

## Review

# ***In vitro* maturation of human primordial ovarian follicles: Clinical significance, progress in mammals, and methods for growth evaluation**

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**Summary.** As cancer treatment improves, more young women of reproductive age are surviving, but they suffer from infertility as a consequence of the radiation and chemotherapy. Human ovarian tissue containing immature primordial follicles has been successfully cryopreserved. The ultimate aim of this technique is to induce ovarian function by re-plantation of ovarian tissue or, further into the future, by *in vitro* maturation (IVM) of the oocytes derived from the cryopreserved-thawed ovarian tissue, followed by routine *in vitro* fertilization. IVM of primordial follicles from young cancer survivors would avoid the risk of cancer re-transmission by the ovarian grafts. The present review discusses the current achievements in IVM of female germ cells and primordial ovarian follicles and the attempts to improve their development by adding various factors to the culture medium. The established methods for the evaluation of survival and growth in culture are also discussed: follicular counts, immunocytochemical methods, transmission electron microscopy, fluorescent viability markers and endocrine assays. Although the development of IVM systems is still in its infancy, researchers need to pursue their approach step-by-step, especially with regard to factors that might be involved in the activation of the ovarian follicles or female germ cells. The final measure of success will be the ability of the *in vitro* matured oocytes to fertilize and produce healthy offsprings. The availability of such treatment will probably lead to its demand not only by cancer patients but by other women as well.

**Key words:** Primordial follicles, IVM, Fertility preservation, Growth evaluation, Growth factors

## Introduction

### *Relevant aspects of early in vivo oogenesis and folliculogenesis*

Human primordial germ cells (PGC) arrive from the yolk sac to the gonad starting from day 26 of pregnancy, and are then termed oogonia (Van den Hurk et al., 2000). Recently, PGC have also been identified in ovaries of adult mice (Johnson et al., 2004) and women (Bukovsky et al., 2004), 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 (Van den Hurk et al., 2000). Meiotic division usually commences gradually in the third month of gestation, and the diploten stage is achieved within weeks of its initiation. At this point, the oogonia enlarge and acquire more intracellular organelles; they are then termed oocytes. Just before birth, the oocytes are arrested in the diploten stage of the prophase of the first meiotic division.

Follicular formation in humans begins in the fourth month of gestation. In rats and mice, this process occurs during the first postnatal days (Ojeda et al., 2000). During follicular assembly, there is a rapid proliferation of the nearby stroma cells, and the oocytes are surrounded by a single layer of flattened somatic cells, termed granulosa cells (GC), enclosed by a basement membrane (Gougeon, 1996; Van den Hurk et al., 2000). These cellular complexes are defined as primordial follicles (30-50  $\mu\text{m}$  in diameter) and can be identified in the human from around 22 gestational weeks (GW) (Fig. 1).

Most of the follicles in human ovaries of adults as well as fetuses remain primordial (Fig. 1) (Gougeon, 1996). Primordial follicles are activated when their GC

become cuboidal, and these are then termed primary follicles (50  $\mu\text{m}$ -0.1 mm in diameter) (Fig. 1). The growth regulation of primordial follicles is not hormonal and the exact stimulating factors are unknown (Van den Hurk et al., 2000) [See "Factors that might be responsible for early oogenesis or folliculogenesis"]. Thereafter, the increased proliferation rate yields a multilaminar granulosa layer, for the formation of secondary follicles (0.1-0.2 mm in diameter). From the secondary stages, follicle-stimulating hormone (FSH) sustains follicular development and growth. In the human, a definitive theca layer is created from the surrounding stroma cells of secondary follicles, and steroid hormones are synthesized through complex interactions between the GC and theca cells (Gougeon, 1996). The final follicular stage consists of the development of antral follicles containing fluid-filled cavities within several layers of cuboidal GC.

#### *Clinical importance of in vitro maturation of primordial follicles*

As cancer treatment improves, more young women of reproductive age are surviving. However, many suffer from ovarian failure and premature menopause, as a consequence of the radiation and chemotherapy (Abir et al., 1998). These patients have limited options for putative fertility restoration, as the cryopreservation of mature oocytes has shown limited success (Fabbri et al., 2001).

Currently, egg donation, offers the only chance for pregnancy in many cases (Van den Hurk et al., 2000; Abir et al., 2001a). However, there is a shortage of donated oocytes worldwide. One possible way to increase the pool of donated oocytes is to use the large number of 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 (Biron-Shental et al., 2004). 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, researchers are seeking methods to preserve self-fertility. Human ovarian tissue containing immature primordial follicles has been successfully cryopreserved after retrieval by a simple laparoscopic operation (Abir et al., 1998). This technique is aimed either at inducing ovarian function by re-plantation of the ovarian tissue or, further into the future, 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 (Donnez et al., 2004; Meirow et al., 2005; Silber et al., 2005). However, some cancers, such as hematological malignancies (Shaw and Trounson, 1997) and breast cancer (Meirow et al., 1998) carry a possible risk of re-transmission by the ovarian grafts. IVM of primordial

follicles would avoid this possibility, because the oocytes do not contain cancerous cells.

Ovarian cryopreservation may also be of benefit to girls with Turner's syndrome (TS), especially those with mosaic karyotypes, whose ovaries often contain follicles, usually at early ages (Abir et al., 2001a; Hreinsson et al., 2002a). 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 after puberty appears.

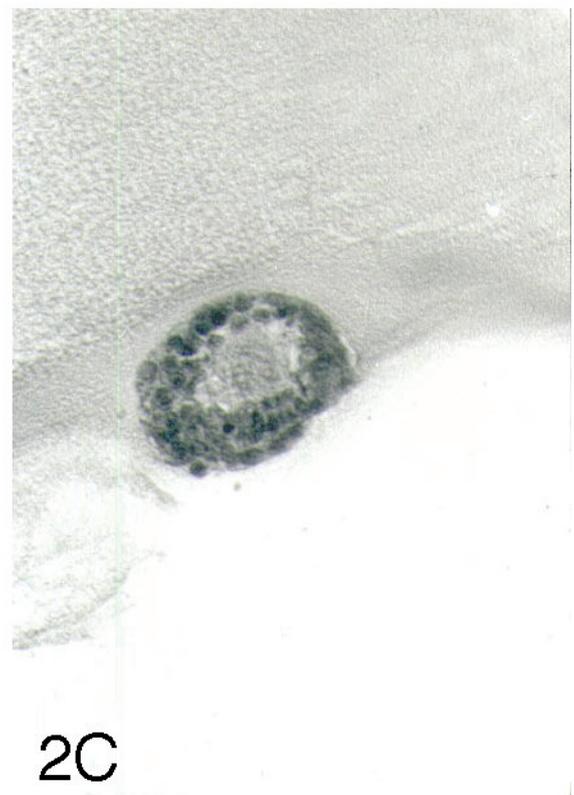
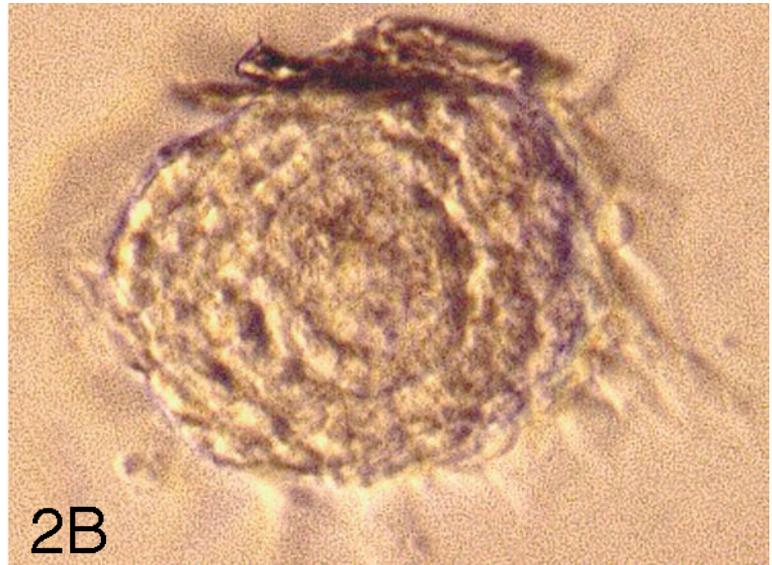
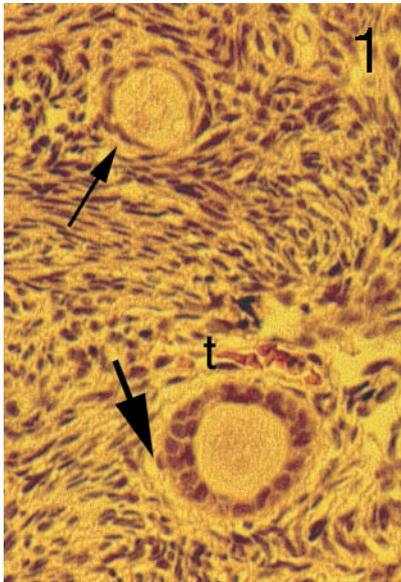
Indeed, once established, fertility restoration by cryopreserved-thawed ovaries may also be preferred by healthy women who choose to postpone childbearing until later in life. IVM of primordial follicles would obviate the complexity, cost, and emotional toll of the artificial reproduction technologies, which require 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 and its attendant side effects to the donor. Finally, IVM of primordial follicles would not only serve human fertility programs but could also enhance efforts at conservation of endangered species.

#### **Culture systems for IVM of primordial follicles**

There are two approaches to the culture of primary and primordial follicles (Van den Hurk et al., 2000) (Table 1). The more popular one involves culturing whole slices of ovarian tissue (organ culture), such that the structural integrity of the tissue is maintained and, hence, the interactions between the surrounding stroma cells and the follicles are retained. Using this method, primordial follicles from cows (Braw-Tal and Yossefi, 1997) and humans (Hovatta et al., 1997; Wright et al., 1999; Louhio et al., 2000; Scott et al., 2004a,b; Zhang et al., 2004) have been grown to secondary stages. The human follicles survived in organ culture for up to four weeks (Hovatta et al., 1997). Attempts to improve the organ culture system for human follicles yielded a reduced atresia rate under serum-free conditions (Wright et al., 1999) and better follicular survival in wells coated with diluted extracellular matrix (Wright et al., 1999; Scott et al., 2004a) and with tissues cut in cubes rather

**Table 1.** Comparison between organ culture and isolated follicles for IVM of primordial follicles.

| Organ culture                        | Cultured isolated follicles         |
|--------------------------------------|-------------------------------------|
| Technically easy                     | Technically difficult               |
| Cellular interactions intact         | Cellular interactions distorted     |
| Monitoring during culture-impossible | Monitoring during culture-possible  |
| Culture of empty specimens-possible  | Culture of identifiable follicles   |
| Not for low follicular numbers       | Suitable for low follicular numbers |
| Ovarian stroma inhibitors remain     | Ovarian stroma inhibitors removed   |

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**Fig. 1.** Ovarian section from a 25-year-old woman. Note the primordial follicle (narrow arrow) and primary-secondary follicle (wide arrow) with its theca layer (t). Hematoxylin and eosin, original magnification x 400

**Fig. 2.** Isolated human follicles. **A.** Two isolated human primordial follicles. Note the central oocytes surrounded by a single GC layer. Original magnification, x 400. **B.** A secondary follicle that developed in culture from a primordial follicle during 24h in collagen gel. Note the central oocyte surrounded by several GC layers. Original magnification, x 400. **C.** Section of an ovarian unilaminar follicle that reached multilaminar stages after 24h of collagen gel culture. Note the multilaminar GC layers surrounding the oocyte. Size at culture: 45 $\mu$ m; and after 24h: 90 $\mu$ m. Hematoxylin and eosin, original magnification, x 400

than in long slices (Scott et al., 2004a).

The second approach to follicular culture involves the use of isolated primordial and primary follicles (Abir et al., 1999, 2001b) (Table 1, Fig. 2). Though whole tissue culturing is much easier than working with tiny follicles of 30–50  $\mu\text{m}$ , 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. By contrast, the recruitment of isolated follicles would make it possible to determine the initial follicular content (Abir et al., 2001a).

Primordial follicles can be isolated enzymatically with collagenase and DNase, as reported in studies for human primordial follicles (Abir et al., 1999, 2001b), or mechanically, as described for bovine primary follicles (Schotanus et al., 1997; Van den Hurk et al., 1998, 2000). Isolated murine (Torrance et al., 1989), bovine (Schotanus et al., 1997; Van den Hurk et al., 1998, 2000) and human (Abir et al., 1999, 2001b) unilaminar follicles were cultured to multilaminar stages in collagen gels (Fig. 2) (Torrance et al., 1989; Abir et al., 1999, 2001b), and isolated rat follicles co-cultured with stroma cells on poly-L-lysine developed to preovulatory stages (Cain et al., 1995). Isolated human primordial follicles developed in culture only within a supporting collagen gel matrix and not on collagen, poly-L-lysine or extracellular matrix (Abir et al., 2001b). Co-culture with stroma cells was not beneficial. Without collagen gel support human primordial follicles survived in culture for up to five days without growth (Oktay et al., 1997). Moreover, although human primordial follicles developed better in organ culture than as partially isolated follicles (with some stroma cells attached) (Hovatta et al., 1999), only fully isolated follicles could grow in collagen gel culture (Fig. 2) (Abir et al., 2001b). Partially isolated follicles survived in culture, but did not grow. As opposed to bovine follicles, which showed only slow and very limited growth in collagen gels without oocyte growth and with a 43% oocyte death rate after seven days of culture (Schotanus et al., 1997); 40% of isolated human follicles cultured for 24h in collagen gel showed an increase in the number of GC layers (to two or three) and in oocyte size (Fig. 2) (Abir et al., 1999, 2001b) [See also “Factors that might be responsible for early oogenesis or folliculogenesis”]. There were no differences in the development of human ovarian follicles derived from fresh or frozen-thawed tissue in organ culture (Hovatta et al., 1999) or cultured as isolated follicles (Abir et al., 2001b).

#### **Established methods for growth and survival evaluation in culture**

To ensure accuracy during evaluation several methods should be used.

#### *Morphometric and histological methods for light microscopy (LM)*

The simplest method to evaluate the growth of isolated follicles is inverted microscopy. This technique enables researchers to observe increase in GC layers (Fig. 2) and oocyte health (dead oocytes=dark; viable oocytes= transparent) (Abir et al., 1999, 2001b). Using an image analyzer or a calibrated eyepiece micrometer (Abir et al., 1997, 1999, 2001b), researchers can measure and compare the diameters of the follicles and the oocytes throughout the culture period. Inverted microscopy, however, is not precise and cannot be used for follicles in organ culture.

Therefore, to determine GC and oocyte viability, ovarian follicles need to be prepared for histologic study, preferably for paraffin sections and staining (usually hematoxylin and eosin) (Fig. 1). In some cases, mitosis can be seen in the GC of these preparations (Hovatta et al., 1997); indicating GC division or DNA replication and follicular and oocyte measurements can be conducted from the histological sections (Hreinsson et al., 2002b).

The number and classification (primordial, primary and secondary) of follicles in the stained sections can be used to evaluate follicular growth and survival in culture (Hovatta et al., 1997; Hreinsson et al., 2002b; Biron-Shental et al., 2004). The follicles are counted in uncultured control samples and periodically throughout culture, with comparison among the follicular classes. Thus, a decrease in the number of primordial follicles and a corresponding increase in growing follicles, from primary stages onwards, signifies follicular growth. However, because different ovarian samples are monitored throughout the culture period, and follicular density may vary by ovarian areas; the count may be inaccurate. Our group uses uniform-sized samples, and the follicles are counted throughout the field (magnification  $\times 100$ ) in two to three levels per specimen (with at least 50  $\mu\text{m}$  between levels to avoid counting the same follicle twice) (Biron-Shental et al., 2004). Alternatively, the follicles can be counted and classified per high power field (magnification  $\times 400$ ) (Hovatta et al., 1997, 1999; Wright et al., 1999). If the section includes large numbers of follicles as in fetal ovaries, a computerized image analyzer is recommended (Biron-Shental et al., 2004). With this, more accurate albeit complicated, method, the follicular density throughout the slice can also be calculated (Lass et al., 1997; Hreinsson et al., 2002a,b). As about 10 sections need to be analyzed for follicular density, however, all the ovarian tissue is used, and additional preparation procedures cannot be performed.

#### *Immunocytochemical/immunofluorescent methods*

Various immunocytochemical methods for the evaluation of DNA division or GC proliferation have been established. The first identifies the expression of

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proliferating cell nuclear antigen (PCNA) in GC during culture (Biron-Shental et al., 2004). PCNA is a 35-kDa nuclear protein that plays an essential role in cell cycle regulation and is an important mediator of cell proliferation (Wandji et al., 1996, 1997). These characteristics make it a useful marker for proliferating cells. The expression of PCNA in GC of primordial follicles correlates with the initiation of folliculogenesis (Fig. 3), and it appears in these cells only when they begin to grow (Wandji et al., 1996, 1997). PCNA expression has been found in the nuclei of GC of cultured fetal bovine (Wandji et al., 1996) and baboon (Wandji et al., 1997) follicles.

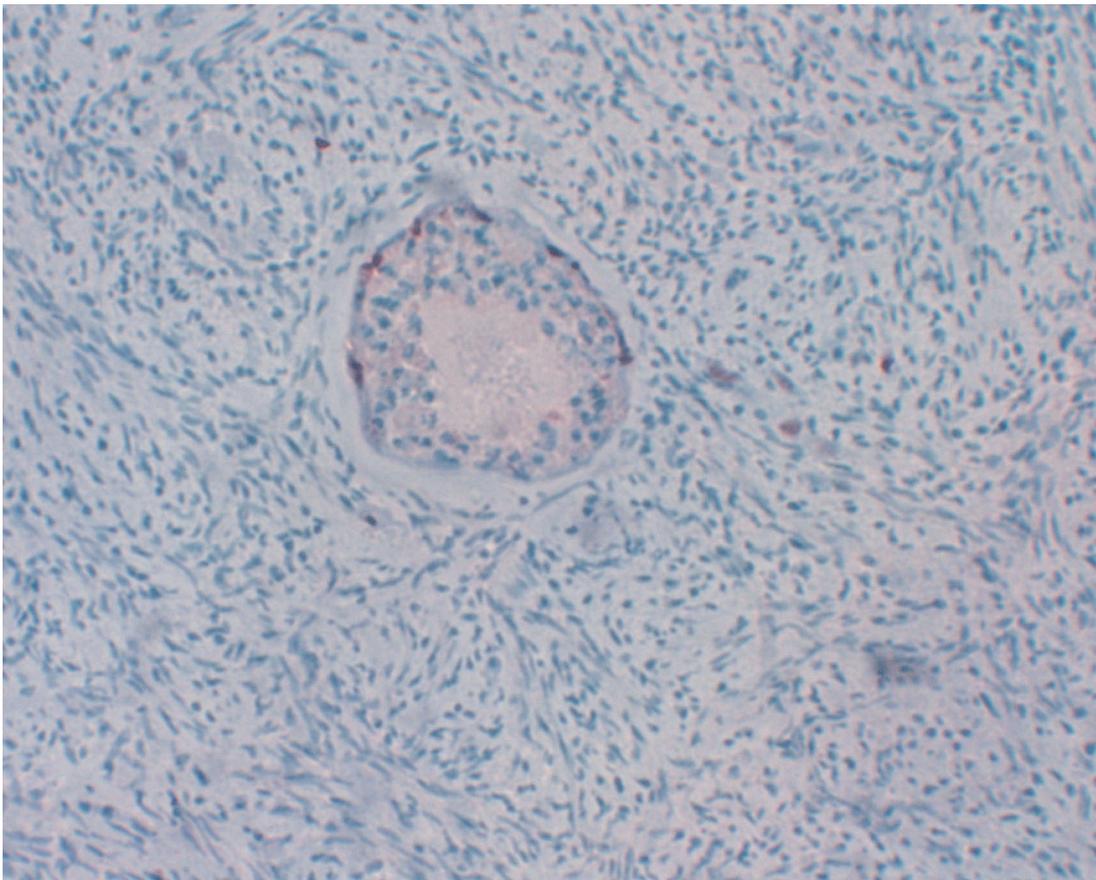
Another marker of DNA proliferation in culture is bromodeoxyuridine (BrdU) incorporation. BrdU is incorporated into the DNA of S-phase cells, and therefore serves as a marker of *in vitro* DNA synthesis (Hulshof et al., 1995). Unlike PCNA, BrdU expression can be detected only if exogenous BrdU is added to the culture medium. BrdU incorporation has been identified in GC of cultured isolated secondary follicles of mice (Cortvrindt and Smits, 1998), cows (Hulshof et al., 1995) and cats (Jewgenow, 1998). However, the duration of incubation with BrdU varied among the studies, from one hour (Cortvrindt and Smits, 1998), to the last culture

day (Jewgenow, 1998) to throughout the culture period (Hulshof et al., 1995; Biron-Shental et al., 2004).

Immunocytochemical methods can also detect the meiotic progression of fetal germ cells in culture. This method was applied using anti-meiosis-specific synapsial complex and anti-centromere fluorescent antibodies (Hartshorne et al., 1999; Lyrakou et al., 2002). The authors detected an increase in the number of pachytene cells when murine fetal ovaries were cultured with growth factors (Lyrakou et al., 2002) and a change in the proportion of zygotene and pachytene cells when human fetal ovaries (13-16 GW) were cultured; indicating normal meiotic progression of the first prophase (Hartshorne et al., 1999; Lyrakou et al., 2002).

#### *Transmission electron microscopy*

Intracellular damage under culture conditions can be evaluated only by transmission electron microscopy (TEM) (Fig. 4) (Van den Hurk et al., 1998; Zhao et al., 2001; Raz et al., 2002). TEM revealed a poor ultrastructure after culture of small bovine primary and secondary follicles, especially in the oocytes (Van den Hurk et al., 1998). In another study, human primordial follicles were cultured with and without



**Fig. 3.** Immunocytochemical staining for PCNA. Section of a human ovary from a 38-year-old woman after one week in culture, stained for PCNA expression. Note the secondary follicle, and the red-brown PCNA staining in some of the GC compared with the background hematoxylin blue staining. Original magnification, x 400

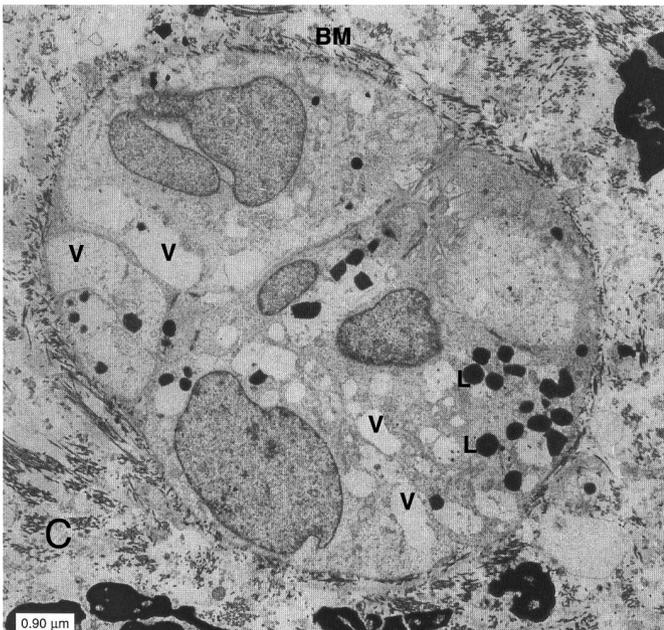
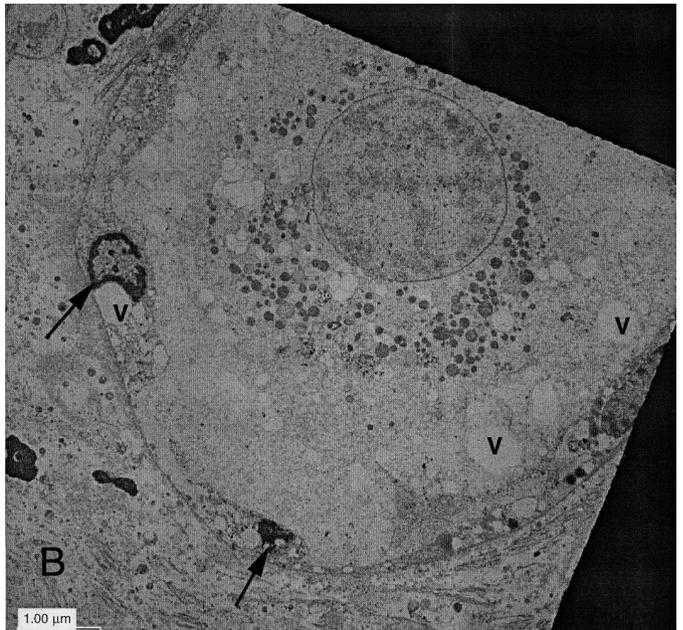
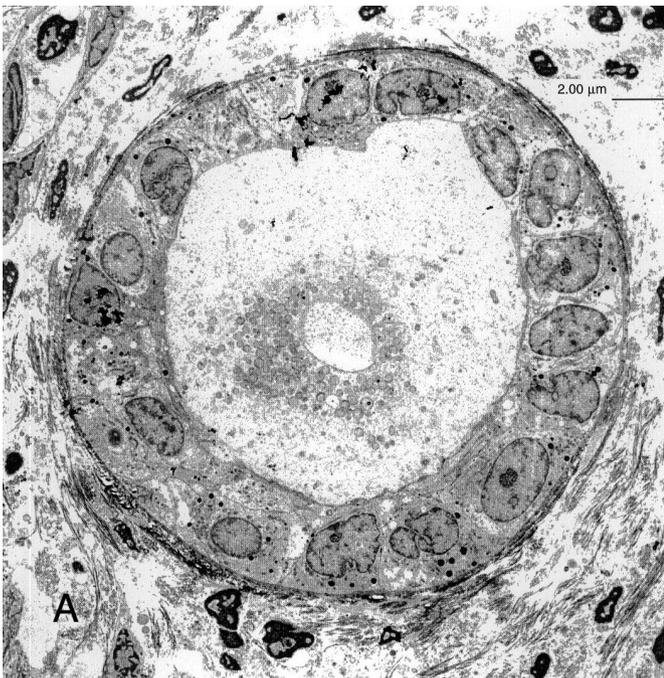
cyclophosphamide and examined by TEM (Raz et al., 2002) (Fig. 4). Follicles cultured with cyclophosphamide had an increased number of damaged GC nuclei and basement membranes (Fig. 4B,C).

TEM can also distinguish morphologically between apoptosis and necrosis (Tilly, 1996). Morphological characteristics of apoptosis include deletion of single cells, cell shrinkage, membrane blabbing, nuclear condensation, cell disruption into small membrane-

enclosed fragments (apoptotic bodies), and phagocytosis by neighboring cells. Necrosis is characterized by death of cell groups, loss of membrane integrity, cell swelling, lysosomal leakage, and clumpy ill-defined aggregation of chromatin.

#### Fluorescent viability stains

Follicular viability after culture can be evaluated by



**Fig. 4.** TEM sections of cultured human ovarian follicles. **A.** Electron microscope section of a primary ovarian follicle from a 38-year-old patient that was cultured without cyclophosphamide for 2h. Note the normal follicular structure with numerous intracellular organelles surrounding the oocyte's nucleus. Original magnification, x 2000. **B.** Electron microscope section of a primordial ovarian follicle from a 24-year-old patient that was cultured with cyclophosphamide (0.5 mg/mL) for 4h. Note the damaged GC nuclei (arrows), the vacuoles (V) in the oocyte and the numerous intracellular organelles surrounding the oocyte's nucleus. Original magnification, x 3000. **C.** Electron microscope section of the outer surface (not mid-section) of an ovarian follicle from a 21-year-old patient that was cultured with cyclophosphamide (0.05 mg/mL) for 24h. Note the damaged feather-like basement membrane (BM), the large vacuoles (V), and the lipid droplets (L) in the GC layer. Original magnification, x 4000

fluorescent markers either, in combination with standard fluorescent microscopy or with confocal microscopy without tissue fixation (Cortvrindt and Smitz, 2001). Fluorescent green signals, produced by staining with calcein (Fig. 5) (Schotanus et al., 1997; Cortvrindt and Smitz, 2001; Hreinsson et al., 2003), or with 5-and 6 carboxyfluorescein diacetate with succinimidyl (Oktay et al., 1997) represent esterase activity in living cells and fluorescent red signals, produced by rhodamine staining represent active mitochondria (Schotanus et al., 1997). Dead cells can be demonstrated by fluorescent red signals produced by staining with ethidium homodimer (Fig. 5) or the DNA stain propidium iodide (Oktay et al., 1997), the result of penetrating only non-viable cells with membrane damage. The technique revealed that the percent of isolated bovine follicles exhibiting esterase activity and active mitochondria in their oocytes decreased after culture, with 43% of the follicles containing dead oocytes (Schotanus et al., 1997; Van den Hurk et al., 1998).

#### *Endocrine methods*

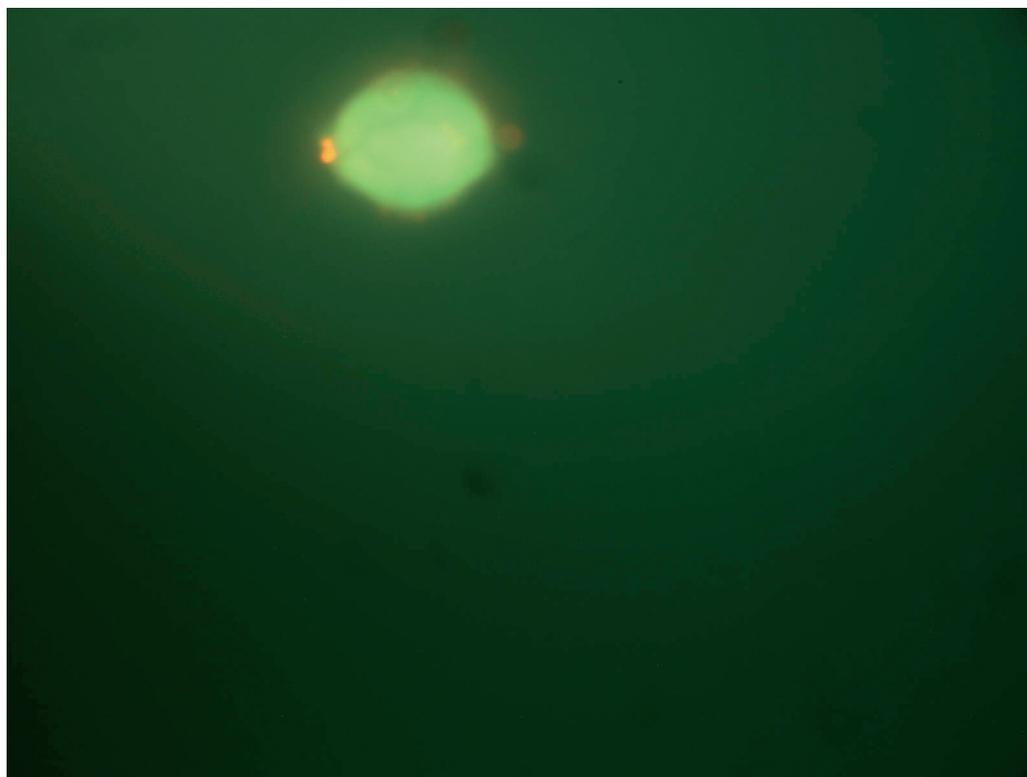
Endocrine methods to evaluate growth include measurement of steroid hormone levels, especially 17- $\beta$ -estradiol (E2), in spent media samples. As primordial and primary follicles do not produce E2, this hormone indicates the presence of secondary follicles. This technique was applied in studies of ovarian follicles

from women (Abir et al., 1997; Scott et al., 2004b) and human fetuses (Biron-Shental et al., 2004). A sensitive double-antibody radioimmunoassay (detection limit=1.4 pg/mL) can be used (Abir et al., 1997; Biron-Shental et al., 2004), although less sensitive methods have also been successful (Scott et al., 2004b).

#### **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 (Eppig and O'Brien, 1996; O'Brien et al., 2003). This research group developed a two-stage culture system: primordial follicles were grown in organ culture to secondary follicles, and 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) were obtained (O'Brien et al., 2003). The first mouse born was extremely obese, and postmortem examination revealed multiple malformations.

In some studies, ovarian tissue was grafted to hosts as an intermediate step in IVM of mammalian primordial follicles (Liu et al., 2000, 2001; Kaneko et al., 2003). Specifically, fresh (Liu et al., 2000) and frozen-thawed (Liu et al., 2001) murine ovarian tissue was transplanted under the kidney capsule of immunodeficient mice (Liu et al., 2000, 2001). The grafts were removed, and the



**Fig. 5.** Calcein and ethidium homodimer staining. Note green calcein staining in the oocyte and GC layer of an isolated human follicle, indicating viability, and the red ethidium homodimer staining in stroma cells attached to the follicle indicating dead cells. Original magnification, x 400

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, in another study, after removal of porcine tissue grafts, initially containing primordial follicles, immature germinal-vesicle stage oocytes were aspirated from antral follicles (Kaneko et al., 2003). Seventeen percent underwent IVM, out of which 55% were fertilized successfully in vitro to two pronuclear-stage embryos. Despite these promising results (Liu et al., 2000, 2001; Kaneko et al., 2003), however, it is very unlikely that IVM of primordial follicles through animal hosts will ever be approved ethically for clinical purposes.

#### **Attempts at IVM of fetal oogonia/oocytes**

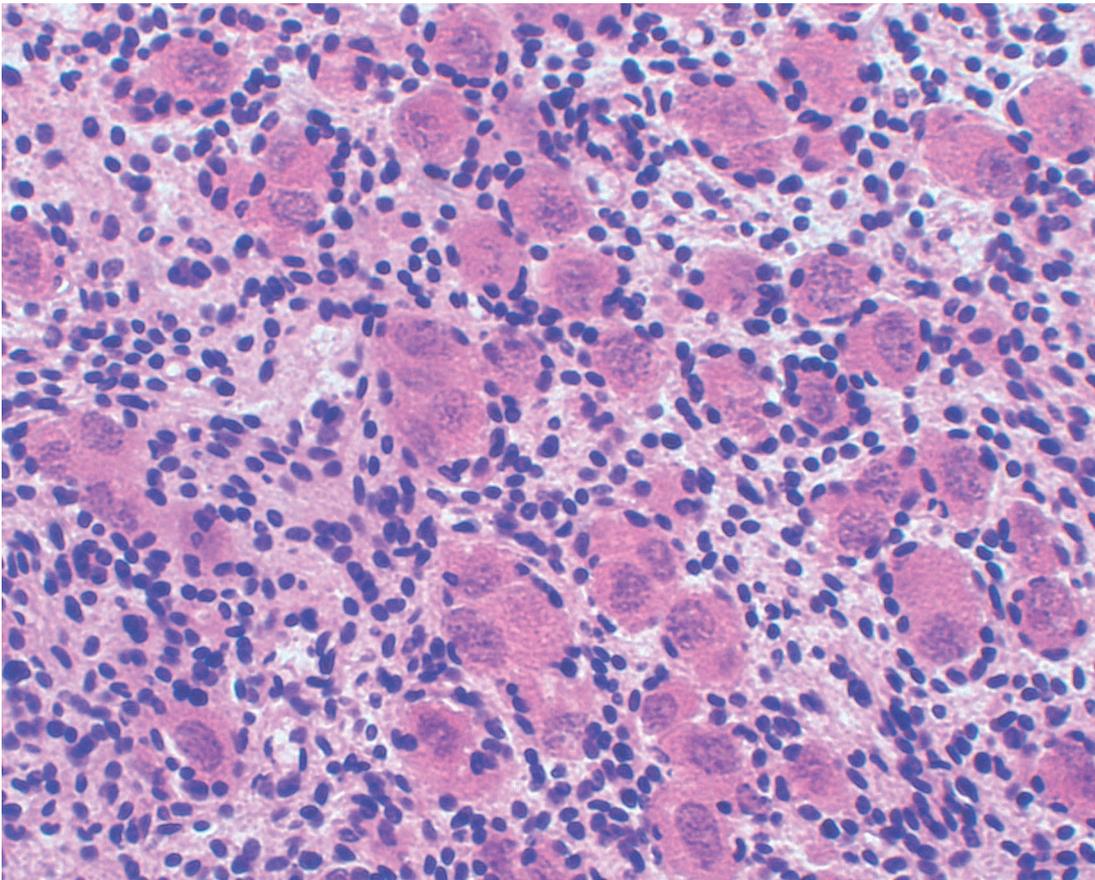
There are several reports on the in vitro growth of oogonia from fetal mice to antral follicles (Obata et al., 2002) or mature oocytes (Klinger and De Felici, 2002). Fetal bovine and baboon follicles developed in vitro into primary and secondary follicles with GC proliferation, as demonstrated by their expression of PCNA (Wandji et al., 1996, 1997; Fortune et al., 1998). However,

although, the diameters of both the fetal bovine follicles and their oocytes increased in culture, about 50% of the follicles were atretic (Wandji et al., 1996).

In another study, human fetal ovaries (13-16 GW) were cultured for 40 days, and after several weeks, the oogonia entered the initial stages of meiosis (Hartshorne et al., 1999). Fresh and frozen-thawed human fetal oogonia and primordial follicles (16-20 GW) were also cultured for two months, and morphologically mature oocytes were obtained (Zhang et al., 1995), although they were smaller than those developed in vivo. Ovarian specimens obtained from second- and third-trimester human fetuses (22-33 GW) were cultured for four weeks (Biron-Shental et al., 2004), and the follicles survived in culture without any apparent increase in the number of primary and secondary follicles (Fig. 6). However, a significant increase in the level of E2 in the spent media samples was detected in the fourth week of culture; indicating steroidogenesis of the secondary follicles.

#### **Factors that might be responsible for early oogenesis or folliculogenesis**

Stem cell factor (SCF) and leukemia inhibiting factor (LIF) are essential for the survival and



**Fig. 6.** Section of human ovarian fetal follicles after two weeks in culture. The tissue was taken from a normal 27- GW-old fetus. Note the primordial follicles and the polyovular primordial follicles, frequently identified in fetal ovaries. Hematoxylin and eosin, original magnification, x 400

proliferation of murine (Pesce et al., 1993; Cheng et al., 1994; De Felici, 2000; Driancourt et al., 2000) and porcine (Shim and Anderson, 1998; Durcova-Hills et al., 1998) PGC, while oncostatin M (OSM) is involved in the survival and proliferation only of murine PGC (Abir et al., 2005a). The addition of SCF to the culture medium of isolated fetal murine oogonia co-cultured with GC resulted in meiotic resumption and folliculogenesis (Klinger and De Felici, 2002).

Growth factors were found to promote the development of primordial follicles only up to the primary-secondary stages (Disson et al., 1995, 2001; Parrott and Skinner, 1999; Driancourt et al., 2000; Louhio et al., 2000; Nilsson et al., 2001, 2002; Hreinsson et al., 2002b; Kezele et al., 2002; Romero et al., 2002; Nilsson and Skinner, 2003; Lee et al., 2004). Treatment of cultured murine (Kezele et al., 2002) and human (Louhio et al., 2000) primordial follicles with insulin induced the transition to the primary-secondary stages and reduced atresia. Bone morphogenetic protein (BMP) -7 not only induced the activation of primordial follicles in mice (Lee et al., 2004), but also stimulated the synthesis of FSH receptor (FSH-R) mRNA, similar to the effect of nerve growth factor (NGF) in rats (Disson et al., 1995; 2001; Romero et al., 2002). Primordial follicles in rats were activated by the addition of SCF (Parrott and Skinner, 1999; Driancourt et al., 2000), BMP-4 (Nilsson and Skinner, 2003), LIF (Nilsson et al., 2002), or basic fibroblast growth factor (bFGF) (Nilsson et al., 2001). A similar effect in humans was achieved with insulin-like growth factor (IGF)- 1 and- 2 (Louhio et al., 2000) and growth differentiation factor (GDF)- 9 (Hreinsson et al., 2002b); however, PCNA expression in the GC was identified only with the addition of IGF-1 (Louhio et al., 2000) or GDF-9 (Hreinsson et al., 2002b). Interestingly, the *in vitro* survival of human primordial follicles, both isolated (Abir et al., 1999, 2001b) and in organ culture (Wright et al., 1999), increased with FSH supplementation.

It is very unlikely that a single growth factor is responsible for the development of PGC or primordial follicles in mammals. Rather, various growth factors probably have similar effects on them, or their growth depends on the combination of growth factors. Indeed, studies have reported that the growth of murine migratory PGC was induced only by the combination of OSM, LIF, SCF, and bFGF, and that the induction of postmigratory PGC growth and survival by OSM was enhanced with the addition of LIF, SCF and bFGF (Cheng et al., 1994; Koshimizu et al., 1996). Similarly, a combination of LIF, SCF, and IGF-1 promoted the survival in culture of murine oogonia (Morita et al., 1999; Lyrakou et al., 2002) and led to a significant increase in the number of meiotic-pachytene cells (Lyrakou et al., 2002).

Others found that the treatment of murine (Kezele et al., 2002) primordial follicles with insulin and SCF, or rat (Nilsson et al., 2002) primordial follicles with insulin and LIF, or human (Louhio et al., 2000) primordial

follicles with insulin and IGF-2, resulted in a significant increase in the number of developing follicles compared with each factor alone (Louhio et al., 2000; Kezele et al., 2002; Nilsson et al., 2002).

Not only growth factors but also intracellular secondary messengers might be involved in the activation of primordial follicles (Morbeck et al., 1993; Scott et al., 2004b; Zhang et al., 2004). GC from isolated porcine primary follicles proliferated in culture with the addition of 8-bromo (8-Br) cyclic adenosine mono phosphate (cAMP) (Morbeck et al., 1993). Culture of primordial follicles from women with 8-Br cyclic guanosine mono phosphate (8-Br-GMP) (Scott et al., 2004b) or cAMP (Zhang et al., 2004) increased growth to secondary stages and decreased atresia rates. Moreover, 8-Br-GMP stimulated E2 production in spent media samples, presumably because of the concurrent increase in the proportion of secondary follicles (Scott et al., 2004b). However, these secondary follicles were smaller than those developed *in vivo*.

The activation of primordial follicles is probably arrested by various inhibiting factors, and the oocyte itself might exert an inhibitory action on the follicle (Gougeon, 1996; Van den Hurk et al., 2000). The finding that the addition of anti-mullerian hormone led to a significant reduction in the rate of growing murine follicles *in vitro* points to its apparent inhibitory effect on the recruitment of murine primordial follicles to the growing phase (Durlinger et al., 2002). There are also reports of growth inhibition of murine follicles with insulin and FSH (O'Brien et al., 2003). Inhibitory effects on primordial follicle activation might also originate from stroma cells. Isolated human primordial follicles were found to develop into secondary follicles already after 24h of culture, but only when they were fully isolated from the stroma layer (Abir et al., 1999, 2001b). Apparently, the complete removal of the stroma layer led to a rapid release of inhibitory factors from the cells surrounding the follicles.

## **Discussion and conclusions**

This review describes the limited progress in the IVM of primordial ovarian follicles and methods of their growth evaluation. In humans and other species, follicular development from primordial stages onwards is complex and lengthy (Gougeon, 1996). It is possible that the optimal culture medium for growth promotion needs to be very rich in supplements and species-specific. Various sequential media may also be necessary for every follicular stage, until a mature, healthy and fertilizable human oocyte is obtained.

The use of fetal follicles as a putative source of oocyte donations for IVF raises several ethical dilemmas (Biron-Shental et al., 2004). So far, this possibility is remote, owing to the scarcity of *in vitro* studies of fetal follicles and oogonia. Studies conducted in our laboratory identified fewer receptors for various growth factors in GC of fetal primordial follicles than in those

from adults (Abir et al., 2004a,b, 2005a,b; Ben-Haroush et al., 2005), suggesting that it will be harder to develop a successful IVM system for fetal follicles than for primordial follicles from women.

Taking into account the important clinical significance of these findings, researchers need to continue pursue their studies step-by-step until a successful culture system is obtained. Although various methods for the evaluation of development in culture have been established, the ultimate test will be the ability of the in vitro matured oocytes to fertilize and produce healthy offspring. Once IVM of human primordial follicles is established, the availability of such treatment will probably lead to its demand not only by cancer patients, but also by other women as well.

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