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Current perspective on primordial follicle cryopreservation and culture for reproductive medicine

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Mature oocytes are rare and precious cells. A technology which generates larger numbers would be very welcome in clinical practice, animal production technology and research. Since de-novo formation of female germ cells has ceased by the time of birth, the most attractive strategy, in theory, is to harvest and culture primordial follicles, the most abundant stage in the ovary at all ages. So far, there has been more success with cryopreservation of primordial follicles than with culture, and frozen–thawed ovarian tissue grafts have restored fertility to a number of species after oophorectomy. However, in-vitro development of isolated follicles is not sustained beyond the primary follicle stage. To meet their requirements for growth, metabolism and differentiation, a multistage protocol will probably be required for the prolonged period of development to maturity. The mouse is the only model, to date, in which a live offspring has ever been produced after growing follicles completely *in vitro*. A triple-stage process was required, involving culture of ovarian explants followed by isolation of granulosa–oocyte complexes and, finally, suitable conditions for completing meiotic maturation. Achievement of this goal for the larger and more slowly developing follicles from human and farm animal ovaries is still a remote possibility.

Key words: cryopreservation/culture/follicle/meiosis/oocyte

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Background

Once the technology of IVF had been established for laboratory animals and humans in the 1960s and 70s, it was logical to attempt to culture immature oocytes to the metaphase II stage. Such work had in fact begun in the 1930s with the demonstration that germinal vesicle oocytes from unstimulated rabbit follicles spontaneously resume meiosis in culture (Pincus and Enzmann, 1935). Some three decades later, Edwards confirmed the phenomenon in several other species, including humans (Edwards, 1965). The results indicated that meiotic prophase is under inhibitory control until it is alleviated by the LH surge. Thousands of calves as well as other farm and laboratory animal species have now been born from embryos transferred after IVF of oocytes that had been matured *in vitro* (IVM) (Telfer, 1998). There are a number of advantages of IVM, including lower drug treatment costs and avoidance of ovarian hyperstimulation syndrome, and the theoretical risk of gonadotrophin therapy promoting ovarian cancer (Whittemore, 1994). Many authorities now stress the importance of striving to minimise the dose and frequency of gonadotrophin stimulation in assisted reproduction, and IVM goes towards serving that goal (Fauser *et al*., 1999). Whilst not yet widely practised in clinical medicine, pregnancy rates after IVM have exceeded 30% in some centres (Cha *et al*., 2000; Chian *et al*., 2000), but this is only the first step towards revolutionizing IVF practices. Far more oocytes are potentially available from the store of small follicles at the base of the pyramid of follicle stages in the ovary.

Typically, 8–12 oocytes are harvested after controlled stimulation for IVF, but only half as many are available from normal ovaries which are unstimulated (though rather more from polycystic ovaries). The numbers and quality of oocytes available sets a limit on success rates for assisted reproductive technologies, and cryopreservation depresses the chances of success further. Below the sonographically detectable threshold of ~3 mm, there are hundreds of small antral and pre-antral follicles in the ovary, but they are laborious to harvest and easily damaged. However,

because they are abundant in young ovaries and localized in the ovarian cortex, primordial follicles can be obtained in relatively large numbers. In this article, we address the feasibility of generating fertile oocytes from primordial follicles, either from fresh or cryopreserved tissue. As will become apparent, this is one of the most challenging goals in reproductive biology and medicine, but still on the distant horizon.

Character of primordial follicles

Primordial follicles consist of a small oocyte surrounded by a single layer of flattened pregranulosa cells resting on a delicate basement membrane. When follicle growth is initiated, the oocyte enlarges and the somatic cells expand clonally to produce mural and cumulus granulosa cells in the Graafian follicle (Boland and Gosden, 1994; Gougeon, 1996; van Wezel and Rodgers, 1996). A basement membrane separates them from the stroma cells, including precursors of the theca layers. Small size (35 µm in humans), paucity of cytoplasmic organelles and the absence of a spindle apparatus distinguish these oocytes from the mature metaphase II stage and are advantageous for low temperature banking (Table I).

Primordial follicles are abundant in young ovaries, decline exponentially with age and vary allometrically with body weight in different species (Gosden and Telfer, 1987). Age-specific numbers can vary enormously between individuals and even between a pair of organs. At menarche, there are ~2×10^5 small follicles in a pair of human ovaries. This irreplaceable store has fallen to 10^3 by the time menopause is imminent some 35 years later, and the rate of attrition doubles during the last decade of menstrual life (Faddy *et al*., 1992; Faddy and Gosden, 1996). Thus, the number of follicles available for recruitment is progressively attenuated, and by the fifth decade of life only a small proportion remains, even though cycles usually continue for another decade before halting at the menopause. Oocyte quality is also compromised in older ovaries and the higher incidence of aneuploidy increases pregnancy wastage and the time to establish a viable pregnancy in women of late reproductive life (Munné *et al*., 2000; Nybo Andersen *et al*., 2000). On these grounds, the ovary has been regarded as the most rapidly ageing organ in the body.

The great majority of primordial follicles never develop far or fail to reach ovulatory size. In prepubertal rodent ovaries, approximately half of these follicles degenerate by a process which is often referred to as 'atresia', though the term more accurately describes the closure of the antrum in larger stages. The process involves apoptosis (Tilly, 1996). It is probably initiated in the germ cell of primordial follicles independently of gonadotrophin levels, whereas large pre-antral and antral stages are gonadotrophin-dependent and hormone withdrawal initiates apoptosis in the granulosa cells before the oocyte is affected. Since the dynamics of small follicles are difficult to study directly in human ovaries, the contribution of apoptosis to the age-related decline in primordial follicles is hard to estimate. According to a compartmental model fitted to the numbers of follicles at different ages and stages, the death rate is low at these stages in young adults, though there is some evidence that it rises after 37 years of age until the store is exhausted (Faddy and Gosden, 1995). One of the key questions that remains is whether the probability of follicle death is independent of the potential quality of its oocyte, for otherwise a technology to rescue follicles may be doomed to inefficiency or even to giving rise to excess birth defects. Oocyte quality is apparently compromised by superovulation treatment in animals, which rescues antral stage follicles from atresia (Ertzeid and Storeng, 2001), but children born after IVF are evidently as healthy as normal. Another reassuring observation is that when programmed cell death in primordial follicles is inhibited by targeted mutagenesis of the *Bax* gene, pregnancy wastage is not affected (Perez *et al*., 1999). Despite these findings, stringent testing of oocyte quality must be carried out before it is safe to launch a radically new culture technology.

Table I. Primordial follicle characteristics favourable for low temperature banking

Biological characteristic	Practical advantage
Small size	Rapid equilibration during cryopreservation
Relatively undifferentiated	More robust during manipulation and treatment
Cortical distribution	Recovery from thin cortical slices
Low capillary density	Indicative of tolerance to hypoxia
Abundance	Potentially maximises the production of gametes

Primordial follicles are localized in a narrow cortical band in primate ovaries under a poorly vascularized tunica albuginea. To recover the follicle-rich layer, a cortical biopsy ~1 mm thick is sufficient, and this causes little bleeding because the larger blood vessels lie deeper in the medulla (Meirow *et al*., 1999). There are patches of cortex which are follicle-rich but, unfortunately, there are no external indicators to serve as guides for biopsying these areas.

The fraction of the follicle store that begins to grow each day is approximately constant throughout young adult life and is independent of the stage of the ovarian cycle and gonadotrophin stimulation (Peters *et al*., 1973; Oktay *et al*., 1997a; McGee and Hsueh, 2000). However, the process of initiation is not absolutely fixed because the fraction rises when the store is substantially depleted by age or after treatment with alkylating agents to destroy some of the follicles (Hirshfield, 1994). There are many possible explanations for this phenomenon, including non-specific changes in metabolites and the influence of growth/ inhibitory factors from neighbouring cells. It also exists in culture, which is fortuitous for a prospective technology to grow small follicles under controlled conditions.

Growth *in vitro*

Generation of fertile oocytes from primordial follicles developing entirely *in vitro* has only been achieved in a single study so far (Eppig and O'Brien, 1996). Only one live offspring was produced, and it was not long-lived. Nevertheless, this result serves as proof of principle and refinements of the protocol will surely lead to improvements for this model species and for others. This achievement was made possible by progress over many years in growing follicular oocytes at progressively earlier stages—starting with the pioneering studies of meiotic maturation and followed by intermediate follicle stages before attempting primordial follicles.

Pre-antral follicles contain a partially grown oocyte surrounded by incompletely grown and relatively undifferentiated granulosa and theca cells. They can be recovered intact by dissection or as granulosa–oocyte complexes denuded of theca–stroma after enzymatic disaggregation in medium containing collagenase. In either case, the intimate relationship between the germ cell and its granulosa cells is preserved, which is undoubtedly important for metabolite trafficking across the extracellular fluid or via gap junctions (Juneja *et al*., 1999).

Granulosa–oocyte complexes grow optimally at moderate densities (~200/well) on collagen-impregnated membranes, which enable them to attach without much spreading. Many complexes extend a stalk of granulosa cells which bears an oocyte like the cumulus oophorus of a Graafian follicle (Figure 1a). Using a defined medium containing albumin, insulin, transferrin, selenium and hypoxanthine (to inhibit premature resumption of meiosis), oocytes from pre-antral follicles of infant mice become mature enough for IVF after incubation for 10 days (Eppig *et al*., 1992). This is currently the best method for obtaining large numbers of oocytes and produces better results than suspending the complexes in collagen gel, perhaps because of changes in the granulosa cells (Torrance *et al*., 1989; Hirao *et al*., 1994) (Figure 1b). Alternatively, intact follicles can be grown on a plastic substrate in the presence of serum, and up to 40% of them produce oocytes reaching metaphase II stage after 2 weeks of culture with FSH and human chorionic gonadotrophin (HCG) stimulation (Cortvrindt *et al*., 1996b). Follicular shape and architecture become altered, but all three follicular cell types remain in contact, pre-antral-like cavities are formed and responsiveness to gonadotrophin stimulation is preserved (Figure 1c). Perhaps the most physiological model is to grow intact follicles on a porous membrane (Nayudu and Osborn, 1992)

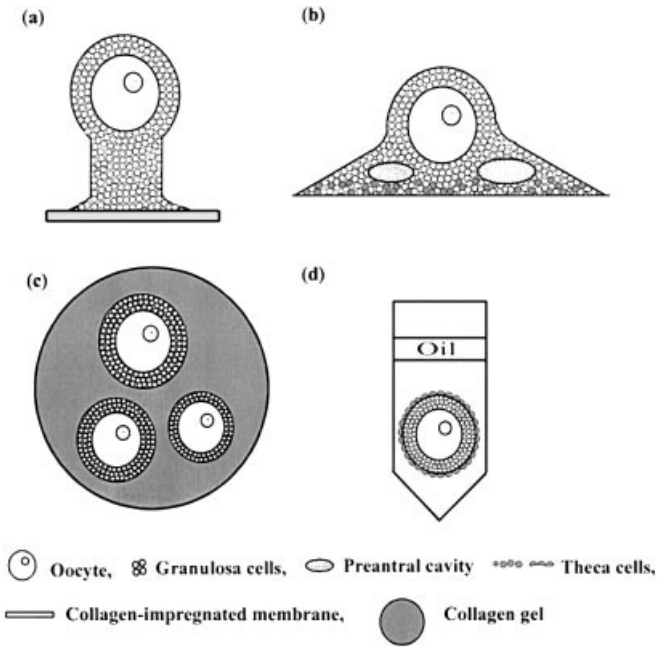

Figure 1. Diagrammatic illustration of four culture systems: (**a**) granulosa–oocyte complexes on a collagen-impregnated membrane; (**b**) granulosa cell complexes suspended in collagen gel; (**c**) follicles attached directly to a plastic culture substratum; (**d**) follicles cultured under mineral oil in V-wells of microtitre plates.

or in microdrops of medium under mineral oil (Figure 1d). Starting at a mid-pre-antral size (~170 µm diameter), follicles develop to the Graafian stage in ~6 days, with normal morphology and steroid production (Boland *et al*., 1994; Hartshorne *et al*., 1994; Spears *et al*., 1994). This method is now so refined that up to 80% of follicles will ovulate *in vitro* in response to HCG (Rose *et al*., 1999).

Progress with other species has been slower, though it is now possible to routinely grow ovine and bovine pre-antral follicles to antral stages in serum-free medium (Newton *et al.*, 1999; Gutierrez *et al*., 2000). With sheep follicles, it was possible to use either fresh or cryopreserved tissue and 20–25% of the follicles reached antral sizes of up to 1 mm after 28 days. The oocytes were, however, incompetent to resume meiosis, and more effort needs to be focused on the end stages as well as on the earliest stages of follicle growth. Studies of human pre-antral follicles have been limited by specimen availability and a small harvest, but some follicles survive in culture for many days (Roy and Treacy, 1993; Abir *et al*., 1997; Hovatta *et al*., 1999; Wright *et al*., 1999; Picton and Gosden, 2000).

A major problem facing development of primordial follicles *in vitro* is the breakdown of basement membrane material and other intrafollicular components during enzymatic isolation from the stroma. This erosion causes the pregranulosa cells to round up and detach from the oocyte, which is an irreversible process that produces oocytes incapable of further development, though they may remain viable for several days (Bachvarova *et al*., 1980). Consequently, the follicles have to be grown in organ explants until they have developed to a stage at which they can be isolated enzymatically as granulosa–oocyte complexes and transferred to collagen membranes for continuing growth (Eppig and O'Brien, 1996). Alternatively, cultured explants can be transplanted to host animals to complete development (Liu *et al*., 2000).

Primordial follicles can be obtained during enzymatic disaggregation of human ovarian tissue obtained at oophorectomy or by biopsy (Roy and Treacy, 1993; Oktay *et al*., 1997b). To minimise exposure to proteases, the procedure is completed by dissection with fine needles, although basement membrane damage still occurs. This problem also applies in sheep and cow ovaries and it is only in species in which follicles are relatively large and the cortical tissue not too fibrous that enzymes can be avoided. In domestic cat ovaries, primordial follicles are large enough to be dissected (45–50 µm diameter), and isolated follicles remain intact and immediately commence growth in culture medium without gonadotrophins. The granulosa cells and oocyte grow in tandem in these cultures, and after 5 days of linear growth the diameter will have increased by 50% and the volume by 3-fold (Figure 2a). Growth is not sustained long-term, however, and the follicles degenerate, though serum helps to preserve them slightly longer (Figure 2b). The general conclusion is that follicle integrity can be preserved by avoiding enzyme treatment, though a medium that permits growth initiation is not necessarily sufficient for continued development. In future, attention should be directed more to the role of growth factors to promote mitosis and inhibit apoptosis, prime candidates being epidermal growth factor (EGF), fibroblast growth factor (bFGF) and follicle-specific factors such as growth differentiation factor-9 (GDF-9), kit ligand and anti-Müllerian hormone (AMH) (Dong *et al*., 1996; Durlinger *et al*., 1999; Parrott and Skinner, 1999), as

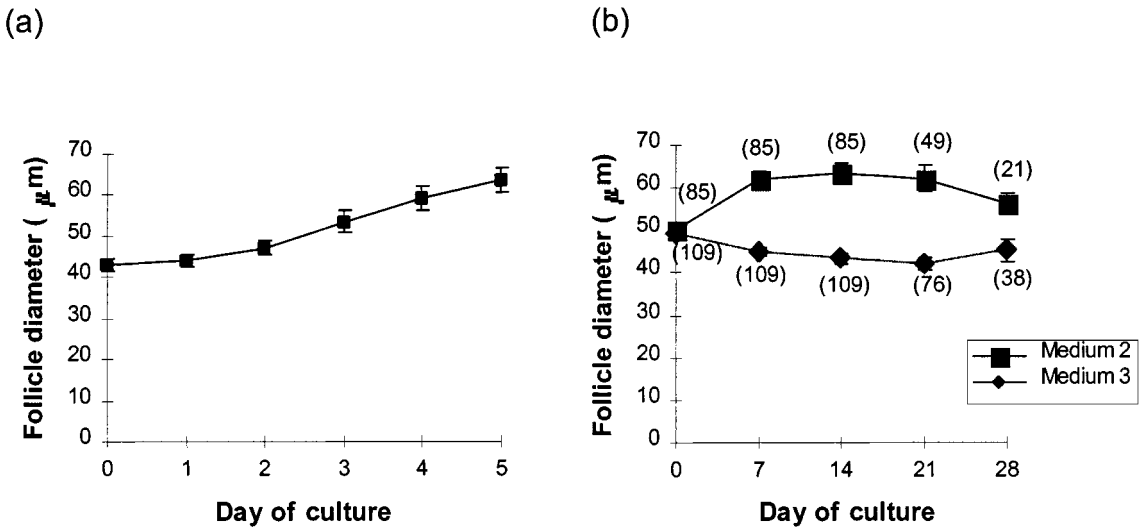

Figure 2. Primordial follicles isolated from cat ovaries for growing *in vitro*: (**a**) 5 day culture in serum-supplemented medium ($n = 73$, $P < 0.001$ comparing days 0 and 5); (**b**) 4 week culture comparing serum-supplemented (2) and unsupplemented (3) media (numbers of observations in parentheses).

well as activin interactions with the extracellular matrix (Oktay *et al*., 2000a).

The observation that virtually 100% of feline follicles start growing after isolation is tentative evidence that they are under inhibitory control *in situ*. This conclusion is supported by general evidence of upregulated growth under certain conditions. When tissue from either ungulate or primate ovaries was cultured, there was a marked increase in the fraction of growing follicles indicated by a nuclear marker of cycling cells (proliferating cell nuclear antigen; PCNA) (Wandji *et al*., 1996; Fortune *et al*., 1998, 1999). Proof is required that this is a physiological response and that follicles can progress beyond granulosa cell layers. Nevertheless, these are hopeful first steps towards the ultimate goal of growing primordial follicles *in vitro*. As in the mouse model (Eppig and O'Brien, 1996), it will probably be necessary to develop a multi-stage protocol to reflect changing physiological conditions and overcome the limitations of diffusion through solid tissue.

Cryopreservation of follicles

Whilst much effort has been devoted to optimizing protocols for cryopreserving preimplantation embryos and mature oocytes, until recently less investment has been made with small follicles. After the pioneering experiments in London in the 1950s (Parrott, 1960), the field lay fallow for three decades because the technology did not have any applications until fertility conservation for patients and animal conservation biology came to the fore (Oktay *et al*., 1998). Whilst autografting is currently the only practicable strategy, culture of frozen–thawed follicles is a more attractive goal in the long term because it would eliminate any risk of re-implanting residual cancer cells and could, in theory, produce more mature oocytes by avoiding follicle wastage created by ischaemia or normal atresia.

Follicles are not ideal objects for cryopreservation because they are multicellular and heterogeneous. To protect them from injury during cooling, much of the water must be replaced by a cryoprotectant agent (CPA), yet many of these agents are toxic at the high concentrations required. As elsewhere in cryobiology, a balance has to be struck in optimizing the concentration of CPA and immersion time, and the rates of cooling and rewarming. Fortunately, primordial follicles in ovarian tissue have turned out to be more tolerant than expected, and success has been reported in many species. In most of the early studies, rat and mouse ovaries were used as models, with the intact ovary being preserved and isografted after thawing (Harp *et al*., 1994; Cox *et al*., 1996; Candy *et al*., 1997; Gunasena *et al*., 1997). Results are now being obtained in which the litter sizes from these animals are comparable with fresh ovarian grafts (Candy *et al*., 2000). Evaluation of cryopreservation has seldom been monitored in culture, but those studies have also produced excellent results (Cortvrindt *et al*., 1996a; Newton *et al*., 1999). In only one study were isolated primordial follicles transferred to a sterile host ovary after freeze-banking, but this too produced fertile gametes (Carroll and Gosden, 1993). The cryotechnology used was based on methods developed for mouse embryos and oocytes, and include permeating CPAs, such as dimethyl sulphoxide, propanediol or ethylene glycol, and usually a non-permeating sugar. In most cases, equilibrium cooling has been adopted, with ice nucleation induced at –7°C and a cooling rate of 0.3–0.5°C min^{-1} down to –30 to –40°C followed by a rapid descent to liquid nitrogen temperatures. Following successes with embryos and oocytes, attempts are now underway with ovarian tissue using ultra-rapid freezing, or vitrification (Sugimoto *et al*., 2000), though solid tissue presents a permeation problem for the higher concentrations of CPA needed.

Similar protocols have been tested on ovarian tissue from farm animals and humans but, instead of using whole ovaries, cortical slices ~1mm thick were cryopreserved and transplanted as xenografts (Oktay *et al*., 2000b) or as autografts (Baird *et al*., 1999; Radford *et al*., 2001). Estimates of follicle survival have ranged from 10 to >70%, according to the CPA used (Newton

et al., 1996), confirming the tolerance of primordial follicles to thermal, chemical and osmotic insults during cryopreservation and the phase of ischaemia after transplantation (Table I).

Perspective

Growth and maturation of oocytes *in vitro* from the primordial follicle stage is a major technical challenge for reproductive science. If it has taken so long to produce adequate—and still imperfect—conditions for human embryos to undergo a few cleavage divisions, how much more difficult will it be to safeguard normal growth and differentiation for oocytes during weeks or months in culture? Developing a safe and effective culture technology for them is likely to be much more difficult than optimizing freezing and thawing methods, yet the potential benefits for reproductive medicine and animal breeding technology could be enormous (Table II).

Put into perspective, ~120 small follicles can be harvested from a 4 mm diameter disk of ovarian tissue from a 30-year-old woman. Each germ cell is capable, in theory, of producing a mature gamete. A single biopsy could suffice for all the IVF treatment cycles that a woman would ever need, as well as providing spare oocytes for donation to other patients and for research. This technology is, of course, only a dream at present, but it may be needed if some cancer patients banking ovarian tissue before undergoing sterilizing treatment are to keep their options open for future fertility (Gosden *et al*., 1999). Follicle culture could avoid the risk of transferring malignant cells, because the zona pellucida excludes cells apart from the fertilizing spermatozoon. This anterior technology for IVF would have a revolutionary impact on reproductive medicine, and there would be more applications for animal breeding and cloning technology because oocyte supplies are frequently limiting.

We close this article on a cautionary note. First, to emphasise the difficulty of the technology and, second, to point out that it carries greater theoretical risks than culturing embryos. During the first 2–3 days of post-fertilization development, embryos depend on molecules and organelles inherited from the egg, and the cytoplasm divides into daughter cells without differentiating until the late morula stage. The human oocyte, on the other hand, enlarges 100-fold in volume over a time-frame of several months (Gougeon, 1996), and its highly specialized character evolves during follicle growth from an undifferentiated germ cell. Epigenetic modifications of the oocyte's genome are notable because imprinting of sex-specific alleles occurs during oocyte growth (Obata *et al*., 1998). Moreover, culture conditions for mouse embryos impact on the monoallelic expression of the *H19* locus, where the paternal allele is normally hypermethylated and the maternal one is preferentially expressed (Doherty *et al*., 2000). Equally, there should be concern about the formation and integrity of imprints in oocytes growing and ripening *in vitro*, because defects could manifest as embryonic death or unhealthy offspring. Indeed, there are already indications that imprinting defects underlie large offspring syndrome in lambs and calves generated as embryos in culture with or without nuclear transfer (Young *et al*., 1998). What is more, the premature death and abnormalities of the one mouse ever produced from a primordial follicle *in vitro* serves as a warning (Eppig and O'Brien, 1996). These concerns demand vigilance, and so we should expect that in the years ahead, this technology will be used first as an aid for research before it is safe to be launched for clinical service.

Table II. Applications of follicular oocyte culture

1. Routine treatment of infertility with minimal or no gonadotrophic stimulation
2. Production of gametes after cryopreservation of ovarian tissue for patients undergoing sterilizing treatment
3. Conservation of rare breeds of animals/endangered species
4. Generation of oocytes for animal breeding technology and cloning
5. Research on oocyte biology and pathology

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