytes Collected from Early Antral Follicles

General description of the substratum-adhering system

Several culture systems have been developed for *in vitro* growth (IVG) of oocytes growing in preantral follicles or early antral follicles [23]. In the culture of oocytes col-

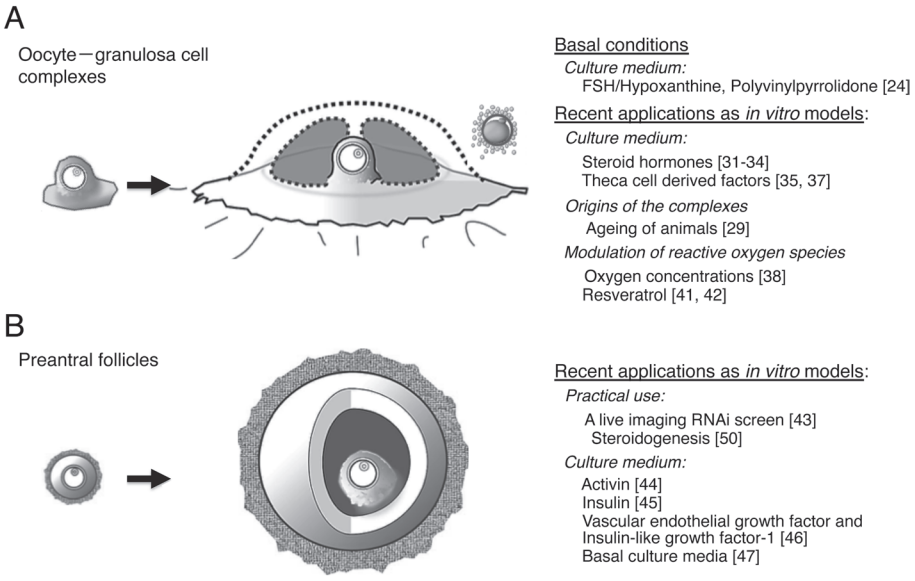

Fig. 2. A summary of recent studies of *in vitro* growth of oocyte–granulosa cell complexes collected from early antral follicles (A) and preantral follicles (B). Studies included in group A were conducted with an emphasis on oocyte competence after growth *in vitro*, while those included in group B placed more emphasis on the growth of follicles.

lected from early antral follicles, it is relatively easy to establish the conditions under which oocytes and associated granulosa cells can establish independent units that are capable of supporting oocyte growth. Extensive studies have been conducted for over a decade using mainly bovine oocytes. Recent findings are summarized in Fig. 2A. Oocyte–granulosa cell complexes usually spread on a substratum (the substratum-adhering system), and the culture media used in most studies have been supplemented with high concentrations of polyvinylpyrrolidone (PVP; molecular weight: 360,000) [24]. Only the complexes comprised of an oocyte and associated granulosa cells, not the whole follicle, have been cultured, because the follicles of large animals are much larger than those of mice and difficult to maintain for a long period of time *in vitro*. A recent finding in pigs suggests that oocyte–granulosa cell complexes can be reconstituted during growth, even after they have been disassociated [25].

The importance of the "timetable" of culture

Although bovine oocytes can grow *in vitro* for the latter half of their growth period [24], further improvements to existing culture systems are necessary, because oocytes grown *in vitro* continue to be smaller than those grown *in vivo*. In a study, in which basic conditions were reexamined, the importance of the appropriate lengths of culture periods for oocyte growth, and of the presence of a "lead-time" (pre-IVM) prior to meiotic maturation was suggested [26]. In the mouse, it has been shown that the oocyte size at the beginning of culture influences the appropriate length of the culture period [27]. Therefore, we need to pay careful attention to the size of oocytes at the beginning of culture. The implementation of pre-IVM before oocyte maturation is considered beneficial for oocytes having reached their full size *in vivo* [28]. Therefore, as a logical extension, allowing a period of time for pre-IVM should be beneficial for oocytes grown *in vitro* to undergo maturation in sequence. Besides the culture conditions, the age of cattle providing oocytes for IVG also negatively affects oocyte growth [29].

Ovarian steroids and theca cells

A characteristic of the culture system used for growing bovine oocyte–granulosa cell complexes is the absence of theca cells. Taking advantage of the absence of theca cells and factors produced by theca cells, the growth-promoting effects of androgen [30] and estrogen [31] on oocytes and granulosa cells have been investigated. It is of interest that the addition of androstenedione promotes the acquisition of meiotic competence by oocytes toward the end of growth *in vitro* [32, 33]. When 17β-estradiol and androstenedione were added together to the medium, the resulting oocytes were able to undergo fertilization and preimplantation development at rates

comparable to those of oocytes grown *in vivo* [34]. The cytoplasm of bovine oocytes grown *in vitro* was able to perform the specialized activities of nuclear reprogramming, as evidenced by the production of fertile offspring after nuclear transfer [35]. Moreover, oocytes grown *in vitro* after vitrification-warming were capable of undergoing meiotic maturation [36]. On the other hand, growth of bovine oocytes was somewhat diminished by the supplementation of bone morphogenetic protein 4, a transforming growth factor *β* superfamily ligand, which is produced by theca cells [37].

Modulation of reactive oxygen species

Oxygen concentration is another important environmental factor that we can control during oocyte culture. In a study, in which growing bovine oocytes were cultured for 14 days, the largest population of fully-grown oocytes was obtained when oxygen concentrations were altered from 5% to 20% in accord with the growth of oocyte–granulosa cell complexes [38]. In more recent attempts, resveratrol, a potent scavenger of reactive oxygen species (ROS), was used to counter the negative effects of ROS being generated during culture. Although the effects of resveratrol on bovine oocytes already grown *in vivo* have varied among studies [39, 40], some positive effects of 20 *μ*M resveratrol have been found for oocytes that were collected from aged cattle and then grew *in vitro* [41]. In the pigs, at a concentration of 2 *μ*M resveratrol increased the amount of ATP in oocytes during growth *in vitro* [42].

In Vitro Growth of Primordial/Preantral Follicles and Oogenesis from Primordial Germ Cells

Culture of preantral follicles

Studies of *in vitro* growth of preantral follicles have been conducted using various species, and most have focused on the growth of follicles, i.e. their size, formation of an antral cavity, and steroidogenesis, not on the growth of oocytes, except for studies using mice. Recent findings are summarized in Fig. 2B. Culturing mouse preantral follicles is now an established research tool for examining the mechanisms involved in oogenesis that cannot be studied in *in vivo* oocytes, such as in live-imaging RNAi screens of important genes for oocyte development [43].

Almost all recent studies that have used bovine or equine preantral follicles have focused on the growth of follicles rather than oocytes. The factors that have recently been examined are the following: activin [44], insulin [45], vascular endothelial growth factor and insulin-like growth factor-1 [46], different basal culture media [47], and heat shock [48]. The same can be said for studies of ovine and caprine preantral follicles, although some observations were made of the early development of oocytes after IVG [49]. The main processes examined after follicle culture were, for example, steroidogenesis [50] and the expression of connexin 37/43 [51]. Tasaki *et al.* [52] have also reported a growth promoting effect of 17*β*-estradiol on porcine preantral follicles *in vitro*.

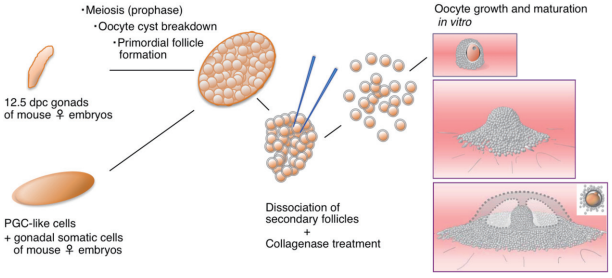

Fig. 3. A schematic diagram of complete *in vitro* recapitulation of oogenesis in the mouse from primordial germ cells (PGCs) and PGC-like cells. The whole procedure comprises three main steps: (1) formation of primary oocytes, which soon become arrested at the prophase of the first meiosis followed by primordial follicle formation; (2) follicular growth toward secondary follicles, which are dissociated and treated with collagenase; and (3)*in vitro* growth of oocyte–granulosa cell complexes.

Culture of primordial follicles

Primordial follicles in the mouse ovary have also been the subject of studies of IVG of oocytes. The mouse 2-step follicle culture system developed by Eppig and colleagues [53, 54] has made it possible to support oocyte IVG, from the primordial follicle stage to the competent mature stage. Production of fertile oocytes from primordial follicles *in vitro* was recently confirmed by Morohaku *et al.* [55]. Similar studies have been conducted on bovine and human ovarian tissues [44, 56], though at the present time significant improvements will be necessary to achieve full oocyte growth in those species.

In vitro oogenesis from primordial germ cells

Reconstituting gametogenesis *in vitro* from primordial germ cells is a key goal for reproductive biology and regenerative medicine. Recently, the first reconstitution of the entire process of mouse oogenesis *in vitro* from primordial germ cells was achieved using a novel culture system that promoted normal follicle formation [57] (Fig. 3). All the events crucial in oogenesis were reproduced

in the culture system, as evidenced by the birth of fertile offspring [57]. Cryopreserved gonads yielded functional oocytes and offspring when the newly developed culture system was used [57]. Most recently, PGC-like cells, originally produced from mouse embryonic stem (ES) and induced pluripotent stem (iPS) cells, have developed into functional oocytes *in vitro* with the help of gonadal somatic cells of female mouse fetuses [58] (Fig. 3). These systems should assist in increasing our understanding of the mechanisms of oogenesis and also lead to the creation of new gamete resources for mammals.

Conclusions

The long-standing question regarding the regulatory mechanisms of meiotic arrest and resumption in mammalian oocytes has finally been resolved, and complete *in vitro* production of mature oocytes with a full developmental competence from PGCs and PGC-like cells has become possible in the mouse. It can safely be said that progress in our understanding and manipulation of mammalian oocytes has accelerated rapidly in the past several years. The information on oocytes/follicles in *in vivo* and *in vitro* systems described in this review should enable both innovative approaches leading to a deeper understanding of oogenesis and the development of new methods for the creation and preservation of female germ cells.

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