CHAPTER 1

Oocyte growth and developmental competence

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INTRODUCTION

While great progress has been made in the treatment of infertility by assisted reproduction technologies (ART), the quality of human oocytes retrieved for infertility treatment is still surprisingly low, with only 10–20% of human eggs producing pregnancy. In order to improve treatment, we must better understand how healthy oocytes develop and what can go wrong during oocyte growth and maturation. The ability to grow mature oocytes from immature oocytes in vitro would mean that women would not require the expensive drug regime and monitoring that they currently have to undergo, as this could all be controlled in vitro. However, progress has been slow in developing these techniques for use in women, with the major problem being a lack of knowledge of how the oocyte acquires developmental competence during its growth within the follicle. The overall aim of current research should be to gain an understanding of how to produce quality oocytes and to elucidate the consequences of impaired oocyte health. The key processes required to produce healthy mature oocytes should be identified, as well as the identification of non-invasive markers to distinguish healthy from unhealthy oocytes. Once we have a better understanding of the factors that are required during development to make a good oocyte, then perhaps we will be able to develop in-vitro growth systems for clinical application.

OVERVIEW OF OOCYTE DEVELOPMENT

Ovarian follicles begin their development as primordial structures, which consist of an oocyte arrested at the diplotene stage of the first meiotic division, surrounded by a few flattened granulosa cells. Once the pool of primordial follicles has been established, and in response to an unknown signal, follicles are gradually and continuously recruited to grow. This initial growth is independent of the pituitary gonadotropins. During early follicular development, the oocyte grows and the granulosa cells proliferate to form a multi-laminar structure called a preantral follicle. Once the follicle reaches a species-specific size, it forms a fluid-filled space called an antrum. When this stage has been reached, follicles become acutely dependent on gonadotropins for further growth and development.

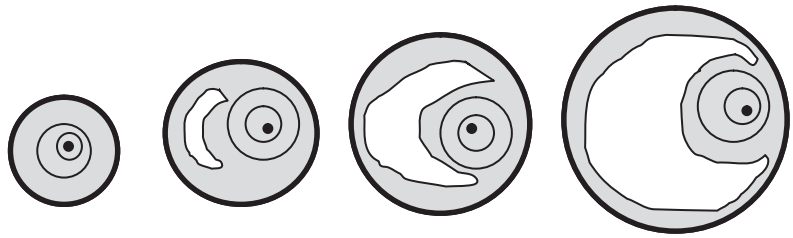
The growth phase of the oocyte allows development of the zona pellucida and production of mRNA and proteins required for subsequent fertilization and early embryonic development.

These factors must be stored within the oocyte, as resumption of meiosis results in transcriptional silencing[1]. Oocyte developmental competence, defined as the ability of the oocyte to resume and complete meiosis, and support preimplantation embryonic development after fertilization, is acquired gradually during folliculogenesis, and oocytes must grow in order to become competent (Figure 1.1). During growth, oocytes also differentiate; a complex cytoplasmic organization is required, dependent on production of new gene products and organelles and the modification and redistribution of existing ones[2]. Since the oocyte chromosomes and cytoplasm have many roles during this process, functional distinctions between nuclear, cytoplasmic, and molecular maturation have been made[3]. Nuclear maturation reflects the transformation of chromatin status from dictyate (germinal vesicle, GV) to metaphase II stage, whereas cytoplasmic maturation encompasses changes in the distribution and organization of the individual organelles such as the cortical granules from germinal vesicle to metaphase II stages. Lastly, molecular maturation can be described as the legacy of the instructions accumulated during the GV stage that control both nuclear and cytoplasmic progression[3].

Oocytes may reach a diameter of approximately 120 μm in early antral follicles of humans, while in rodents, the maximum diameter of 70 μm has already been reached at the end of the preantral stage and the oocyte acquires the competence to resume meiosis. Once competence is achieved, the oocyte remains in meiotic arrest until the preovulatory gonadotropin surge. The competence of an oocyte to resume and complete meiosis and, after fertilization, to develop into a blastocyst is markedly increased during preovulatory development through a process called oocyte capacitation[4]. Thus, after termination of oocyte transcription, the oocyte does not enter quiescence but prepares itself for a possible continued development as an embryo after fertilization.

MAINTENANCE OF OOCYTE MEIOTIC ARREST

Throughout oocyte growth, prophase arrest is thought to be maintained by inherent factors in



Follicle stage	Preantral	Early antral	Late antral	Preovulatory
Oocyte growth	Growing	Late growth	Fully grown	Fully grown
GVBD	Incompetent	Competent	Competent	Competent
MII	Incompetent	Incompetent	Competent	Competent
Embryogenesis	Incompetent	Incompetent	Limited	Competent

Figure 1.1 Oocyte developmental competence is defined as the ability of the oocyte to resume and complete meiosis, i.e. to undergo germinal vesicle breakdown (GVBD) and progression to metaphase II (MII), and to support preimplantation embryonic development after fertilization. This acquisition of oocyte developmental competence is acquired gradually during follicular development

the oocytes, and is correlated with low levels of cell cycle regulatory proteins such as $p34^{cdc2}$ and cyclin B[5]. Once oocytes become meiotically competent, maintenance of meiotic arrest requires the activity of signaling molecules within the oocyte such as the heterotrimeric G protein, Gs[6,7]. A G protein-coupled receptor, named Gpr3, which is present in oocytes, has recently been identified as a negative regulator of meiotic resumption[7]. A high proportion of oocytes within antral follicles in mice lacking Gpr3 contained metaphase chromosomes, indicating the resumption of meiosis. The predominant expression of Gpr3 mRNA in the oocyte, compared with somatic cells, and the dependence of meiotic arrest on Gs, supports the conclusion that the Gpr3 receptor in the oocyte is required to maintain meiotic arrest[7] (Figure 1.2). However, the putative ligand for the Gpr3 receptor remains unidentified, but may be produced by the granulosa cells, since competent oocytes spontaneously resume meiosis upon removal from the surrounding granulosa cells[8].

The level of cyclic adenosine monophosphate (cAMP) in the oocyte plays a critical role in resumption of meiotic maturation, as pharmacologic manipulation to increase cAMP levels in competent mammalian oocytes results in inhibition of meiosis[9]. It has been hypothesized that a breakdown in gap junctional communication between the oocyte and granulosa cells at the time of the preovulatory LH surge results in a decrease in cAMP levels within the oocyte, leading to the inactivation of the PKA pathway[10] (Figure 1.2). However, germinal vesicle breakdown (GVBD), the first stage of meiotic resumption, occurs prior to any detectable ionic or metabolic uncoupling between the oocyte and cumulus cells, supporting the idea that a cumulus or granulosa cell factor could override the inhibitory effects of cAMP and promote meiotic resumption[11]. On

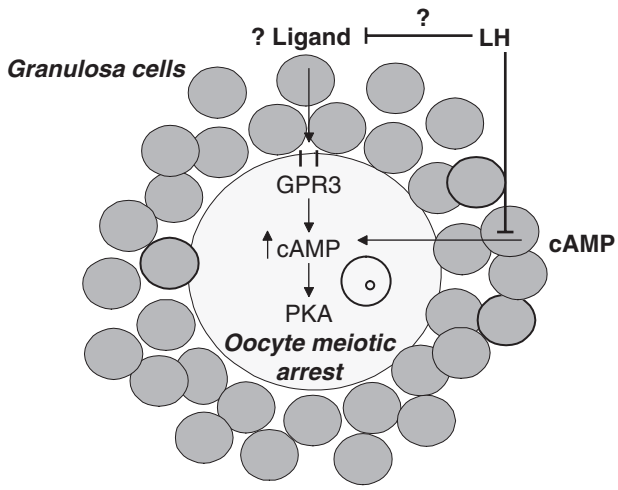


Figure 1.2 The level of cyclic adenosine monophosphate (cAMP) in the oocyte plays a critical role in resumption of meiotic maturation. Breakdown in gap junctional communication between the oocyte and granulosa cells at the time of the preovulatory LH surge is thought to result in a decrease in cAMP levels within the oocyte, leading to the inactivation of the PKA pathway. The Gpr3 receptor in the oocyte has been recently identified and is required to maintain meiotic arrest. However, the putative ligand for the Gpr3 receptor remains unidentified, but may be produced by the granulosa cells, since competent oocytes spontaneously resume meiosis upon removal from the surrounding granulosa cells

the other hand, the oocyte and granulosa cells may respond separately to LH in terms of their production of cAMP. This idea is supported by experiments using cell type-specific phosphodiesterase (PDE) inhibitors, where inhibition of PDE3A, expressed exclusively in oocytes, completely blocks oocyte maturation in vitro and in vivo[12,13], whereas inhibition of PDE4, restricted to granulosa cells, has no effect[12,14,15].

IN-VITRO MODELS FOR THE STUDY OF OOCYTE GROWTH AND DEVELOPMENTAL COMPETENCE

Culture systems for preantral follicles, or their oocyte–granulosa cell complexes (OGCs), are important for studying oocyte development, as well as for analysis of follicular development and function. Techniques for isolation of immature follicles and OGCs have been established in rodent and domestic animal species, as well as in humans[16–18]. The viability of the rodent systems has been demonstrated by the production of live offspring from in-vitro grown OGCs from preantral follicles[19] and from the culture of whole preantral follicles[20]. More recently, oocytes from primordial follicles activated in vitro have acquired competence to be matured and fertilized, resulting in the production of live offspring[21,22].

The limited success of these rodent systems has resulted in attempts to develop similar methods to be applied in humans[23–25]. Less densely packed follicles, fibrous stromal tissue, larger follicles, and slow follicular growth have all played a role in delaying a successful system for isolation and culture of preantral follicles from women. In addition, difficulty in obtaining a sufficient amount of quality human ovarian material has limited the progress of development of culture systems for human follicles. Both mechanical[24] and enzymatic isolation techniques[23,26] have been applied to human preantral follicles. Another potential method of obtaining human follicles is the use of aspirates obtained during oocyte retrieval for IVF, as has been reported by Zhang et al.[27]. However, the number of follicles recovered was low, and it was concluded that this was not a useful source of human follicles[27]. Thus, research should focus on improving techniques for the isolation and growth of human follicles. Another potential option for the preservation of fertility in women at risk of premature ovarian failure, such as those about to undergo chemotherapy, is the cryopreservation and subsequent transplant of ovarian tissue. Ten years after the first demonstration of restoration of fertility in sheep[28], a healthy child was born after transplant of a frozen-thawed ovarian autograft into a woman cured of Hodgkin's disease[29]. However, despite further success in this area[30], this technology is still very much at an early stage and more research is required to improve clinical outcome. In addition, as the risk of reseeding cancer cells from the graft poses a serious problem, research should focus on improving in-vitro methods for growing and maturing early stage follicles present in the cryopreserved ovarian tissue.

In-vitro systems for rodent and domestic animal species have been instrumental in the progression of knowledge of oocyte growth and developmental competence. This review will highlight some of the factors that have been shown to participate in oocyte development in animal models, as well as the relevance to human fertility.

PARACRINE CONTROL OF OOCYTE DEVELOPMENT

Within the follicle, the three-dimensional organization of the cells and the paracrine interactions between the oocyte and surrounding granulosa cells are critical for normal cell development and function[31–33] (Figure 1.3). Intrafollicular paracrine factors mediate oocyte–granulosa cell interactions and are essential for the production of healthy oocytes. The importance of some of these factors is described in the following sections.

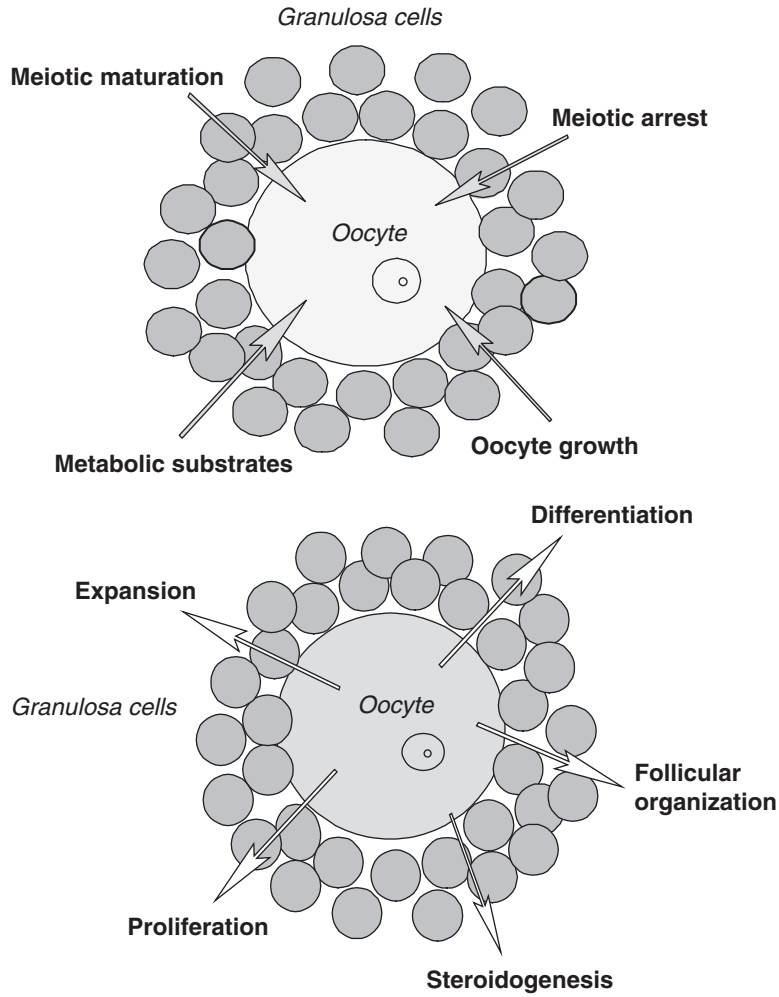


Figure 1.3 Within the follicle, the three-dimensional organization of the cells and the paracrine interactions between the oocyte and surrounding granulosa cells are critical for normal cell development and function. Via gap junctions and paracrine factors, granulosa cells influence a number of oocyte activities (upper diagram), while oocytes are capable of controlling and/or influencing several granulosa cell functions (lower diagram)

Granulosa cell factors

Kit ligand

The tyrosine kinase receptor Kit and its ligand, Kit ligand (KL; also known as KitL and SCF), have been localized to oocytes and granulosa cells, respectively[34]. KL has been shown to stimulate oocyte growth[35,36], and increased KL in follicular fluid in in-vitro fertilization (IVF) patients has been correlated with successful pregnancies[37]. KL is expressed in granulosa cells as either membrane-bound or soluble proteins arising from alternatively spliced mRNAs[38].

Soluble KL (KL-1) can be cleaved due to the presence of an 84 base pair exon (exon 6), which encodes a proteolytic cleavage site, allowing the extracellular domain to be released as a soluble product. Membrane-bound KL (KL-2) lacks this exon, is not efficiently cleaved, and thus remains more stably on the membrane[38]. The ratio of KL-1/KL-2 mRNA differs between tissues[38], between ovaries of mice of different ages[39], and between granulosa cells of preovulatory and ovulatory rat follicles[40], suggesting that these transcripts are differentially regulated.

Kit, the receptor for KL, is expressed in oocytes at all stages of follicular development in the mouse ovary[41], and several studies have demonstrated that female mice with naturally occurring mutations in KL or Kit are infertile due to developmental abnormalities[41,42]. For example, mice homozygous for the *Sld* allele, which only produce KL-1, are sterile due to a deficiency in germ cells[43]. However, mice that exclusively produce KL-2 are fertile[44], suggesting that KL-2 may be the principal isoform required for oocyte development. Indeed, KL-2 has been reported to induce a more persistent activation of Kit receptor kinase than the soluble form of KL[45,46], and thus is likely to be the more potent isoform for regulation of oocyte growth.

In order to determine the specific role of each KL isoform in promoting murine oocyte growth and maintenance of meiotic arrest in vitro, experiments using oocytes in co-culture with fibroblasts expressing either KL-1 or KL-2 have been performed in our laboratory. The data suggest that KL-2 is the principal isoform required to regulate oocyte growth and prevent spontaneous GVBD in isolated growing oocytes[47]. In addition, using immunofluorescence, it was found that KL-2 maintained the expression of Kit on the oocyte surface, whereas treatment with soluble KL-1 resulted in downregulation and/or internalization of the Kit receptor[47]. This phenomenon has previously been shown in mast cells[48]. Previous data from our laboratory have also shown that, in response to human chorionic gonadotropin (hCG), there is a shift in steady-state mRNA from KL-2 to KL-1 in rat mural granulosa cells[40], and a rapid depletion of both isoforms in cumulus cells, which suggests differential functions of the KL isoforms during oocyte development and meiotic progression.

Oocyte factors

Growth/differentiation factor-9 (GDF-9)

GDF-9 has been shown to be expressed in human and mouse ovaries, and appears to be localized exclusively to oocytes at all stages of follicular growth, except primordial follicles, in neonatal and adult mice[49]. The pattern of GDF-9 expression as well as results from GDF-9 gene knockout studies suggest that this factor may play an autocrine role in the regulation of oocyte development and maturation and/or a paracrine role in the regulation of granulosa cell proliferation and differentiation[50,51].

In GDF-9-deficient mouse ovaries, follicular development does not progress beyond the primary stage, but the oocytes within these follicles grow larger than normal[50,52]. In addition, oocytes from GDF-9-deficient mice do not acquire full developmental competence[51]. Interestingly, these mutant mice also have elevated levels of KL mRNA[51,52], which suggests that GDF-9 regulates KL expression. Differential regulation of the two KL transcripts is likely to be a vital component of regulation of KL expression during oocyte and follicular development. Since downregulation of KL-2 expression coincides with the cessation of oocyte growth[36], oocytes in GDF-9-deficient mice may exceed the normal maximum diameter due to continued elevation of KL-2 expression. Thus, a failure to downregulate KL-2 at the appropriate stage of development may impair oocyte growth and acquisition of developmental competence.

With respect to human fertility, a decrease in GDF-9 mRNA expression has been reported in human polycystic ovaries[53], thus deregulation

of GDF-9 expression may contribute to aberrant folliculogenesis in women with polycystic ovary syndrome (PCOS). In addition, recombinant GDF-9 has been shown to promote the development of human primordial follicles to the secondary stage in culture, as well as improving follicular survival[54]. However, more studies are required to determine the significance of GDF-9 for oocyte growth and developmental competence in humans.

Bone morphogenetic protein-15 (BMP-15)

BMP-15 is an oocyte-specific homolog of GDF-9, and has been cloned in mice[55]. In sheep, where this factor has been well studied, the Inverdale fecundity gene (FecX) carries an inactivating mutation in BMP-15[56], implicating this factor in the control of ovulation rate. In rodents, both GDF-9 and BMP-15 promote proliferation of granulosa cells from small antral follicles[57–59], and BMP-15 has been reported to inhibit FSH-stimulated progesterone production by rat granulosa cells[58]. Evidence of interactions between GDF-9, BMP-15, and KL in vitro has been reported[60,61]. For example, recombinant GDF-9 inhibits KL mRNA expression in mouse preantral granulosa cells[60], whereas BMP-15 promotes KL expression in monolayers of granulosa cells from rat early antral follicles[61] (Figure 1.4). Recently, work in our laboratory has provided evidence of communication between BMP-15 and KL at the molecular level in intact murine OGCs in vitro[36]. By inhibition of Kit activity within OGCs in vitro, we are able to propose a mechanism whereby FSH regulates BMP-15 expression in a dose-dependent manner via Kit signaling. Thus interactions between these oocyte and granulosa cell factors, regulated by FSH, are likely to play a role in oocyte development (Figure 1.5).

The relevance of BMP-15 in human ovarian development remains unclear; one study has reported no aberrant BMP-15 mRNA expression in human polycystic ovaries[53], whereas preliminary data have recently been presented showing a loss of BMP-15 protein expression in ovaries from women with PCOS[62]. Significant species differences may exist in the relative importance of BMP-15, and much remains to be elucidated about the roles of BMP-15 and GDF-9 in the human ovary.

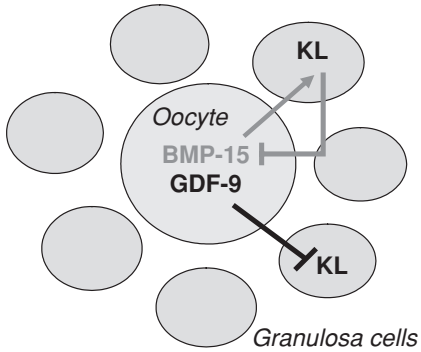


Figure 1.4 Interactions between GDF-9, BMP-15, and KL in vitro. Recombinant GDF-9 has been shown to inhibit KL mRNA expression in mouse preantral granulosa cells, whereas BMP-15 promotes KL expression in monolayers of granulosa cells from rat early antral follicles. In addition, a negative feedback loop can be proposed, as an increase in KL expression results in suppression of BMP-15 in mouse oocyte–granulosa cell complexes grown in vitro

THE ROLE OF FSH IN OOCYTE DEVELOPMENT

Endocrine control of follicular development by FSH rests on a network of intrafollicular paracrine interactions[63]. For example, FSH promotes proliferation and differentiation of preantral follicles via paracrine factors such as IGF-1 and activin[64,65]. In addition, FSH regulates KL expression in granulosa cells from murine preantral follicles[66]. We have recently investigated the role of FSH in the regulation of KL

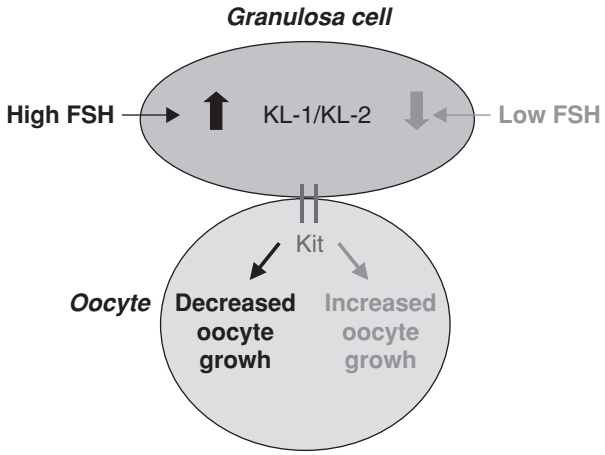


Figure 1.5 The correct concentration of FSH is required to modulate intrafollicular levels of KL mRNA, which subsequently controls oocyte growth. Low concentrations of FSH promote oocyte growth by increasing KL-2 expression, thereby reducing the ratio of KL-1/KL-2. Increased KL-1/KL-2 expression, stimulated by high concentrations of FSH, enhances follicle development but impairs oocyte growth

expression during the development of mouse preantral oocyte–granulosa cell complexes in vitro[36]. It was demonstrated that a low concentration of FSH decreased the ratio of steady-state KL-1/KL-2 mRNA by increasing KL-2 mRNA levels, and this was associated with increased oocyte growth in culture[36] (Figure 1.5). These results suggest that the correct balance of KL-1/KL-2 production is necessary for optimum oocyte growth in vitro. In addition, the correct concentration of FSH is crucial for appropriate regulation of paracrine factors to promote oocyte development. In granulosa-luteal cells obtained after oocyte harvest from patients undergoing IVF, a decrease in KL mRNA expression was reported in response to FSH and hCG in a time- and concentration-dependent manner in vitro[67], suggesting that KL is hormonally regulated in humans, and is likely to participate in follicular function during the human menstrual cycle.

Several studies provide evidence that a cautious approach is required when using FSH to stimulate oocyte and granulosa cell development. For example, repeated ovarian stimulation of mice with gonadotropins in vivo has been reported to reduce subsequent in-vitro meiotic competence of oocytes[68]. Eppig et al.[69] reported that a relatively high concentration of FSH, in the presence of insulin, promoted precocious differentiation of granulosa cells within cultured preantral OGCs. These OGCs were also found to contain oocytes with reduced competence to undergo fertilization and preimplantation development[69] (Figure 1.6). A subsequent study by the same authors used a much lower concentration of FSH during follicular growth in vitro, and the rate of oocyte fertilization and blastocyst development was significantly improved[22]. Therefore, FSH is important for the acquisition of oocyte developmental competence, but the correct concentration of FSH is required to prevent premature follicle differentiation that would impair oocyte developmental competence. Recently, Roberts et al.[70] demonstrated that FSH impaired metaphase I chromosome alignment and increased aneuploidy in oocytes from mouse antral follicles matured in vitro, and these abnormalities were exacerbated

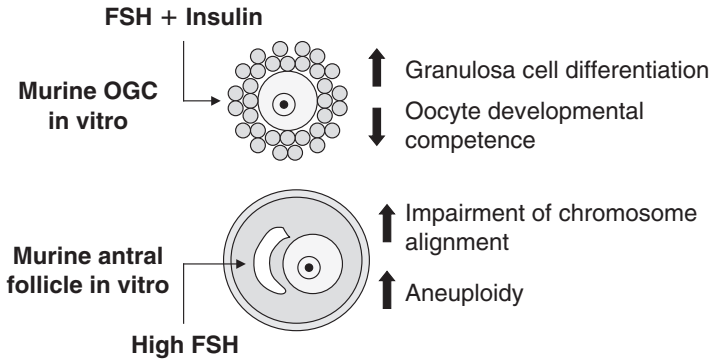


Figure 1.6 Murine oocyte–granulosa cell complexes (OGC) and antral exposed to high concentrations of FSH in vitro result in aberrant granulosa cell differentiation and oocyte development

with increased concentrations of FSH (Figure 1.6). Interestingly, the authors hypothesized that exposure of oocytes to high levels of FSH during meiotic maturation increases PKB/Akt phosphorylation, resulting in global phosphorylation and inactivation of spindle GSK-3. The resulting increased spindle stability provides an explanation for the greater chromosomal dispersion in oocytes exposed to high concentrations of FSH[70]. Importantly, the need for appropriate use of FSH in the treatment of human infertility has been demonstrated by Leader et al. (unpublished observations), who have shown that follicular growth and ovulation rate are increased using a low-dose FSH protocol for ovulation induction in women with anovulatory infertility, compared with a higher dose of FSH.

The association between oocyte and embryo culture and fetal abnormalities in animal models[71,72] is a growing cause for concern. The increasing trend to culture embryos for longer periods of time before uterine transfer so that the 'best' embryos can be used for transfer could also pose a problem[73]. The contribution of embryo culture to perturbations in metabolism and gene expression and the long-term consequences on development and behavior have been examined by Ecker et al.[73], who demonstrated that adult mice derived from cultured embryos exhibited specific behavioral alterations in anxiety/locomotor activity, as well as spatial memory. Another study also demonstrated that specific culture conditions have the ability to change the pattern of gene expression in preimplantation mouse embryos[74]. It is therefore of the utmost importance to investigate this phenomenon during the growth and maturation of immature oocytes in vitro, as well as during embryo culture, if in-vitro technology is to be applicable to the preservation of human fertility.

GAP JUNCTIONS AND TRANSZONAL PROCESSES (TZPS) PARTICIPATE IN THE CONTROL OF OOCYTE DEVELOPMENT

Efficient delivery of factors to and from the oocyte at critical stages of development is essential for the coordination of oogenesis and folliculogenesis. Gap junctions, a form of oocyte–granulosa cell communication mediated by connexins, are also necessary for normal follicular development. A stage-specific pattern of distribution of different types of connexins during murine follicle development has been established[75]. Another study has shown that KL induces the onset of in-vitro growth of isolated fetal mouse oocytes, in the absence of gap junctional communication with granulosa cells[76]. However, these oocytes

were unable to progress to the final stages of growth, and there was a lack of synchrony between nuclear and cytoplasmic maturation. It was noted that these oocytes had characteristics resembling oocytes from connexin-43- and -37-deficient mice, which have impaired follicular development beyond the preantral and early antral stages, respectively[77,78]; thus it was hypothesized that the preantral/antral transition is a critical stage of oocyte development requiring the coordinated differentiation of the oocyte with the granulosa cells[76]. The maintenance of adequate communication between these two cell types during the preantral and early antral stages is therefore necessary to ensure subsequent oocyte developmental competence. Gittens et al.[79] have provided evidence for interplay between paracrine signaling and gap junctional communication. In that study, expression of KL, Kit, and GDF-9 were analyzed in connexin-43-deficient mice, and the expression of connexin 43 was analyzed in GDF-9-deficient mice. The results suggest that, although gap junctional coupling among granulosa cells is not required to sustain expression of these paracrine factors, and GDF-9 is not required to sustain gap junctional coupling among granulosa cells, granulosa cells must be coupled via connexin 43 gap junctions in order to optimally respond to GDF-9[79].

As discussed earlier, paracrine factors secreted by oocytes and somatic cells regulate many important aspects of oocyte and follicular development, and there is substantial evidence which supports a model for bidirectional paracrine communication, based on the developmental regulation of the delivery and reception of paracrine factors at the oocyte–granulosa cell interface[80]. TZPs, which are granulosa cell extensions that traverse the zona pellucida and terminate on the oocyte cell surface, have been characterized in many mammals by electron microscopy[80,81]. These TZPs have been shown to undergo dynamic alterations in form and number during the course of follicular development[81]. TZPs are most numerous at the preantral stage, forming adhesive and gap junctional contacts at the oolemma. During peak periods of oocyte growth, TZPs extend as deep invaginations, impinging on the oocyte germinal vesicle[81]. FSH has recently been shown to regulate the ability of granulosa cells to make connections with the oocyte[82]. In that study, it was shown that FSH treatment of prepubertal or FSHβ-knockout mice decreased the density of TZPs, which coincided with changes in chromatin remodeling and acquisition of oocyte meiotic competence[82]. Given the importance of oocyte–somatic cell interactions during early follicular development, investigation into regulation of the structural integrity of this interface during growth in vitro is required if culture systems for the production of competent oocytes are to improve significantly.

CONCLUSIONS

At present, the major barrier to developing and optimizing in-vitro techniques for alleviation of infertility in women is our lack of knowledge of how the oocyte acquires developmental competence during its growth within the follicle. The overall aim of current research should be to gain an understanding of how to produce quality oocytes and to elucidate the consequences of impaired oocyte health. Currently, research is under way to identify cross-species (including human) determinants of oocyte quality using a variety of techniques, from culture systems to animal models of infertility. Once we have a better understanding of the factors that are required during development to make a good oocyte in these animal models, then perhaps we will be able to develop in-vitro growth systems for clinical application. Importantly, any factors identified that promote oocyte health, or are indicators of oocyte quality, will be investigated in humans with correlations to pregnancy outcome and offspring health.

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