The earliest stages of folliculogenesis in vitro

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In recent years several follicle culture systems have been pioneered in different mammalian species for studying ovarian folliculogenesis and culturing immature oocytes. Applications of these in vitro techniques include fertility preservation for humans, conservation of rare animals and development of oocyte banks for research purposes. Immature female gametes in the ovarian cortex can be cryopreserved for later use if culture techniques are available afterwards to promote growth and maturation. This review focuses on biochemical and biophysical factors related to oocyte culture in mice, the only animal in which live offspring have been produced after folliculogenesis in vitro. The advantage of using mice for these studies is that, in parallel to development of follicle culture systems, essential knowledge on folliculogenesis can be obtained from knockout mouse models. Recent experiments in mice stressed the principal role of the oocyte in follicle development and the strict timing of the biological processes underlying oogenesis in vitro. In large domestic animals and humans, study of oocyte culture is confounded by the constitutively prolonged nature of ovarian follicle development. In humans, only some aspects of follicle development have been studied because of the limited availability of suitable material for experimentation, technical difficulties related to manipulation of very small structures and lack of knowledge on physiological regulation of the early stages of follicle growth. Only a few reports describe ovarian follicular growth in vitro. In this review, relevant information on hormonal and growth factor regulation of the earliest stages of follicle growth in mammals is reviewed. Techniques are becoming available for the precise isolation of distinct classes of follicle and powerful molecular biology techniques can be used in studies of ovarian tissue culture.

The availability of oocytes is the limiting factor in the development of new reproductive techniques. The duration of the fertile period in a female’s life is determined by the size of the primordial follicle pool formed during fetal life and by the rate of depletion of the pool after birth. Mechanisms of regulation of follicle formation, oocyte attrition and follicle development and atresia are only partially understood and more information on these processes could be obtained by culturing follicles in vitro at the earliest stages of formation and growth. In small mammals, the ovarian physiology of mice and knockout mice models has been studied extensively and culture of well-defined stages of preantral and antral follicles has provided some insight into the complex interplay between somatic and germ cells during growth and maturation. It is possible to grow female gametes fully in vitro: in rodents, live offspring have been obtained after extended follicle culture periods of a few weeks. However, even in mice, much effort is still required to refine the culture systems.

For domestic animals and humans, the technology is still far from successful; there are only a few reports of limited success using in vitro culture of large preantral follicles that have progressed further developmentally. These larger follicles contain oocytes that are almost fully grown; however, the meiotic competence of these follicles remains low, indicating that the culture methods require optimization. As the store of female gametes is maintained within the primordial follicles, the real challenge is to establish the cultures of primordial follicles. Unravelling the regulation of the transition from primordial to primary follicle is the key to accessing an almost unlimited source of oocytes for biomedical use. This review summarizes current knowledge on mammalian folliculogenesis in vivo and discusses achievements of in vitro systems for culture of preantral follicle stages, focusing on mice and humans.
Formation of the primordial follicle

Primordial germ cells arise at about 3 weeks after fertilization and migrate by amoeboid movements from the epithelium of the yolk sac via the connective tissue of the hindgut to the genital ridge. During their journey, the primordial germ cells multiply rapidly. In humans, the genital ridge is formed at about 3.5–4.5 weeks of gestation by a knot of mesenchyme overlaid by coelomic epithelium. At about week 7 of gestation the cells derived from the mesonephros and the coelomic epithelium migrate inward from the genital ridge, forming primitive medullary cords and sex cords, respectively. The cords, which are anatomically less well defined in females than in males, are compacted in the cortical region of the gonadal ‘anlage’. The primitive cords are colonized by the primordial germ cells. In the female fetus, the primordial germ cells are named oogonia upon arrival in the primitive gonad. The oogonia are still interconnected by cytoplasmic bridges. The primitive medullary cords degenerate to be replaced by highly vascularized ovarian stroma. The cord cells proliferate and mesenchymal cells condense around the oogonia to form the individual primordial follicles. Follicle formation in humans begins in the inner part of the ovary, near the rete ovarii, between week 16 and week 18 of fetal life. In the primordial follicles, the mesenchymal cells secrete an outer basement membrane and the same cells will give rise to granulosa cells in the growing follicle. Meanwhile, the mitotic activity of the oogonia ceases and the oogonia enter meiosis (Byskov, 1986). In mammals, this process of envelopment of the primordial germ cells by highly vascularized ovarian stroma enables more clinics to offer fertility preservation to women undergoing treatments, such as chemotherapy, radiotherapy or ovariectomy, that have devastating effects on the follicle (Donnez and Bassil, 1998; Yeung et al., 1998; Smitz and Cortvrindt, 1999). After initial cryopreservation experiments with mice (Carroll et al., 1991; Cortvrindt et al., 1996a; Newton et al., 1996; Oktay et al., 1997; Cook et al., 1999), the cryopreservation technique requires sophisticated equipment such as a programmable freezing machine. Therefore, investigation of new cryopreservation media that permit rapid freezing by simply plunging the tissue into liquid nitrogen would be worthwhile (Zhang et al., 1995). This development would enable more clinics to offer fertility preservation to women of frozen oocytes, but that there is a need for refinement of the cryopreservation conditions before moving to clinical applications. Most primordial follicles die by ischaemia after clamping of ovarian arteries and by the slow revascularization. However, transplantation of ovarian tissue will not be possible for all malignant indications as a result of the risk of re-transferring the cancer cells. For these indications, in vitro systems for folliculogenesis should be developed (Shaw et al., 1996; Meirow et al., 1999).

Cryostorage of ovarian cortical tissue

After the pioneering work by Roger Gosden in the early 1990s (Carroll, 1990), researchers started to investigate storage of immature female gametes (Newton, 1998; Gook et al., 1999). At any stage of premenopausal life, there are only a few follicles at advanced stages of development that could be used for storage. In an ovary of a woman aged 50, there are only approximately 100 follicles in the growing pool and approximately 30 of these are at the antral stage (Gougeon, 1984). Therefore, it is more advantageous to cryopreserve the large number of gametes present at the earliest stages of follicle development: the resting primordial stages (Gougeon et al., 1994; Lass et al., 1997).

By using slow freezing protocols it is possible to recover 70–80% of the initial number of primordial follicles that are frozen (Hovatta et al., 1996; Newton et al., 1996; Oktay et al., 1997; Cook et al., 1999). The cryopreservation technique requires sophisticated equipment such as a programmable freezing machine. Therefore, investigation of new cryopreservation media that permit rapid freezing by simply plunging the tissue into liquid nitrogen would be worthwhile (Zhang et al., 1995). This development would enable more clinics to offer fertility preservation to women undergoing treatments, such as chemotherapy, radiotherapy or ovariectomy, that have devastating effects on the follicle (Donnez and Bassil, 1998; Yeung et al., 1998; Smitz and Cortvrindt, 1999). After initial cryopreservation experiments with mice (Carroll et al., 1991; Cortvrindt et al., 1996a; Newton et al., 1996) and sheep (Gosden et al., 1994; Aubard et al., 1999) the survival and potential for further development of frozen ovarian primordial follicles was evaluated by transplanting ovarian tissue pieces in immunocompromised mice (Newton et al., 1996; Shaw et al., 1997; Oktay et al., 2000). Homologous transplantation was attempted in humans by Nugent et al. (1998) and Oktay et al. (2000). The transplantation experiments on human tissues indicate that cryopreservation does not compromise further development of frozen oocytes, but that there is a need for refinement of the grafting conditions before moving to clinical applications. Most primordial follicles die by ischaemia after clamping of ovarian arteries and by the slow revascularization. However, transplantation of ovarian tissue will not be possible for all malignant indications as a result of the risk of re-transferring the cancer cells. For these indications, in vitro systems for folliculogenesis should be developed (Shaw et al., 1996; Meirow et al., 1999).

Requirements for experimental material for follicle culture

Primordial follicles that will survive the sampling and cryopreservation conditions are suitable for use in the ovarian tissue culture laboratory. Ideally, a sufficiently high
Fig. 1. Overview of follicle formation and growth in the human fetal ovary. (a) Section through a fetal ovary at week 18 of gestation. Follicles are not yet organized. The large clear cells with the largest nuclei, observed mainly in the central portion of the section, are oogonia. (b) Part (a) at larger magnification. Oogonia are dispersed within mesenchymal cells. Cells from the coelomic epithelium form cords. At this stage oogonia are still interconnected by cytoplasmic bridges and appear as strings (arrow). The fetal ovary is delineated by coelomic epithelium. (c) Ovary from a fetus at week 24 of gestation. Oogonia can be observed which are entirely surrounded by pregranulosa cells and form the primordial follicle. The tissue already shows lytic changes. Follicles are embedded within mesenchymal tissue, which is highly vascularized. (d) Ovary from a fetus at week 25 of gestation. Follicles at the primordial stage with flattened pregranulosa cells are present. There is a follicle containing a growing oocyte (arrow) showing the cuboidal transformation of the pregranulosa cells. (e) Fetal ovary (week 30 of gestation) showing lytic changes. Primordial follicles are observed. In the right upper quadrant there is a follicle with three to four layers (arrow) surrounded by a theca interna containing a collapsed oocyte. (f) Fetal ovary (week 34 of gestation). The left half of the figure contains large (atretic) multilayered follicles, the right half contains very dense tissue populated with primordial follicles and oogonia. Scale bars represent (a) 20 μm, (b) 10 μm, (c,d) 25 μm, (e) 30 μm, (f) 150 μm.
of the ovarian vascular tree and use of the tissue should be kept as short as possible. The tissue should be kept in a physiological solution with antibiotics on melting ice to reduce autolytic processes. It is impossible to estimate the number of follicles within the cortical tissue by observation under a dissection microscope, as there are a number of morphological structures that can be mistaken for follicle structures (curled vessels). Cortvrindt and Smitz (2001) reported a method permitting a rapid evaluation of a biopsy in terms of the number of follicles, ovarian tissue viability and even morphology of the follicles (Fig. 2).

### Classification of small ovarian follicles and morphological aspects

Several classifications for mammalian ovarian follicles (human, sheep, mouse) have been published (Pedersen and Peters, 1968; Lintern-Moore et al., 1974; Gougeon, 1996; Lundy et al., 1999). The classification proposed by Gougeon (1996) for human follicles is summarized (Table 1). The primordial follicle is defined as a primary oocyte surrounded by flattened granulosa cells. When a few of the flattened cells become cuboidal the follicle is classified as ‘transitionary’ or ‘intermediary’; these follicles are still considered to belong to the resting pool.

A primary follicle is characterized by a full cuboidal granulosa cell layer surrounded by a basement membrane. Primary follicles are the first stage belonging the growing pool. Preantral follicles can be identified as a growing primary oocyte surrounded by several (up to six or seven) granulosa cell layers. Theca cells are recruited from the interstitial stromal cells and can be recognized as individual cells on the basement membrane in part of the primary follicles (Hirshfield, 1991; Lundy et al., 1999; Parrott and Skinner, 2000). As soon as the follicle reaches the secondary stage (two layers), a distinct theca cell layer is formed in all follicles (Gougeon, 1996; O’Shaugnessy, 1997; Lundy et al., 1999). Preantral follicle growth can be divided into two phases: a vascular and an avascular phase. After seven to eight doublings of the number of granulosa cells, mammalian follicles reach a diameter of 200 μm. Fluid-filled patchy spaces appear within the granulosa cells and the follicles are termed ‘early antral’. Follicles are termed ‘antral’ when the fluid-filled spaces have become coalescent into a large crescent cavity; granulosa cells then differentiate into mural and cumulus cells. Other morphological milestones can be used to describe the developmental stages. Formation of the zona pellucida during transformation of the primordial follicle into a primary follicle is another distinct reference point. The zona pellucida and theca interna layer are formed when the follicles are at the primary stage (Braw-Tal and Yossefi, 1997; O’Shaugnessy et al., 1997; Lundy et al., 1999; Yeh and Adashi, 1999). In a further stage of preantral follicle development, a theca externa is formed as a highly vascularized layer, providing the follicle with systemic endocrine factors that permit its exponential volumetric expansion (Fig. 3).
Do all oocytes from primordial follicles have the capacity to be ovulated?

It is difficult to classify a follicle as being either at rest or at an early phase of growth using strictly morphological criteria. The transformation of flattened pregranulosa cells into cuboidal cells, initiating the growth of the oocytes, is an extremely slow process. Gougeon and Chainy (1987) referred to the transformation as the ‘maturation’ phase instead of the ‘growing’ phase. These authors consider that the follicular growth phase is initiated only when the oocyte of a primary follicle starts to grow rapidly. More recent data presented by Gougeon and Busso (2000) using proliferating cell nuclear antigen (PCNA) immunostaining or bromodeoxyuridine (BrdU) incorporation indicates that up to the primary stage most of these follicles are in the resting stage. It is still a matter of debate whether primordial follicles can be removed directly from the resting pool by atresia. Reynaud and Driancourt (2000) and Yuan and Giudice (1997) could not demonstrate apoptosis in resting follicles in sheep ovaries and in human ovarian specimens, respectively. Lee et al. (1999) studied expression of apoptosis-related genes in 35 human ovaries and described the sequence of expression of genes known to induce cell death by apoptosis for the earliest stages of follicular growth. Lee et al. (1999) could not demonstrate expression of Bcl-2 or Bax in primordial follicles. FAS and FAS ligand were present only in oocytes from primordial follicles, but not in their surrounding granulosa cells. In preantral follicles, neither Bcl-2 nor Bax could be detected in oocyte or granulosa cells, but both types of cell showed weak staining for FAS and FAS ligand (compared with intense FAS staining in primordial oocytes). Atretic follicles showed intense staining for Bax in granulosa and theca cells and moderate staining for FAS in oocyte, granulosa cells, theca cells and stroma. Weak staining for FAS ligand was observed in granulosa and theca cells, but not in stromal cells. There is a consensus that in early preantral follicles in vivo the process of atresia is initiated in the oocytes, whereas in the larger antral follicle population apoptosis emerges in the somatic compartment (Tilly et al., 1991; Yuan and Giudice, 1997).

Recent observations from de Bruin et al. (2001) indicated that a necrotic process is the basis of atresia in resting follicles.

<table>
<thead>
<tr>
<th>Follicle</th>
<th>Follicle diameter (µm)</th>
<th>Oocyte diameter (µm)</th>
<th>Oocyte nuclear diameter (µm)</th>
<th>Mean number of granulosa cells (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primordial</td>
<td>35</td>
<td>32</td>
<td>16</td>
<td>13 (7–23)</td>
</tr>
<tr>
<td>Intermediary</td>
<td>38</td>
<td>32</td>
<td>16</td>
<td>28 (9–50)</td>
</tr>
<tr>
<td>Small primary</td>
<td>46</td>
<td>33</td>
<td>17</td>
<td>76 (23–223)</td>
</tr>
</tbody>
</table>

Adapted from Gougeon, 1996.

**Fig. 3.** The earliest follicle growth stages in the adult human ovary. (a) Initiation of follicle growth in the proximity of a capillary vessel at the corticomedullary border. The transition from the resting to the growing stage of the follicle is shown. In close proximity to the blood vessel there is a primordial follicle (left) and a large primary follicle (right). The upper left quadrant contains a follicle with granulosa cells transforming from flattened to cuboidal morphology. (b) Resting primordial follicle embedded within stromal cells. An oocyte at the germinal vesicle stage in surrounded by flattened pregranulosa cells encapsulated by a thin basal membrane. (c) Growing primordial follicle: the volume of the oocyte has increased as well as the surrounding granulosa cells, which have changed shape to cuboidal. Note the thickening of the basal membrane. (d) Primary follicle: growing oocyte surrounded by a dense layer of cuboidal granulosa cells encapsulated in a basal membrane. Note the proximity of a vessel (arrow). (e) Late preantral follicle with six to eight layers of granulosa cells and a well-organized theca cell layer. Scale bars represent (a) 30 µm, (b,c,d) 12 µm, (e) 20 µm.
If all primordial follicles have an equal potential for development when exposed to optimum culture conditions, initiating an in vitro culture with primordial follicles could provide a very large source of developmentally competent oocytes. Cryopreservation of oocytes (primordial follicles) could circumvent the accelerated disappearance of the finite store of female gametes that occurs in vivo when females are exposed to environmental toxicants, tobacco, alcohol, disease, irradiation or adverse medication.

The question remains whether the intrinsic quality of oocytes in resting follicles decreases with age. Although morphological changes in mitochondria, smooth endoplasmic reticulum and Golgi apparatus from oocytes in resting follicles are correlated with age, it is as yet uncertain whether this finding has any prognostic value for the future lower quality of oocytes (de Bruin et al., 2001).

Functional characteristics of preantral follicle growth

The earliest stages of ovarian folliculogenesis are morphologically similar in different mammalian species. However, each species has its own specific timescale for development. In rodents (mice, rats) the time span between initiation of follicle growth and formation of the antral cavity is a few weeks; in large domestic animals it takes several months. During the preantral growth phase, the oocyte grows rapidly and reaches almost its maximum volume when the first accumulation of fluid is observed within the granulosa. In humans, Gougeon (1986) estimated that the maturation phase from primordial to primary follicle takes >120 days. Once in the growing pool, the follicle requires 65 days to reach the early antral phase (follicle of 2–5 mm diameter), at which point it becomes dependent on gonadotrophins for further growth.

Regulation of early preantral follicle growth stages

The fine regulation of the selection and initiation of growth of the primordial follicles out of the dormant pool remains enigmatic. Primordial follicles are located in a 2 mm thin, poorly vascularized layer under the tunica albuginea. The follicles showing initiation of growth are found in the corticomedullary border, which is highly vascularized (van Wezel and Rodgers, 1996) (Fig. 3). It is known that the earliest growth stage, including the transition from primordial to primary follicle, is gonadotropin-independent and is regulated mainly by intraovarian factors. The first trigger for initiation of growth has not been characterized and might arise in the oocyte or in the surrounding cells. These cells are of widespread origin: some are derived from hematopoietic stem cells, some belong to vascular structures and some to the autonomic nervous system. Oncogenes c-myc and erb-A localized in oocytes of the primordial follicle and pregranulosa cells might play a role in autonomous growth (Li et al., 1994; Sato et al., 1994). C-Kit, which is present in primordial oocytes, plays an important role as inhibition of interaction of C-Kit with Kit ligand, originating in granulosa cells, blocks the initiation of growth (Yoshida et al., 1997). Studies in vitro in mice showed that Kit ligand induces growth in oocytes isolated from prepubertal animals (Packer et al., 1994). A recent review by Driancourt et al. (2000) emphasized the relevance of Kit–Kit ligand interaction as a key regulator of the initiation of growth from the primordial pool and transitions beyond the primary stage.

There is evidence for mechanisms that hold the pool of primordial follicles under growth arrest. The tissues surrounding the primordial follicles undoubtedly play a crucial role in co-ordinated initiation of growth, as removal of the stromal compartment in vitro is accompanied by marked initiation of granulosa cell proliferation (Wandji et al., 1997; Fortune et al., 2000).

Members of the transforming growth factor β (TGF-β) superfamily of secreted growth factors play important roles at several stages of ovarian folliculogenesis. Among factors of the TGF-β family, activin A is secreted by secondary follicles. In an in vitro model using rat follicles, Mizunuma et al. (1999) demonstrated that activin A can prevent the growth of primary follicles despite their exposure to extraovarian growth factors. Recent data from the knockout mouse model for anti-Müllerian hormone (AMH) and from follicle culture indicates that AMH inhibits recruitment of primordial follicles (Durlinger et al., 1999). In the human ovary, AMH protein was not detected in primordial follicles, but was found in the granulosa cells of primary follicles and was abundant in secondary and early antral follicles (De Vet et al., 2000).

Some members of the TGF-β superfamily are expressed by the oocyte: growth differentiation factor 9 (GDF-9) and bone morphogenetic protein 15 (BMP-15), which are similar to GDF-9B and BMP-6 (for a review see Elvin et al., 2000). Although GDF-9 expression has been localized in other extraovarian tissues, BMP-15 mRNA has been found exclusively in the ovary. There is species-dependent variability in the expression of these factors. In mice, there is no GDF-9 expression in oocytes before the type 3a preantral follicle stage, but in pigs and cows GDF-9 has also been found in primordial follicles. The knockout mouse for GDF-9 showed arrest of granulosa proliferation and, despite exaggerated oocyte growth, absence of theca cell formation (Dong et al., 1996). The other TGF-β family members expressed by the oocyte were unable to substitute for GDF-9. These oocytes cannot become cytoplasmically mature.

Factors of vascular origin (pericytes), such as Thy-1 differentiation protein, could signal to the oocyte to remove a hypothetical arresting factor originating in the oocyte and acting upon the pregranulosa cells (Bukovsky et al., 1995).

The hypothesis that oncogenes such as erbA (Maruo, 1995), myc (Li et al., 1994) and Wilms’ tumour gene (Hsu et al., 1995; Chun et al., 1999) may be involved in initiation of follicle growth is based on their localization in oocytes of primordial follicles and granulosa cells of the follicles at the earliest growth stages. Expression of Wilms’ tumour gene
(WT-1) decreases from the primordial pool of oocytes up to the antral stage follicles. Expression of WT-1 suppresses growth and differentiation genes (growth factors and receptors).

There is increasing evidence that the following neurotrophins play a role in the initial stages of follicular development: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophins 3 and 4. The mRNAs encoding these factors and their tyrosine kinase receptors (trk) are transiently present in the ovarian follicle during follicle formation and early follicular growth (Ojeda et al., 2000). A combined deficiency of neurotrophins 3 and 4 and BDNF resulted in a significant reduction in the number of follicles at all preantral stages (Ojeda et al., 1999). Among neurotrophins, vasoactive intestinal polypeptide (VIP) is also a potential growth initiator: VIP is expressed on nerves in the proximity of follicles at the earliest growth stages in cows (Hulshof et al., 1994) and monkeys (Schultea et al., 1992).

In the bovine model, primordial oocytes express basic fibroblast growth factor (bFGF) (Van Wezel et al., 1995), which can interact with granulosa cells from early preantral follicles (Gospodarowicz and Bialecki, 1979). Furthermore, FGF-2 mRNA and FGF receptor have been localized in human fetal ovarian tissue (Yeh and Osathanondh, 1993).

In humans, as soon as formation of a secondary follicle begins, granulosa cells develop FSH receptors (Oktay et al., 1997) and theca cells become differentiated around the basement membrane. As soon as theca cells form, LH receptor is expressed (mice: Sokka et al., 1996; O’Shaughnessy et al., 1997). During formation of the secondary follicle there is a migration towards the medullary part of the ovary, in which early theca externa will be acquired, and vascularization of the follicle is initiated. Gonadotrophin receptors and steroid receptors also appear (Fig. 4). LH stimulates the theca cells to proliferate, differentiate and initiate androgen production. Although theca cells are an important target of LH action, the survival of theca cells is not dependent on LH, as demonstrated recently in LH receptor knockout mice (Zhang et al., 2001). It is mainly androstenedione, which is LH-dependent, which is derived from theca cells and acts in granulosa cells to cytodifferentiate the responses to FSH (Magoffin and Erickson, 1994). Androgen receptor immunoreactivity has been demonstrated in human and primate granulosa and theca cells (Hild Petito et al., 1991; Horie et al., 1992) and expression of the androgen receptor gene was most abundant in the growing preantral and antral follicle pool (primates: Weil et al., 1998).

From knockout mouse models and diseases in humans involving genes for gonadotrophin subunits and gonadotrophin receptors it was shown that the preantral follicular growth phase is largely gonadotrophin-independent.
Cattanach et al., 1977; Kendall et al., 1995; Dierich et al., 1998; Abel et al., 2000). In humans, follicles of 2–5 mm in diameter can be observed in the ovary in the absence of FSH (Kallman syndrome). However, the efficiency of growth during the preantral phase might be improved when baseline concentrations of gonadotrophins are made available.

The mouse model in follicle culture

Rodent species (mice, rats, hamsters) have been used extensively for investigation of follicle growth in vitro. The advantage of using mice is that they have a short life cycle allowing experiments to be performed within a reasonable time frame.

The production of live offspring after culture of early stage follicles for extended periods has only been documented in mice (Eppig and Schroeder, 1989; Eppig and O’Brien, 1996; Cortvrindt et al., 1998b). It is well established that folliculogenesis and meiotic maturation are strictly timed processes (Fig. 5). Consequently, at isolation, each follicle unit is at a particular developmental stage. Thus follicle selection at isolation should ideally involve a very homogeneous population of follicles to obtain a large yield of competent oocytes at a particular time point after culture. Selection of follicles is possible under the microscope (×400) by measuring follicle diameter and classifying the number of granulosa cell layers surrounding the oocyte. For each initial follicle diameter at the start of culture there is an intrinsic optimum culture period in which to reach the optimum maturation stage. Initiating a

Fig. 5. Experimental evidence on follicle culture periods from published studies. Optimum maturation results are obtained only when the duration of culture is adapted to the stage of follicle development at isolation. Follicle development in vitro follows a strictly timed progression.

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culture with follicles that have a homogeneous diameter will greatly improve oocyte quality at the end of the culture. During the first wave of follicular growth, it is easy to obtain a large homogeneous population of follicles. As follicle formation occurs after birth in mice, the age of the juvenile mice can be selected in relation to the maximum developmental stage of the follicles that are required for each specific experiment.

A group of 20–30 primary follicles per ovary can be isolated mechanically from 7-day-old mice. Approximately 40–50 early secondary follicles can be dissected with fine needles from ovaries of 14-day-old animals.

### Table 2. Characteristics of culture systems for mouse ovarian follicles

<table>
<thead>
<tr>
<th>Culture system (see Fig. 6)</th>
<th>Follicle components</th>
<th>System and follicle integrity</th>
<th>Medium</th>
<th>Protein</th>
<th>Hormones + growth factors</th>
<th>Ovulation stimulus</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>Organ culture</td>
<td>Groups –</td>
<td>Sessile</td>
<td>Waymouth</td>
<td>BSA</td>
<td>EGF</td>
</tr>
<tr>
<td>S2</td>
<td>Oocyte/GC</td>
<td>Groups Open (COC)</td>
<td>Sessile</td>
<td>Waymouth</td>
<td>BSA</td>
<td>–</td>
</tr>
<tr>
<td>S3</td>
<td>Oocyte/GC/TC</td>
<td>Individual Open</td>
<td>(1) Floating</td>
<td>α-MEM</td>
<td>FCS</td>
<td>FSH/LH</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(2) Sessile</td>
<td>α-MEM</td>
<td>FCS</td>
<td>–</td>
</tr>
<tr>
<td>S4</td>
<td>Oocyte/GC/TC</td>
<td>Individual Open</td>
<td>Sessile</td>
<td>α-MEM</td>
<td>FCS</td>
<td>FSH/LH</td>
</tr>
<tr>
<td>S5</td>
<td>Oocyte/GC/TC</td>
<td>Individual Closed</td>
<td>Floating</td>
<td>α-MEM</td>
<td>Mouse serum</td>
<td>hCG + EGF</td>
</tr>
</tbody>
</table>

BSA: bovine serum albumin; COC: cumulus–oocyte complexes; EGF: epidermal growth factor; FCS: fetal calf serum; GC: granulosa cells; hCG: human chorionic gonadotrophin; MEM: minimal essential medium; TC: theca cells.

### Fig. 6. Published culture systems for mouse follicles, designed in relation to the follicle diameter at the start of culture. The figure relates stage of follicle development to mean follicle diameter in mice.

**Culture systems for mouse ovarian follicles**

Different culture systems have been described for mouse ovarian follicles (Fig. 6) depending on the size classes of follicles at isolation.

Details of a variety of culture systems have been published (Table 2) but in terms of culture morphology they
can be divided as follows: (i) cultures in which the aim is to keep the spherical structure intact; and (ii) cultures in which the follicles are allowed to remodel into sessile mound-like structures.

The choice of culture system used should be dictated by the aims of the study. When the purpose is to use the somatic cell compartment as a ‘feeder layer’ for oocytes, a high yield can be obtained by allowing the follicles to remodel (Cortvrindt et al., 1996b) (Fig. 7). When the aim is to determine physiological intrafollicular relationships, it is necessary to maintain the cultured units as multilayered spheres (Nayudu and Osborn, 1992). In addition, when the culture is to be used to study ovulation, it is necessary to retain a normal follicular wall, that is, mural granulosa, basement membrane and theca cell layer (Rose et al., 1999).

Choosing an artificial culture system will always imply a compromise. When the aim is to mimic normal physiology and to culture the follicles as multilayered spheres, the supply of essential nutritional and physical factors to the inner centre of the structure becomes critical (Johnson et al., 1995). Nutrients, growth factors and oxygen have to diffuse through a non-vascularized follicular wall. Experience with this culture model shows that decreased oocyte and follicle survival rates ensue, probably because it is impossible to compensate in vitro for the absence of the highly vascularized network that is present within a normal thecal wall.

When the aim is to culture oocytes, the environment should be supportive of the growth of this large type of cell and should supply the essential nutrients. This support is feasible even with a non-spherical ‘open’ structure (Eppig and Schroeder, 1989). The accessibility of nutrients, hormones and gases is much better in an open structure and leads to an improved oocyte survival rate. Open culture systems can be refined by adapting the composition of the medium to preserve the normal differentiation status of the cells surrounding the oocyte. As emphasized by Eppig et al. (1997), the crosstalk between oocyte and cumulus cells is of major importance in determining oocyte developmental competence. Fine tuning of the culture components, including the types of cell (with or without thecal cells), gonadotrophin concentration and ratio, growth factors (insulin, IGF-I, epidermal growth factor (EGF)), protein source (albumin, synthetic serum supplements, homologous serum, fetal bovine serum) and concentration, can lead to optimization of the culture system (Table 3). Maintaining the physical parameters such as temperature and pH changes under strict control is of significant importance to guarantee good culture outcome.

When optimizing the culture conditions, it is important to recognize the balanced interrelationships between the biochemical and physical components of the system. It has been demonstrated that there is close interaction between the oxygen requirements of a particular system, the hormonal and growth factor composition and the protein source (Gosden and Byath-Smith, 1986; Eppig and Wigglesworth, 1995; Smitz et al., 1996).

Follicle survival and oocyte competence are obviously interdependent. Oxygen supply to the deepest layers of the follicle is dependent on the requirements of the somatic cells, which are driven into proliferation by the relative amounts of insulin, IGFs and FSH. Follicle survival in vitro is FSH-dependent but the FSH concentration in the culture medium determines the metabolic rate at which the granulosa cells function. The metabolic requirements for oxygen will differ in relation to the basal FSH and insulin tonus. In the mouse follicle culture system described by Cortvrindt et al. (1996b), oocytes do not survive under reduced oxygen (5%) when the FSH concentration in the medium is 100 miu ml\(^{-1}\) (Smitz et al., 1996). Oocyte survival under low oxygen tension is possible only by reduction of the FSH concentration to 10 miu ml\(^{-1}\) (the minimum effective dose) (R Cortvrindt and J Smitz, unpublished). Oxygen provision also influences the generation of free radicals, which can be neutralized by serum components and scavengers. The study of Eppig and Wigglesworth (1995) illustrates the relationship between oxygen tension and the potential to generate normal blastocysts. It should be emphasized that, in this system, oocyte–granulosa cell complexes are cultured without serum and gonadotrophins. These conditions are compatible with a lower metabolic rate and hence lower oxygen requirements for membrane and steroid biosynthesis. Applying a supraphysiological oxygen tension to the culture system of Eppig and Wigglesworth (1995) might lead to production of free oxygen radicals, which interfere with key developmental processes and lead to arrest in development.

Fig. 7. In vitro growth of early preantral follicles, in vitro ovulation, preimplantation embryo culture and production of live offspring in mice. (a) A secondary follicle (diameter 115 μm) containing an oocyte at the germinal vesicle stage (diameter 55 μm) after isolation from a 14-day-old mouse. (b) At day 2 of culture the theca cells have attached the follicles to the bottom of the culture dish and somatic cells are proliferating. (c) At day 4 granulosa cells have pierced through the basal membrane and are overgrowing the theca cell monolayer. (d) At day 6 the original spherical structure is completely undetectable. The oocyte is maintained within a mound of granulosa cells surrounded by distally located theca cells. (e) At day 8 of culture the antral-like cavities are formed showing clear differentiation of granulosa cells in mural and cumulus cells. (f) On day 10 the antral-like cavity is recognized as a clear halo around the cumulus–oocyte complex. (g) On day 12 the antral-like cavity is expanded and an individual cumulus complex is visible. (h) On day 13 the expanded and mucified cumulus–oocyte complex has detached from the cultured follicle, which received 1.5 iu hCG l\(^{-1}\) on day 12. (i) Denudation of the somatic cells on day 13 reveals a first polar body extruded in the perivitelline space. Oocyte diameter is 72 μm. (j) An embryo at day 1 obtained from a fertilized oocyte grown in vitro. (k) Hatching blastocyst at day 5 after fertilization. (l) Normal live young obtained after culture of early preantral follicles.
Endpoints in follicle culture can vary widely: intact follicle survival, germinal vesicle breakdown, extrusion of the first polar body, fertilization, hatching blastocyst formation or live offspring. It is evident that for those performing oocyte culture the only valid endpoint on which to evaluate culture conditions is achieving a reasonable yield of healthy offspring. However live offspring is a distant endpoint. Hence there is a need to determine valid predictors of normal developmental competence at the oocyte. Further studies are required to evaluate the point at which developmental competence is determined by the source and the endocrine environment at harvesting. How important are factors such as the origin of the follicle (fetal, prepubertal, postpubertal, aged female), priming of the animal with gonadotrophins before follicle isolation, and nutritional and seasonal influences? Do all primordial follicles have the potential to generate healthy offspring or do some primordial follicles have certain inherent defects which are eliminated by the natural selection processes during further development? To what extent is the marked reduction in the number of immature female gametes determined by the hormonal environment? Is there an optimum environment for the oocyte in which it could progress from the resting stage to a fully grown oocyte that is developmentally competent? Can the natural selection processes be bypassed by artificially designed growth conditions in vitro? The use of ovaries from fetal or prepubertal animals for research purposes to answer these questions is very attractive since these ovaries contain a large population of primordial follicles.

Suggestions of a low development competence of oocytes harvested from prepubertal animals originate from studies using antral follicles as the starting material, which have already progressed much further in development and possibly are already conditioned irreversibly towards atresia (Driancourt et al., 2001).

The experiments of Eppig and O’Brien (1995) and Cortvrindt et al. (1998b) reported the production of live offspring from follicles isolated from prepubertal animals (follicles were isolated from 12–14-day-old mice). As the mouse primordial follicle is formed between day 1 and day 4 after birth, these experiments prove that developmentally competent oocytes can be generated within 3–4 weeks after the formation of the follicle in which they are harboured. The first waves of growing follicles might not require the long transition phase (as seen in adult animals) for transformation of the resting primordial follicle into a growing primary follicle. For adult rats, this maturation period is estimated to be 30 days (Hirshfield, 1991; Hirshfield and DeSanti, 1995; for a review see McGee and Hsueh, 2000). The shorter period for maturation and growth of follicles from the first cohort could be related to the more advanced stage of the oocytes retained in the primordial follicles of the first wave. Data from Lundy et al. (1999) in sheep demonstrated the differences in the diameter of oocytes in primordial follicles. Alternatively, the differences observed in follicle growth characteristics could be explained by the difference in origins of the somatic cells (precursors of follicular cells: mesenchymal and mesangial cells of the mesonephros) during the process of oocyte envelopment ending in formation of the primordial follicle (McNatty et al., 2000). This observation provides the important message that within the same ovary follicle heterogeneity in somatic composition might promote growth at a faster rate. As suggested earlier by Eppig and O’Brien (1995), follicle culture could circumvent an ‘inadequate’ hormonal environment related to the age of the animal (prepubertal or perimenopausal). Experiments from our laboratory indicate that mouse follicles isolated at the early preantral stage from prepubertal animals perform equally well during culture in vitro (in terms of blastocyst formation) as do follicles of a similar developmental stage isolated from adult females (D Nogueira, R Cortvrindt and J Smitz, unpublished), whereas oocytes grown in vivo are less competent when they originate from prepubertal animals.

Similar work using follicles from aged perimenopausal

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**Table 3.** Percentage success illustrated by stepwise implementation of components in basal culture medium for culture of mouse ovarian follicles

<table>
<thead>
<tr>
<th>Components of culture medium</th>
<th>Days</th>
<th>Follicle survival</th>
<th>Meiotic competence</th>
<th>Fertilization</th>
<th>Development</th>
<th>Live young</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rich basal medium</td>
<td>3</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Rich basal medium + 5% FCS</td>
<td>6</td>
<td>20</td>
<td>20</td>
<td>10</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Rich basal medium + 5% FCS + FSH</td>
<td>13</td>
<td>70</td>
<td>80</td>
<td>40</td>
<td>Variable</td>
<td>Variable +/–</td>
</tr>
<tr>
<td>Rich basal medium + 5% FCS + FSH + LH</td>
<td>13</td>
<td>80</td>
<td>90</td>
<td>70</td>
<td>Stable</td>
<td>60</td>
</tr>
<tr>
<td>Rich basal medium + 5% FCS + FSH + LH + GF</td>
<td>13</td>
<td>90</td>
<td>100</td>
<td>80</td>
<td>Stable</td>
<td>70 ++</td>
</tr>
</tbody>
</table>

GF: growth factors, proteins, peptides, scavengers; GVBD: germinal vesicle breakdown; MII: metaphase II.

*Percentage of plated follicles.

*Percentage of surviving oocytes.

*Percentage of fertilized oocytes.
females could be conducted to test the hypothesis that oocyte quality is determined by the follicular environment rather than by age factors.

In other mammals in which primordial follicles are formed during fetal life the question is even more important as the follicle reserve is largest at that stage of development. As a source for oocyte banking (for research purposes), a fetal ovary is most attractive as it contains the very large number of primordial follicles embedded in a very soft interstitial tissue that makes follicle harvest and extraction much easier.

Although culture of ovarian follicles from mice has shown the potential to generate live offspring, refining the conditions to optimize yields remains a major challenge. The shortened life span of a mouse born after culture of a primordial follicle in vitro should prompt further studies on the life expectancy of such offspring (Eppig and O’Brien, 1998).

The mouse model has great potential for use in the pursuit of the essential knowledge that will underpin more longlasting and extensive experiments in larger mammalian species.

**Culture of preantral follicles from other mammalian species**

Several researchers have published results on follicle culture in other mammalian species such as rats, hamsters, pigs, cats, sheep and cows.

When species that are larger than rodents are used, the first problem encountered is the isolation of small follicles, which are located within a dense interstitial tissue. For isolation, either an enzymatic (Greenwald and Moor, 1989; Roy and Treacy, 1993) or a mechanical approach is chosen, or enzymatic pretreatment can be followed by mechanical isolation using fine needles (Figueiredo et al., 1993; Abir et al., 1999).

Although enzymatic digestion combined with other selection techniques (centrifugation or sieving) permits the harvest of large numbers of small follicles, survival rates of unilaminar follicles after *in vitro* culture are low (Figueiredo et al., 1993; Van den Hurk et al., 1998). The low survival rate may be due to the uncontrolled activity of the proteolytic enzymes, which can ultimately cause release of thecal cell layers, rupture of basement membrane and damage to or alteration of surface protein molecules. This damage leads to dispersion of granulosa cells, which will attach to the culture vessel and break their contact with the oocyte.

The mechanical isolation of multilaminar preantral follicles using small needles is difficult and time consuming. In large mammals, there are a limited number of follicles at the large preantral stages within the ovary and these are embedded within a fibrous interstitial matrix, which precludes easy dissection. However, mechanical isolation has the advantage that it is possible to control the cell population included in the culture (Ralph et al., 1995; Abir et al., 1997). As first reported by Wu et al. (1998), it is also possible to obtain small preantral follicles from the regular needle aspirates during oocyte retrievals for *in vitro* fertilization (IVF). Depending on the physical aspiration procedure and the type of ovary, between 1 and 40 (average 13) follicles with a diameter between 30 and 95 μm can be harvested (Mitchell et al., 1999). Wu et al. (1998) reported that such follicles could be cultured to antral stages and became meiotically competent, but such extensive development could not be repeated in another laboratory using similar or modified conditions (L. M. Mitchell and G. M. Hartshorne, personal communication).

Attempts to culture primordial and primary follicles from cows (Van den Hurk et al., 1998) and humans (Abir et al., 1997; Hovatta et al., 1997, 1999) have resulted in massive follicle degeneration after a few days in culture. This led researchers to the idea of culturing the very small follicle units (primordial, transitional, primary) *in situ* first. For this purpose ovarian cortex is sliced into pieces 100 μm in thickness and cultured just beneath the surface of the medium (to allow good oxygen and nutrient penetration and avoid necrosis) (Hovatta et al., 1997, 1999; Wright et al., 1999). This type of initial tissue culture could lead to initiation of growth and the subsequent culture steps could be performed using isolated follicle units.

A review of published studies reveals that, at present, such systems do not lead to normal follicle development *in vivo*. The limited successes with culture of early preantral follicles from large mammals compared with the results achieved with follicles of equivalent stages from rodents may be explained by the fact that, initially, oocyte volume is proportionally much smaller in larger species than it is in rodents. This effect is revealed by comparison of the percentage of the maximum volume of the oocytes, for the same classes of follicle, for different species (Fig. 8).

Use of several culture systems involving different culture media, growth factors and serum supplements provided the same observation: the resting follicles are activated, but degeneration occurs mainly within a few days (Braw-Tal and Yossefi, 1997; Hovatta et al., 1997; Wandji et al., 1997). Typical morphological changes are observed when culturing small ovarian tissue pieces *in vitro* (Fig. 9). *In vivo*, the transition and growth of primordial to primary follicles is characterized by slow transformation of the granulosa cells (a process of 120 days) from a flattened to a cuboidal appearance and initial growth of the nucleus; *in vitro*, these changes occur within a few days (Fortune et al., 2000). The regulatory mechanisms that co-ordinate growth of oocyte and cell division in the surrounding somatic cells are largely unknown. The culture environments and media compositions tested to date appear incapable of creating the right environment for inducing growth changes that are compatible with physiological growth patterns. Chaotic proliferation and altered differentiation of pregranulosa cells is observed in histological preparations from cultured tissue blocks. Oocyte growth is often insufficient or even undetectable and the development of a thecal cell layer at
the secondary follicle stage is rarely observed. In cultures of ovarian slices from baboon fetuses many follicles were observed in which the oocyte had increased in diameter but the surrounding cells had remained flattened (Fortune et al., 2000).

The limited success to date testifies that signalling pathways are as yet inappropriately installed. Physical (temperature, pH, oxygen supply) or chemical (for example nutrients, growth factor balance) aspects of the culture systems are still inadequate and require optimization.

In mice and rats, factors such as FSH and activin induce physiological responses that promoted follicle survival (Li et al., 1995; Cortvrindt et al., 1997; McGee et al., 1997; Yokota et al., 1997; Smitz and Cortvrindt, 1998). This effect was not observed unequivocally in larger mammals and humans. Although Fortune et al. (2000) found no evidence of growth-promoting effects of FSH or activin during the culture of bovine or baboon follicles from primary to secondary stages in vitro, Hovatta et al. (1997) and Wright et al. (1999) obtained improved follicle development by addition of FSH.

Other factors added to culture media of human follicles that promote development in vitro include insulin (Wright et al., 1999) and GDF-9 (Hreinsson et al., 2001). Addition of Steel factor did not improve the growth of human follicles in vitro (Hovatta et al., 1997).

The hypothesis was put forward that the media used for culture of cortical tissue pieces are too rich and lead to wholesale activation of primordial follicles. The normal environment of primordial follicles is poorly vascularized and oxygen tension is low in this compartment (Van Wezel and Rodgers, 1996). Fortune et al. (2000) recently developed a technique of grafting cortical tissue pieces between the chorioallantoic membrane and the yolk sac membrane of chicken eggs. This ‘in ovo’ grafting technique prohibited the massive activation of the primordial follicles which is observed when follicles are cultured in vitro, providing evidence that the growth arrest of primordial follicles is not solely dependent on a medullary inhibitor, but is possibly linked to factors induced during the rapid neovascularization of the graft or to the environment created by bursal fluid within chicken embryonic membranes. This model could provide clues to the mechanisms regulating initiation of follicle growth.

Culture of preantral follicles from the growing pool in large mammals and humans

Some studies have focused on the use of follicles isolated from ovarian tissue, which have already reached the early preantral growth stage.

Ovaries from large mammals do not contain many follicles at this stage of growth. Hence the use of growing follicles as starting material will not be of major importance in oocyte storage programmes. As unilaminar follicles are present in large numbers in the human ovary, development of a culture system for these follicles would be more useful. Attempts to promote growth of secondary follicles in culture have been more successful: promising results have already been obtained in sheep. Newton et al. (1999) and Cecconi et al. (1999) reported growth of late preantral follicles in vitro and an increase in the volume of the oocytes, which were occasionally capable of resuming meiosis. Pig cumulus–oocytes complexes from preantral follicles have been grown for 2 weeks in collagen matrix and oocytes that reached 100 μm in diameter became meiotically competent in vitro (Hirao et al., 1994). However, the final yield from this culture system was very low: < 2% of oocytes completed the meiotic process. Attempts by Telfer et al. (2000) to design a culture system for pig follicles revealed that serum is an essential survival factor and that only 19% of follicles with a starting diameter of 210–260 μm could become meiotically competent. In cows, Gutierrez et al. (2000) maintained late preantral follicles in long-term culture for 1 month and reported an increase in follicle diameter but did not provide evidence of oocyte meiotic capacity. To date, few studies have focused on oocyte quality and there are insufficient data on oocyte meiotic competence after culture in vitro. In humans, although late preantral follicles can be kept viable in a culture dish over periods of a few weeks, there is as yet no consistent data documenting oocyte meiotic competence (Abir et al., 1997; Picton et al., 1999).

Conclusions and prospects

Establishment of techniques for the culture of follicles in vitro will enable important progress in different fields of biology and medicine. Growth of primordial follicles in vitro followed by in vitro maturation of oocytes will provide mature oocytes for nuclear transfer programmes and cloning. Cryopreservation of female gonadal tissue is
already an established technique, which allows survival of > 50% of the stored gametes. Combination of cryostorage and follicle culture will offer new possibilities for preserving rare species. Strategies will be developed to protect the oocyte store from insults leading to sterility (environmental agents, chemotherapy, radiotherapy). The developments will bring new hope to young women undergoing cancer therapy who may be able to preserve their fertility.

The ability to impact on the pace of spontaneous growth of follicles brings the potential to prolong the reproductive lifespan and prevent some of the secondary effects of the menopause and ageing.

Today, the only available culture techniques are for animals such as mice, in which folliculogenesis is relatively short. Experiments to date with ovarian cortical pieces from larger animals, for example sheep, cows, pigs and humans, have provided disappointing results. Approaches using culture in vitro lead to the unco-ordinated growth of granulosa cells and oocytes and to the absence of normal differentiation of granulosa and theca cells.

There is ample opportunity for improvement in this area: the technical requirements and the selection of essential biochemical determinants for the preantral stages of follicle development need to be characterized precisely. Mapping these stage-related biochemical determinants of follicle growth requires investigation of follicles at well-characterized growth stages using the new powerful molecular biology techniques such as gene chip technology. Interaction between those working in the fields of cell culture and molecular biology will lead to more prompt advances in this area.

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