CHAPTER 7

In-vitro development of small ovarian follicles

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RELEVANT ASPECTS OF EARLY IN-VIVO OOGENESIS AND FOLLICULOGENESIS WITH EMPHASIS ON HUMANS

The in-vivo development of germ cells and follicles from fetal to adult life will be discussed briefly, focusing on topics essential for the understanding of the later sections on the in-vitro processes. Despite the numerous studies performed on ovarian development, much is still unknown, especially regarding the early stages of oogenesis and folliculogenesis[1,2].

Human primordial germ cells (PGC) arrive from the yolk sac to the gonad from day 26 of pregnancy, and are then termed oogonia[2,3]. Recently, PGCs have also been identified in ovaries of adult mice[3,4] and women[3,5], but this issue needs to be further investigated.

Three events induce the development of human female fetal germ cells in the gonad (ovary): mitotic division cycles of the oogonia, meiotic division, and follicular assembly[2,3]. The number of female germ cells in the fetal ovary peaks at about 7 million in mid pregnancy and then drops drastically during the third trimester. Meiotic division usually commences gradually in the third month of gestation, and the diplothen stage is achieved within weeks of its initiation. At this point, the oogonia enlarge and acquire more intracellular organelles and are then termed oocytes. Just before birth, the oocytes are arrested in the diplothen stage of the prophase of the first meiotic division[2,3]. They have completed genetic recombination and do not undergo any additional nuclear maturation until puberty.

Follicular formation in humans begins during the fourth month of gestation. In rats and mice, this process occurs during the first postnatal days[3,6]. During follicular assembly there is a rapid proliferation of the nearby cells, and the oocytes become surrounded by a single layer of flattened somatic cells, termed granulosa cells (GC), enclosed by a basement membrane[1–3]. These cellular complexes are defined as *primordial follicles* (30–50 μm in diameter) and can be identified in the human from around 22 gestational weeks (GWs).

Most of the follicles in human ovaries of adults as well as fetuses remain primordial and reach ovulatory sizes (18–20 mm in diameter) within 6 to 9 months[1,3]. Primordial follicles are activated when their GC become cuboidal, and these are then termed *primary follicles* (50 μm–0.1 mm in diameter). Thereafter, the increased proliferation rate yields a multilaminar granulosa layer[1–3], *secondary follicles* (0.1–0.2 mm in diameter). In the

human, a definitive theca layer is created from the surrounding stroma cells around the follicle when a secondary follicle contains three to six GC layers[1–3]. Steroid hormones are synthesized through complex interactions between the GC and theca cells. During the secondary follicular stage the oocyte starts to grow, while forming a glycoprotein coat, the *zona pellucida* (ZP), between itself and the innermost layer of the GC. The final follicular stage consists of the development of *antral follicles* (early antral follicle: 0.2–0.4 mm in diameter) containing fluid-filled cavities within several layers of cuboidal GC; the innermost layers surrounding the oocytes are termed *cumulus cells*. At ovulation, the first meiotic division is completed with the extrusion of the first polar body enclosed between the oocyte and the ZP, forming a mature oocyte.

The growth regulation of primordial follicles is not hormonal. Follicle-stimulating hormone (FSH) sustains follicular development and growth only from secondary stages. The exact factors that stimulate growth of primordial follicles are unknown and various growth factors have been suggested for this role[2,3] (see the section 'Factors that might be responsible for early oogenesis or folliculogenesis').

CLINICAL IMPORTANCE OF IN-VITRO MATURATION (IVM) OF PRIMORDIAL FOLLICLES

As cancer treatment improves, more young women of reproductive age are surviving[3]. However, many suffer from ovarian failure and premature menopause, as a consequence of the radiation and chemotherapy[3,7]. Ovarian failure has been recorded mostly after malignancies that affect younger patients: Hodgkin's and non-Hodgkin's lymphoma, leukemias, Ewing and bone sarcomas, brain and breast tumors. These patients have limited options for putative fertility restoration, as the cryopreservation of mature oocytes has shown limited success[3,8].

Currently, egg donation promises, in many cases, the only choice for pregnancy[2,3,9]. However, there is a shortage of donated oocytes worldwide. One possible solution to increase the pool of donated oocytes is to use immature oocytes from ovaries of aborted human fetuses. However, before this becomes clinically feasible, methods to mature fetal follicles in vitro need to be developed[3,10]. Moreover, the use of oocytes from aborted fetuses is highly controversial and is forbidden in many countries.

Be that as it may, most women prefer to use their own oocytes and, therefore, methods to preserve self-fertility are required. Human ovarian tissue containing immature primordial follicles has been successfully cryopreserved after retrieval by a simple laparoscopic operation[3,6,7]. This technique is aimed either at inducing ovarian function by re-plantation of ovarian tissue or, further into the future, by in-vitro maturation (IVM) of oocytes derived from the cryopreserved-thawed ovarian tissue, followed by routine in-vitro fertilization (IVF) and embryo transfer (ET). To date, three live births have been reported after transplantation of ovarian tissue[3,11–13]. However, some cancers, such as hematologic malignancies[3,14] and breast cancer[3,15], carry a possible risk of disease re-transmission by the ovarian grafts.[2,3] Although IVM of primordial follicles would avoid this possibility, because the in-vitro matured oocytes will not contain cancerous cells, this method has so far shown limited success (see the section 'IVM of primordial follicles from adults').

Ovarian cryopreservation could also perhaps benefit girls with Turner's syndrome (TS), especially those with mosaic karyotypes, whose ovaries often contain follicles, usually at early ages[3,9,16] (see also the section 'IVM of primordial follicles from adults'). In these cases, ovarian biopsies should be preferably cryopreserved even before the first signs of puberty, since most or all of the follicles might be lost once signs of puberty appear.

Indeed, once established, fertility restoration by cryopreserved-thawed ovaries may be

preferred also by healthy women who choose to postpone childbearing until later in life[3]. IVM of primordial follicles for artificial reproduction technologies would obviate the complexity, cost, and emotional toll of the procedure, which requires superovulation and extensive monitoring during the follicular phase of the cycle. Furthermore, it would make oocyte donation simpler, because an ovarian biopsy could be retrieved by a simple operation without any hormonal stimulation with its attendant side-effects to the donor. Finally, IVM of primordial follicles would not only benefit human fertility programs but could also enhance efforts at conservation of endangered species.

ATTEMPTS AT IVM OF FETAL OOGONIA/OOCYTES

It is unknown whether oocytes from fetuses have the same developmental capacity as those from adults, and relatively few studies to date have investigated this possibility in mammals.[3] There are several reports on the in-vitro growth of oogonia from fetal mice to antral follicles[3,17] or mature oocytes[3,18]. Fetal bovine and baboon follicles have developed in vitro into primary and secondary follicles with GC proliferation, as demonstrated by their expression of proliferating cell nuclear antigen (PCNA)[3,19–21], which plays an essential role in cell cycle regulation[3,10,19–21]. However, although the diameters of both the fetal bovine follicles and their oocytes increased in culture, about 50% of the follicles were atretic[19].

In another study, human fetal ovaries (13–16 GWs) were cultured for 40 days and, after several weeks, the oogonia entered the initial stages of meiosis[3,22]. Fresh and frozen-thawed human fetal oogonia and primordial follicles (16–20 GWs) were also cultured for 2 months, and morphologically mature oocytes (with a first polar body) were obtained[3,23], although they were smaller than those developed in vivo. In a study conducted in our laboratory[3,10], ovarian specimens obtained from second- and third-trimester human fetuses (22–33 GWs) were cultured for 4 weeks (Figure 7.1). The follicles survived in culture without any apparent increase in the number of primary and secondary follicles, in the expression of PCNA or bromodeoxyuridine (BrdU) incorporation (another marker for dividing cells) in the GC, or in the number of atretic follicles. However, a significant increase in the level of estradiol in the spent media samples was detected in the fourth week of culture, indicating steroidogenesis of secondary follicles.

IVM OF PRIMODIAL FOLLICLES FROM ADULTS

Experiments to mature human oocytes in vitro from primordial follicles have had limited

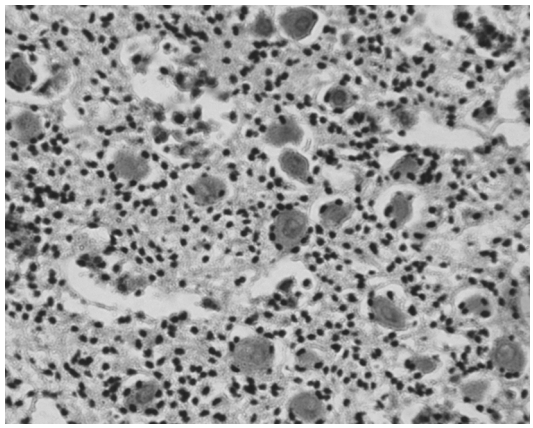


Figure 7.1 Micrograph of cultured human fetal follicles[10]. Section of human ovarian fetal follicles after 3 weeks in culture, stained for bromodeoxyuridine (BrdU) incorporation (DNA division). The tissue was taken from a 33 GWs fetus with achondroplasia. Note the normal primordial follicles, lack of brown BrdU staining in the GC indicating no proliferation, and brown staining only in the oocytes. Background blue staining is hematoxylin. Magnification × 400 (see color plate section)

success[2,3]. Researchers developing such maturation systems face two main problems: the signals involved in the transition of primordial follicles are unknown and there is a need to maintain the long-term survival and growth of the ovarian follicles under culture conditions.

Culture systems

There are two approaches to the culture of primary and primordial follicles[2,3]. The more popular one involves culturing whole slices of ovarian tissue (organ culture), such that the structural integrity of the ovarian tissue is maintained and, hence, the interactions between the surrounding stroma cells and the follicles are retained.

Using this method, primordial follicles from cows have been grown in organ culture to secondary stages[3,24]. Several studies also described the use of organ culture for human primordial follicles[3,25–31]. Although the follicles survived for up to 4 weeks, they did not develop beyond the secondary stages. There were no differences in the developmental capabilities of human ovarian follicles derived from fresh or frozen-thawed tissue[25]. Attempts to optimize the organ culture system yielded a reduced atresia rate[27,29] under serum-free conditions[27] and improved follicular survival in wells coated with diluted extracellular matrix[25,29] and with tissues cut in cubes rather than in long slices[3,29].

The second approach to follicular culture involves the use of isolated primordial and primary follicles[3,32,33] (Figures 7.2 and 7.3). Though whole tissue culturing is much easier than working with tiny follicles of 30–50 μm, the latter method enables researchers to directly monitor follicular growth during the culture period. This is of special importance considering the poorly populated human ovarian tissue in adults. For example, because of the low follicular content of ovaries from patients with TS, organ culture as well as ovarian transplantation could lead to the use of empty ovarian specimens, whereas the recruitment of isolated follicles would make it possible to determine the initial follicular content[2,3].

The first stage of isolation is incubation of the ovarian tissue with a cocktail of digesting enzymes (2 h for human tissue), usually of collagenase and DNase. This softens the stroma tissue and makes the isolation process easier[3,32,33]. The DNase breaks the sticky DNA ends, eliminating the risk of unprocessable digested ovarian tissue. The enzymatic treatment is followed by mechanical microdissection of the ovarian follicles in combination with repeated pipetting. Isolated mouse unilaminar follicles have been cultured to multi-laminar stages in collagen gels[34], and isolated rat follicles co-cultured with stroma cells on poly-L-lysine coated plates developed to preovulatory stages[35]. GC from isolated primary porcine follicles were found to proliferate in culture[36].

One group cultured bovine primary follicles isolated without enzymatic digestion[2,3,37]. The bovine ovarian tissue was cut into small fragments with a tissue chopper. The fragments were then transferred through nylon filters and morphologically normal primary follicles were isolated. These follicles were embedded in collagen gels and cultured. Follicular growth was slow and very limited (up to 24% in follicular diameter) and there was no oocyte growth; 43% of the oocytes were dead after 7 days of culture[37].

In another study, morphologically normal isolated primordial human follicles from fresh and frozen-thawed ovarian tissue survived for up to 5 days in culture, but without indications of actual growth[38]. Our group isolated human primordial and primary follicles from both fresh and frozen-thawed ovarian tissue[32,33] (Figures 7.2 and 7.3). Apart from an increase in lipid droplets in their GC, the isolated follicles appeared ultrastructurally normal compared with follicles in intact tissue blocks. After 24 h culture in collagen gels, 40% of the follicles showed an increase in the number of GC layers (to two or three) and in oocyte size (Figure 7.2). However, the follicles developed only within a supporting collagen

gel matrix and not on collagen, poly-L-lysine, or extracellular matrix[33], or even within agar (Figure 7.3). Although others have demonstrated that human primordial follicles developed better in organ culture than as partially isolated follicles (with some stroma cells attached)[26], we found that only fully isolated follicles could grow in collagen gel culture. Partially isolated follicles only survived in culture, with no signs of GC proliferation or increase in oocyte diameter[33] (Figures 7.2 and 7.3).

The most promising studies of IVM of mammalian primordial follicles

Only in the mouse has the production of live young from cultured primordial follicles been successful[39,40]. This research group developed a two-stage culture system for murine primordial follicles. Primordial follicles were grown in organ culture to secondary follicles. The secondary follicles were then isolated enzymatically and cultured further to mature oocytes, followed by routine IVF and ET. However, to date, only 59 live offspring (5.7% of embryos transferred) have been obtained[40]. The first mouse born was extremely obese and a postmortem examination revealed multiple malformations[39,40].

In some studies, ovarian tissue was grafted to hosts as an intermediate step in IVM of mammalian primordial follicles[41–43]. Specifically, fresh[41] and frozen-thawed[42] murine ovarian tissue was transplanted under the kidney capsule of immunodeficient mice[41,42]. The grafts were removed, and the secondary follicles were isolated and cultured until mature oocytes were obtained, and then further fertilized. Live born mice were reported after IVF and ET.

Similarly, porcine tissue containing primordial follicles was grafted[43], and when the grafts were removed, immature germinal vesicle (GV)-stage oocytes were aspirated from antral follicles. Seventeen percent underwent IVM, out of which 55% were fertilized successfully

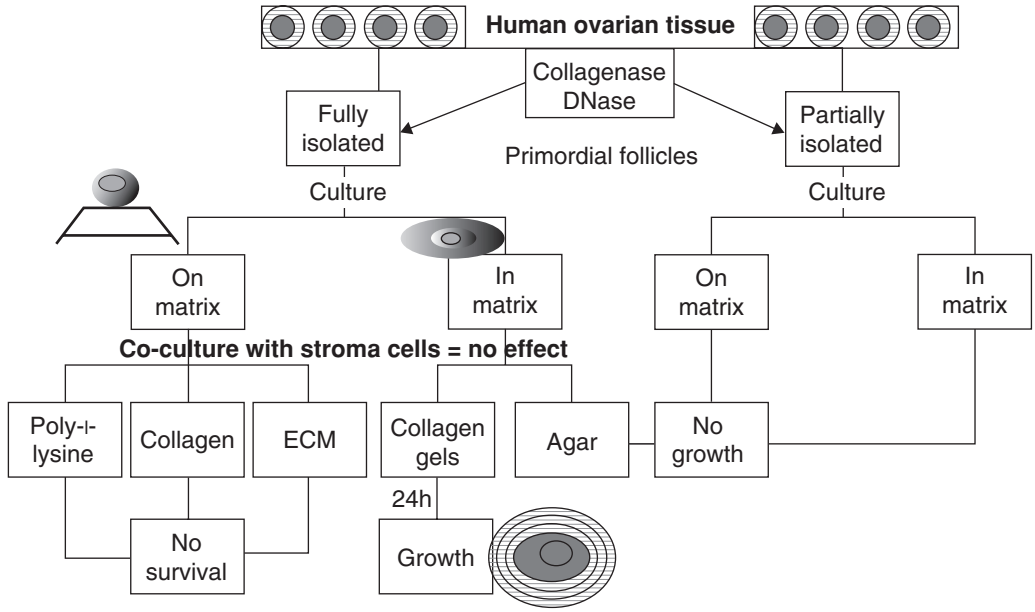
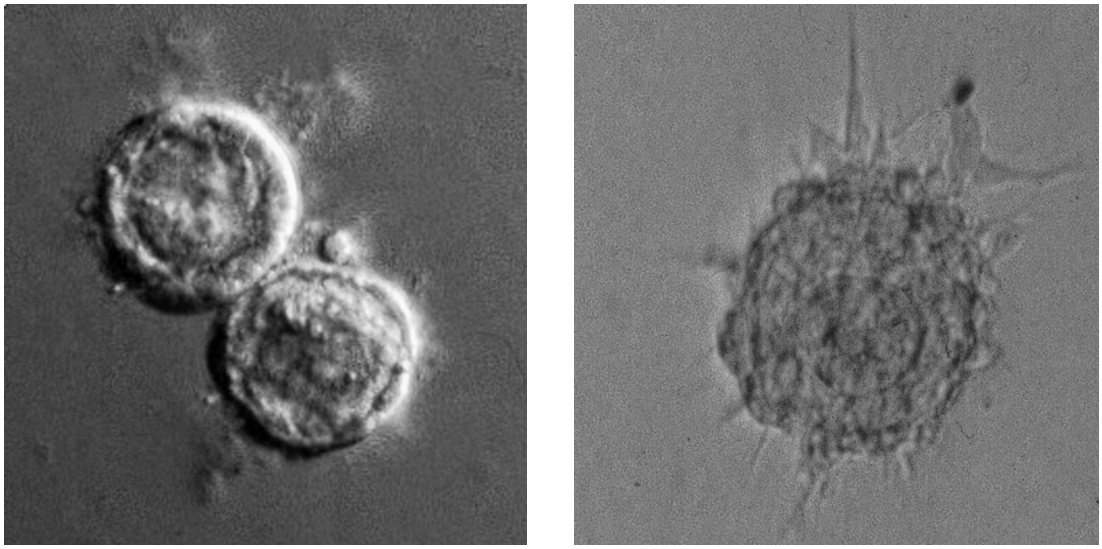


Figure 7.2 Strategies and results for culture of partially versus fully isolated human primordial follicles[32,33]. ECM: extracellular matrix



(a) **(b)**

Figure 7.3 Micrograph of growing isolated human follicles[32,33]. (a) Two partially isolated human primordial follicles. Note the central oocytes surrounded by a single GC layer. Magnification × 400. (b) A secondary follicle developed in culture from a primordial follicle during 24 h in collagen gel. Note the central oocyte surrounded by several GC layers. Magnification × 400

in vitro to two-pronuclear-stage embryos. Despite these promising results[41–43], however, it is very unlikely that IVM of primordial follicles through animal hosts will ever be approved ethically for clinical purposes of production of human fertilizable oocytes.

FACTORS THAT MIGHT BE RESPONSIBLE FOR EARLY OOGENESIS OR FOLLICULOGENESIS

The stimulus that initiates the growth of primordial follicles into secondary, multilaminar follicles remains unknown[2,3]. Locally produced oocyte- and GC-derived paracrine factors have been found to mediate cell–cell interactions that are related to the initiation and progression of follicular development. Studies have localized to very small follicles certain cytokines, oncogenes, neurotropins, growth factors, or their receptors, and have identified growth factors whose deficiency or that of their receptors in transgenic mice resulted in impaired PGC development or early folliculogenesis[2,3,44–48]. Therefore, these factors might possibly be candidates for initiation of early folliculogenesis. In the current chapter we will emphasize only on a portion of these factors: those that have actually affected mammalian PGC or follicular development in vitro or in vivo.

Growth factors that seem to promote growth of oogenia or primordial follicles

Table 7.1 summarizes the effects of various growth factors in activating primordial follicles in different mammalian species. *Insulin* or the *insulin like growth factor (IGF)* family probably activates mammalian primordial follicles in a species-specific manner[28,40,49]. The effects of

Table 7.1 Effects of growth factors on mammalian primordial follicles

GF	*Species*	*Growth*	*FSH-R*	*Other*	*Effect Ab or Ab-R*	*References*
BMP-4	Rat	To 1st follicles			Ab: *decrease* in oocytes, follicles, ovarian weight. *Increase*: apoptosis	51
BMP-7	Mouse	To 1st and 2nd follicles	Increase			54
bFGF	Rat	To 1st and 2nd follicles		*Increase*: stroma proliferation	Ab: *decrease* in growth	68
GDF-9	Rat	To 1st follicles		*Increase*: ovarian weight		52
	Human	To 1st and 2nd follicles		*Decrease*: atresia		53
Insulin	Human	To 1st follicles		*Decrease*: atresia		28
IGF-1	Human	To 1st follicles		(1) *Decrease*: atresia (2) PCNA expression in GC		28
IGF-2	Human	To 1st follicles		*Decrease*: atresia		28
LIF	Rat	To 1st and 2nd follicles		LIF + insulin >> LIF	Ab: *decrease* in growth	67
NGF	Rat	To 1st and 2nd follicles	Increase			56–58
SCF	Rat	To 1st and 2nd follicles			Ab-R: *decrease* in growth	63

GF: growth factor; FSH-R: follicle stimulating hormone receptor; 1st: primary; 2nd: secondary; Ab: antibody against the specific growth factor; Ab-R: antibody against the receptor for the specific growth factor; BMP-4: bone morphogenetic protein 4; BMP-7: bone morphogenetic protein 7; bFGF: basic fibroblast growth factor; GDF-9: growth differentiation factor 9; IGF-1: insulin like growth factor 1; IGF-2: insulin like growth factor 2; LIF: leukemia inhibiting factor; NGF: nerve growth factor; SCF: stem cell factor; PCNA: proliferating cell nuclear antigen; GC: granulosa cells

insulin on murine ovaries are unclear: one group described insulin inhibition of primordial follicles[40], whereas another showed that insulin, but not IGF-1, promoted the transition from the primordial to primary stages[49]. In humans, insulin, IGF-1, or IGF-2 reduced follicular atresia, and IGF-1 and IGF-2 induced follicular growth to primary stages[28]. However, PCNA expression in the GC was identified only with the addition of IGF-1.

Various members of the *transforming growth factor beta* (TGF-β) superfamily, such as *growth differentiation factor 9* (GDF–9), *bone-morphogenetic protein* (BMP)-4, 7, and 15, and *neurotropins* have been implicated in early folliculogenesis[2,3,47,50–54]. Treatment of neonatal rats with *GDF-9* increased ovarian weight and decreased the proportion of primordial follicles, with a concomitant increase in the proportions of primary and secondary follicles[52]. Treatment

of cultured human primordial follicles with GDF-9 promoted their survival and progression to secondary stages with an increase in PCNA expression in the GC[53].

When rat primordial follicles were cultured with *BMP-4*, a significantly higher proportion of developing primary follicles and fewer arrested primordial follicles were observed[51]. By contrast, treatment with an anti-BMP-4 antibody resulted in a progressive loss of oocytes and primordial follicles, increased cellular apoptosis, and decreased ovarian size. Treatment of cultured murine primordial follicles with *BMP-7* stimulated the primordial–primary transition and the synthesis of the FSH receptor (FSH-R) mRNA[54]. Similarly, in-vivo injection of BMP-7 into the ovarian bursa of rats increased the number of primary, secondary, and antral follicles in parallel with a decrease in the number of primordial ovarian follicles[50]. Injecting *BMP-8b* into mice deficient in the BMP-8b gene rescued defective PGC[55].

Neurotropins are involved not only in ovarian innervation but also in follicular assembly and initiation of folliculogenesis[3,6,48]. Treatment of cultured rat primordial follicles with *nerve growth factor (NGF)* initiated the growth of primordial ovarian follicles, increased FSH-R mRNA expression, and increased the ovarian capacity to respond to FSH with cAMP formation and growth to secondary stages[56–58]. When fetal murine ovaries (containing oogonia), neonatal murine ovaries (containing primordial follicles), and human fetal ovaries (from fetuses aged 13–16 GWs) were cultured with a potent inhibitor of the neurotropins' receptors, the survival of oogonia and oocytes decreased[6,59].

Stem cell factor (*SCF, kit ligand*) regulates the survival and differentiation of migratory PGC and also of postmigratory PGC that are already settled in the murine gonad, primarily through its anti-apoptotic function[60–62]. The addition of SCF to the culture medium of isolated fetal murine oogonia co-cultured with GC resulted in meiotic resumption and folliculogenesis[18]. The addition of SCF to culture medium of postnatal rat ovaries containing primordial follicles induced follicular growth. By contrast, the addition of an anti-SCF-R antibody reduced growth and inhibited the activation of primordial follicles and the development to secondary follicles[62,63]. In pigs, SCF was found to be essential for the survival and proliferation of PGC[64].

Leukemia inhibiting factor (*LIF*) and *oncostatin M* (*OSM*) are members of the *interleukin 6* (*IL-6*) family of cytokines[44,46], and are involved in the survival and proliferation of murine PGC. LIF has been found to regulate the growth, differentiation, and survival of the premigratory and postmigratory PGC in the developing murine[61,65] and porcine gonad[64,66], primarily through its anti-apoptotic function[60]. Antibodies against LIF receptor abolished PGC survival in culture[65]. Furthermore, LIF promoted the growth of rat primordial follicles in culture, whereas the addition of an anti-LIF antibody slightly decreased the number of developing follicles[67].

Basic fibroblast growth factor (bFGF, FGF-2) is a member of the *FGF* family[48]. It has been shown to promote the development of rat primordial follicles in vitro[68]. Specifically, the addition of bFGF to the culture medium induced a significant decrease in the number of primordial follicles with a corresponding increase in the number of primary and secondary follicles.

FSH and primordial follicles

The effect of *FSH* on mammalian primordial follicles is controversial and is probably species-dependent. In the mouse, FSH inhibited the activation of primordial follicles[40] and, in the cow, it had no effect on cultured primordial follicles[21]. Although FSH-R can be identified in human follicles only from the primary stages[69], in-vitro studies of isolated human primordial follicles[33] and primordial follicles in organ culture[27] suggested that FSH acts as a survival factor[27,33].

Nucleotide derivatives (secondary messengers) and primordial follicles

Nucleotide derivatives, such as *8-bromo guanosine 3′,5′-cyclic monophosphate (8Br-cGMP)* and *cyclic adenosine monophosphate (cAMP)*, are important intracellular secondary messengers that might also be involved in the activation of primordial follicles[30,31,36]. GC from isolated porcine primary follicles proliferated in culture with the addition of 8Br-cAMP[36]. Slices of human ovaries cultured with 8Br-cGMP[30] or cAMP[31] showed increased growth to secondary stages; the proportion of viable follicles increased significantly in parallel with a decrease in atresia rates. 8Br-GMP also induced an increase in estradiol production in spent media samples, presumably because of the concurrent increase in the proportion of secondary follicles. However, these secondary follicles were smaller than those developed in vivo.

Possible involvement of multiple growth factors in early oogenesis or folliculogenesis

It is very unlikely that only a single growth factor is responsible for the development of PGC or primordial follicles in mammals.[3] Rather, various growth factors probably have similar effects on them, or stimulation from a combination of growth factors is required for their growth.

Indeed, studies have reported that growth promotion of murine migratory PGC was induced only by the combination of OSM, LIF, SCF, and bFGF[66,70]. Furthermore, OSM induction of the growth and survival of murine postmigratory PGCs was enhanced with the addition of LIF, SCF, and bFGF. Similarly, a combination of LIF, SCF, and IGF-1 promoted the survival in culture of murine oogonia[71,72] and led to a significant increase in the number of meiotic pachytene cells[72]. The addition of an antibody against glycoprotein 130, a receptor unit of various growth factors including LIF and OSM, blocked survival of the postmigratory colonizing murine PGC[70]. Likewise, in pigs, either LIF or SCF[64], or their combination[66], was essential for the survival and proliferation of PGC.

SCF or bFGF or insulin, or a combination of insulin with bFGF, or insulin with SCF and bFGF affected murine primordial follicles in a similar manner: a 25 to 30% increase in the level of primordial-to-primary follicle transition[49]. By contrast, treatment with insulin and SCF resulted in a 40% increase in the number of primary follicles. In the rat, the addition of LIF with insulin to cultured primordial follicles led to a greater number of developing follicles compared with LIF alone[67].

In mice, an inhibitor of the neurotropins' receptor decreased the survival of oocytes in newly formed primordial follicles, but this effect was rescued by the addition of bFGF to the culture medium[59]. In humans, there was a greater increase in the transition of primordial follicles to primary stages when insulin was added together with IGF-2 than when insulin and IGF-2 were each added alone[28].

Inhibitory factors on primordial follicles

Anti-Müllerian hormone (*AMH*), another member of the TGF-β superfamily, seems to inhibit the recruitment of primordial follicles for the growing phase[73]. Murine primordial follicles cultured with AMH showed a 40–50% reduction in the rate of growing follicles.

Some authors have suggested that *the oocyte* has an inhibitory action on the follicle, which can be overcome by the stimulation of factors arising from vesicular pericytes, such as Thy-differentiation protein[1,2].

Inhibitory effects on primordial follicle activation might also originate from *stroma cells*. Isolated human primordial follicles developed into secondary follicles already after 24 h of culture, only when they were fully isolated from the stroma layer[32,33]. Apparently, complete removal

of the stroma layer leads to a rapid release of inhibitory factors from the cells surrounding the follicles.

DISCUSSION AND CONCLUSIONS

This review shows that the development of systems for the maturation of primordial as well as primary follicles is still in its infancy, and success so far is low.[3] Therefore, researchers need to continue their studies step-by-step using various follicular stages and culture systems (Figure 7.4). In humans and other species, follicular development from primordial stages onwards is complex and lengthy[1–3]. It is possible that the optimal culture medium for growth promotion needs to be very rich in supplements and species-specific. Growth factors should be added to the culture media of human primordial follicles to assess their in-vitro effect on folliculogenesis, alone and in combination. Various sequential media

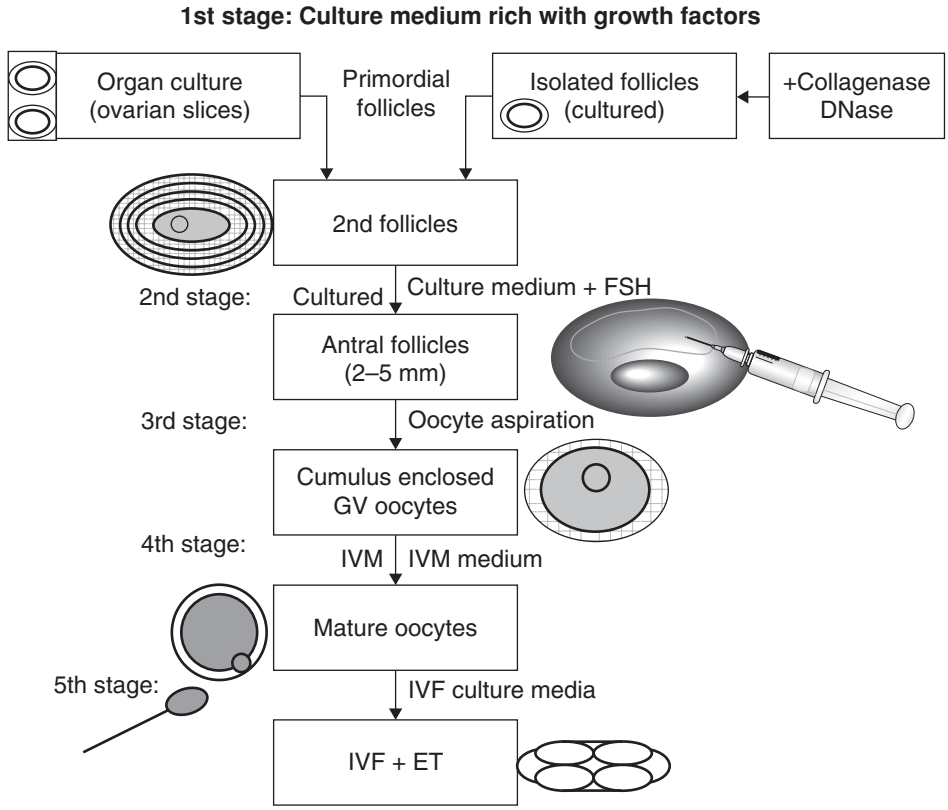


Figure 7.4 A possible strategy for the development of cultured human primordial follicles for an ultimate production of mature fertilizable oocytes. Note the five-stage strategy with sequential media: first stage – culture of primordial follicles (isolated or in organ culture) to secondary follicles with a medium rich in growth factors; second stage – culture of secondary follicles to small antral follicles with a medium containing FSH[1,2]; third stage – aspiration of cumulus enclosed GV-stage oocytes; fourth stage – culture of GV-stage oocytes to mature oocytes (with first polar body extrusion) using IVM medium[74]; fifth and final stage – standard IVF and ET

may also be necessary for every follicular stage, until a mature, healthy, and fertilizable human oocyte is obtained (Figure 7.4). It is very unlikely that human follicles could be brought in culture to an ovulatory size of 18–20 mm[1]; we expect that cumulus-enclosed GV-stage oocytes will be aspirated from small antral follicles obtained in culture and matured in vitro (Figure 7.4)[74].

The use of fetal follicles as a putative source of oocyte donations for IVF raises several ethical dilemmas[3,10]. So far, this possibility is remote, owing to the scarcity of in-vitro studies of fetal follicles and oogonia. In addition, most human abortions occur at early stages, and the respective oogonia from these ovaries develop very slightly in culture[22]. Studies conducted in our laboratory identified fewer receptors for various growth factors in GC of fetal primordial follicles that in those from adults[3,44–48], suggesting that it will be harder to develop a successful IVM system for fetal follicles than for primordial follicles from women. At the same time, a more in-depth understanding of the mechanisms involved in fetal folliculogenesis would assist researchers in improving the technology of IVM of small follicles from adults.[3] Therefore, studies on both mammalian fetal and adult follicles should be continued.

Currently, women who are about to receive anti-cancer treatment face limited options for fertility preservation[3,7]. Once the IVM technology of human primordial follicles is established, it will be possible to use oocytes that were matured and fertilized in vitro and thereby to avoid the dangers of malignancy re-transmission. The availability of such treatment will probably lead to its demand not only by cancer patients, but also by healthy women who choose to postpone childbearing until later in life. Moreover, taking into account the multiple malformations found in the first live mouse developed from a primordial follicle[2,3,40], after a successful acquisition of an IVM technology for primordial follicles, extensive research on their subsequent embryonic development will be necessary.

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