human reproduction update

Current achievements and future research directions in ovarian tissue culture, *in vitro* follicle development and transplantation: implications for fertility preservation

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BACKGROUND: Female cancer patients are offered 'banking' of gametes before starting fertility-threatening cancer therapy. Transplants of fresh and frozen ovarian tissue between healthy fertile and infertile women have demonstrated the utility of the tissue banked for restoration of endocrine and fertility function. Additional methods, like follicle culture and isolated follicle transplantation, are in development.

METHODS: Specialist reproductive medicine scientists and clinicians with complementary expertise in ovarian tissue culture and transplantation presented relevant published literature in their field of expertise and also unpublished promising data for discussion. As the major aims were to identify the current gaps prohibiting advancement, to share technical experience and to orient new research, contributors were allowed to provide their opinioned expert views on future research.

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RESULTS: Normal healthy children have been born in cancer survivors after orthotopic transplantation of their cryopreserved ovarian tissue. Longevity of the graft might be optimized by using new vitrification techniques and by promoting rapid revascularization of the graft. For the *in vitro* culture of follicles, a successive battery of culture methods including the use of defined media, growth factors and three-dimensional extracellular matrix support might overcome growth arrest of the follicles. Molecular methods and immunoassay can evaluate stage of maturation and guide adequate differentiation. Large animals, including non-human primates, are essential working models.

CONCLUSIONS: Experiments on ovarian tissue from non-human primate models and from consenting fertile and infertile patients benefit from a multidisciplinary approach. The new discipline of oncofertility requires professionalization, multidisciplinarity and mobilization of funding for basic and translational research.

Key words: fertility preservation / tissue culture / transplantation / oocyte / follicle

Introduction

The banking of ovarian tissue has become widespread in countries that have already well-established fertility centres (Jeruss and Woodruff, 2009). Some research programmes are exploring small adaptations of conventional cryopreservation methods applied specifically to ovarian tissue. Despite the fact that there has been a growing interest in banking ovarian tissue, with samples collected from several hundred patients, there are very few research initiatives on how to ensure the function of the thawed tissue once patients return with fertility treatment requests. Major advances in this technology have occurred in the past 6 months and a number of transplants in cohorts of infertility patients provide a rationale for banking cancer tissue and transplants (Silber *et al.*, 2005, 2008b; Silber and Gosden, 2007). Thus far, approximately 30 autotransplantations have led to the birth of eight healthy children, which is a very encouraging evolution of the technique (Donnez *et al.*, 2004; Meirow *et al.*, 2005; Demeestere *et al.*, 2007; Andersen *et al.*, 2008; Silber *et al.*, 2008b). The advantage of this technology is that the patient will have restoration of both endocrine and fertility function. The disadvantage is that some cancers have an elevated risk of metastatic spread to the ovary which may re-introduce aggressive cancer cells to the recent survivor of their disease. Thus, additional methods for use of the stored ovarian tissue are needed to minimize risk of cancer potentiation, whereas maximizing the likelihood of a take home baby.

In contrast, research efforts to establish efficient *in vitro* culture methods for growing follicles at various stages have lagged, primarily due to the inability to access primate (human and non-human) tissues. There were very few original research articles published in the last 2–3 years on this topic, which suggests that barriers to tissue acquisition, low levels of research funding and gaps in our knowledge about early follicle growth contribute to this phenomenon. Indeed, the number of institutes worldwide focusing on the culture of human tissue can be counted on one hand and addressing the barriers will significantly increase the pace and quality of research that is urgently needed to improve the future fertility of cancer patients (Jeruss and Woodruff, 2009). The most important need is for robust *in vitro* follicle culture methods as an alternative to homologous transplantation, as the risk for re-introducing cancer cells via the transplant can pose significant and undue risk to the patient. The growth of follicles *in vitro*, starting from a primordial follicle and ending in the production of an ovulatory follicle with a developmentally competent oocyte, has only been achieved in mice thus far. The mouse model demonstrates that long-term culture *in vitro* is compatible with healthy offspring (O'Brien *et al.*, 2003). Proactive experiments in mice using different *in vitro* culture methodology have shown that long-term culture of follicles does not harm the primary imprinting process (Anckaert *et al.*, 2009a). The mouse model has also been very valuable for understanding some basic principles of follicle assembly, initiation of follicle growth and the role of specific growth and differentiation factors (for reviews see Van den Hurk and Zhao, 2005; Picton *et al.*, 2008).

However, the physical and anatomical differences in the ovaries of humans, cows, sheep, pigs and mice make it very difficult to apply the successful culture method used for growing mouse primordial (Eppig and O'Brien, 1996) or primary follicles (Lenie *et al.*, 2004) to larger species (Smitz and Cortvrindt, 2002). Overcoming the growth arrest and follicle death observed in current *in vitro* culture systems requires a better comprehension of the physiology of follicle growth initiation, the interplay of important growth factors, the basic metabolic needs of early growing follicles and the physical environment of the follicle.

Due to the limited availability of human and primate ovarian tissue for research, follicle culture technology has progressed more quickly in some domestic animals, such as the cow, sheep, goat and pig, than in primates and humans. The data generated from these animal models are valuable for translational and clinical research, as human tissue is less accessible. Research in non-human primate models is also scarce, although it will be necessary in order to identify the optimal culture protocol for human follicles. Similarities in the physiology of folliculogenesis and in the aetiology of common gynaecological diseases in primates (D'Hooghe *et al.*, 2009) could justify their preferential use as a model to more rapidly advance the work done with human tissues.

Outside of their natural environment, follicles must cope with physical restrictions such as oxygen tension, temperature and uptake of nutrients. Such complex problems have pushed ovarian follicle culturists to seek help from tissue engineers. Furthermore expertise in basic physiology and biochemistry is of great value when designing and implementing artificial matrices and monitoring basic metabolite consumption from conditioned media. Some very promising initial data on the use of new matrices in human and non-human primate follicle culture are presented in this synopsis (Xu *et al.*, 2009a, b). Observations from the most recent experiments using this matrix have introduced new concepts in follicle biology; in particular, these studies have challenged the premise that hormones are the only factors that

control follicle development; rather, that the three-dimensional (3D) architecture of the oocyte and surrounding somatic cells are critical to the appropriate development of the oocyte. Further, this work shows that the physical environment of the ovary is a regulating force and this biomechanical signal can be pheno-copied by an appropriate support matrix (West-Farrell et al., 2009).

This manuscript brings together data and opinions from directors of well-established American and European research laboratories who are recognized leaders in their field. It gives a factual overview of where female fertility preservation and oncofertility research stands today and identifies challenges and new directions for the field in the near future.

Methods

Contributors to this article agreed to focus on one particular area of their expertise within the larger topics of either follicle culture/oocyte maturation or ovarian tissue transplantation. The main goal of this article was not to extensively review all existing literature on each topic, but rather to provide a concise overview of where the field stands today and the current opinion of experts in the field. Contributors were asked to pinpoint some relevant research areas for the near future and were encouraged to discuss any new unpublished data from their own research. The article aims to provide ESHRE and its readership with an unbiased view of where the field stands today and to indicate new directions that might advance the field.

All contributors have been performing research in the area of ovarian function and assisted reproductive technologies and have published extensively on their research. The panel members were representatives of European and American Academic Reproductive Research Centres and their work reflects current practise in their home countries. It was decided that the article would include rich and diverse expertise from scientists working with large mammalian models (cow, sheep, primate, cat) as the basis for developing translational and clinical methodologies, and in order to discuss the potential use of current technologies for the preservation of endangered animal species. All members of the panel had been invited by ESHRE's Task Force on Basic Reproductive Science.

Although it is recognized that the work on rodent species has, and is, playing a tremendously important role for our understanding of reproductive physiology, the organizers chose not to include experts working with this model as it was a pre-set goal of the panel to focus on particular problems with culture and transplantation of human ovarian tissue. Nevertheless, proper referencing of work performed with the mouse model and with certain knockout models was included in this article. Although the technologies described in this article are intended to be used on tissues and cells that will be cryopreserved, the panel preferred not to expand on cryopreservation or vitrification technologies, as most members of the panel are self-described users of the technology rather than experts in this research area. Where applicable, appropriate referencing of cryopreservation research was used.

Results

Ovarian tissue culture techniques

Models for ovarian cortical tissue culture: from mouse to large mammalian species
The production of one live mouse in 1996 from an oocyte grown entirely in vitro showed that oocyte development from the primordial follicle stage to the stage of developmental competence is possible in in vitro culture (Eppig and O'Brien, 1996). This inspired the development of methods for culturing primordial follicles from cattle and baboons (Wandji et al., 1996a, b; Braw-Tal and Yossefi, 1997; Wandji et al., 1997). Eppig and O'Brien used a two-step culture system, comprised of an 8-day culture of whole newborn mouse ovaries, with primordial follicles just beginning to form, followed by enzymatic dissociation of the ovary, and isolation and further culture of oocyte–granulosa cell complexes from the secondary follicles that had developed during the 8-day organ culture. Subsequent efforts improved the culture system and produced multiple, normal offspring (O'Brien et al., 2003). These techniques work well for mice because their follicles form during the first few days after birth, providing an ovary with a fairly uniform population of primordial follicles. In addition, the ovaries are small enough to be cultured intact, and soft enough to be dissociated enzymatically.

In contrast, in primates and most domestic species, follicles form over a number of weeks during fetal life, the ovaries are too large to be organ-cultured, and the stromal tissue is tough, making enzymatic dissociation without damage to oocytes difficult (Wandji et al., 1996a). To avoid these problems, an in vitro system was developed for culturing small pieces of ovarian cortex obtained from fetal bovine and baboon ovaries during the last third of gestation. Fetal ovarian cortex is rich in primordial follicles, since massive follicular attrition has yet to occur, and the tissue is much softer than adult ovarian tissue, facilitating dissection of the cortex from the inner medulla. Cortical pieces were cultured in Waymouth's MB 752/1 supplemented with antibiotics, pyruvic acid (25 mg/l) and ITS+ [6.25 μg insulin, 6.25 μg transferrin and 6.25 ng selenium plus 1.25 mg bovine serum albumin (BSA) and 5.35 μg linoleic acid/ml]. In this medium, primordial follicles of fetal baboons and fetal or adult cattle activate to become primary follicles within the first 2 days of culture and follicular and oocyte growth ensues (Wandji et al., 1996b, 1997; Braw-Tal and Yossefi, 1997). Initially this activation was considered to be 'spontaneous' because the same medium and culture conditions promoted an apparently normal percentage of follicles to be activated in the study by Eppig and O'Brien (1996). Because almost all the bovine and baboon follicles activated in medium with ITS+, this system is not appropriate for studying factors that might promote activation in vivo, but it was used to show that anti-Mullerian hormone (AMH) can inhibit activation and growth of primary follicles in bovine cortical pieces (Cushman and Fortune, 2003 and unpublished results). In more recent studies, culturing bovine cortical pieces in TS+ (i.e. ITS without insulin) maintained follicles at the primordial stage for at least 10 days, implicating insulin as an activator of primordial follicles in cattle. Culturing cortical pieces in TS+ has provided a new culture system that allows the testing of other putative follicle activators and use of this system has thus far suggested a role for kit ligand in follicle activation in cattle (Muruvi and Fortune, 2009).

Although bovine and baboon follicles activate readily in the cortical culture systems detailed above, very few primary follicles progress to the secondary follicle stage (i.e. follicles with a theca layer surrounding two or more layers of granulosa cells with no antral cavity). Attempts to stimulate the primary to secondary follicle transition by adding fetal bovine serum (FBS), follicle-stimulating hormone (FSH) or activin to the culture medium or by lowering the oxygen tension in the system detailed above were found not to be effective (Fortune et al., 1998, 1999, 2000; Gigli et al., 2006). These negative results

led to the development of an 'in ovo' culture system, in which cortical pieces from fetal cattle or baboons were grafted beneath the chorioallantoic membrane (CAM) of 6-day-old chick embryos, to test the hypothesis that they would become vascularized and that this would allow not only activation, but also development to the secondary stage. Although the grafts vascularized rapidly and remained healthy until their removal 10 days later, follicle activation did not occur (Cushman *et al*., 2002). Further transplantation studies with whole mouse ovaries provided evidence that the AMH secreted by the gonads of male and female chick embryos (Hutson *et al*., 1981; Teng, 1987; di Clemente *et al*., 1992) inhibits activation of mouse primordial follicles *in ovo*, since follicles in mouse ovaries lacking the AMH type II receptor activated *in ovo*, whereas follicles in wild type ovaries did not (Gigli *et al*., 2005). CAM-grafting of bovine cortical pieces after gonadectomy of the host chick embryo also allowed activation, providing indirect evidence that AMH is responsible for the lack of activation in bovine and baboon cortical pieces *in ovo* (Gigli *et al*., 2005). In the future, the *in ovo* model could be used to test putative stimulators of activation to determine which can overcome the inhibitory effect of AMH when applied to a CAM graft of ovarian tissue, but such experiments are technically difficult.

In summary, a new *in vitro* culture system for bovine ovarian cortical pieces has been developed that can maintain follicles in the primordial stage. The culture system can be used to identify follicle activators, and insulin and kit ligand have thus far been identified. *In vivo* activation is probably regulated by a balance between stimulatory and inhibitory factors impinging on an individual follicle and the *in vitro* culture system can also be used to test the effects of putative inhibitors, such as AMH, on the action of various stimulators. The *in vitro* system is not very effective at promoting development to the secondary follicle stage; however, an important goal is to determine how that might be achieved. Follicles in whole newborn mouse ovaries quickly reach the secondary stage under similar culture conditions, so it is important to study species that are good models for human follicular development, such as cattle and non-human primates. In the *in ovo* culture system, follicles are held at the primordial stage by circulating AMH and this system could be used to test various factors for their ability to overcome the inhibition by AMH and to stimulate the primary to secondary transition. The drawbacks of this culture system are that it is only practical to leave the grafts in place for about 10 days because of developmental changes to the CAM, and the techniques are difficult to master.

### Growing human oocytes from primordial follicles

The development of a culture system for human tissue has always been perceived as being problematic because of the prolonged period of follicle development required. Recent work from Telfer's lab (Telfer *et al*., 2008) has shown that human primordial follicles grow well within mechanically loosened cortical pieces, which contain growing follicles with the underlying stroma removed, and can develop to multilaminar pre-antral (secondary) stages within 6 days. The culture conditions in this new system differ from those described in previous studies (Hovatta *et al*., 1997, 1999) as no serum is present and no supporting matrix is used. Most of the underlying stromal tissue is removed so that the cultured pieces consist of predominantly ovarian cortex containing primordial and primary follicles. Multilaminar (pre-antral) follicles grown within human ovarian

cortical strips can be isolated and have the potential to grow to the antral stage of development if cultured individually within a total culture period of 10 days (Telfer *et al*., 2008). This time scale makes the complete *in vitro* development of oocytes from human tissue a practical and viable prospect. However, whether this altered growth rate affects subsequent oocyte development is a question that needs to be addressed.

It is assumed that complete follicle development from primordial to the pre-ovulatory stage in human takes up to 8 months and Gougeon (1986) calculated the time needed for a follicle to grow from the primary to the pre-ovulatory stage to be 84 days. However, there is no good evidence to show that this is a continuous period of growth; indeed, it is likely that follicles grow *in vivo* in a 'start-stop' manner in response to local influences. This hypothesis is supported by studies showing that advanced-stage follicles can arrest the growth of follicles at the pre-antral stage without compromising their subsequent ability to proceed further (Mizunuma *et al*., 1999). Studies on the underlying molecular mechanisms regulating the initiation of follicle development (Foxo3 and Pten knockout studies) have shown that despite global activation of follicles, the rate at which follicles develop differs (i.e. some initiated follicles take days to reach antral stages although others take weeks or even months; Castrillon *et al*., 2003; Reddy *et al*., 2008). Studies in which the rate of granulosa cell division has been altered *in vivo* (e.g. by deletion of Pten in murine granulosa cells), resulted in rapid follicular growth but had no effect on fertility (Fan *et al*., 2008). Therefore, it appears that oocyte development can be supported within a rapidly developing follicle. Indeed, this 'accelerated' growth has been demonstrated in other (non-human) culture systems and fully grown oocytes have been obtained (porcine: Wu *et al*., 2001; bovine: Telfer *et al*., 2000; Thomas *et al*., 2003a for review). The challenge now is to define the *in vitro* conditions that facilitate a rate of growth that supports normal oocyte development, and current work in Telfer's lab is focusing on this (Johnson *et al*., 2009; Telfer *et al*., 2009).

Cortical strip culture removes follicles from the *in vivo* endocrine and paracrine processes regulating growth rate; however, follicles will still be subject to the effect of follicle interactions and the influence of stromal cell factors. It is clear that tissue shape and stromal density are important factors that regulate follicle growth initiation *in vitro*, as solid cubes of cortical tissue show a lower rate of growth initiation (Hovatta *et al*., 1997). In contrast, when stromal cells are removed and the tissue is cultured as flattened 'sheets', the initiation rate is greater and follicles grow faster (Telfer *et al*., 2008). The physical environment of the follicles within the cortical tissue affects their response to stimulatory and inhibitory factors and therefore influences their ability to grow (McLaughlin and McIver, 2009).

Once follicle growth is initiated within the strip, the follicles can develop to multilaminar stages; at this point, the cortical strip environment becomes inhibitory to further growth. Therefore, strip culture cannot support optimal development of all stages, and a multi-step culture system is required to support complete development (Fig. 1). Pre-antral follicles can be mechanically isolated from the cortex culture system after 6 days and placed within an individual culture system for further development to antral stages (Telfer *et al*., 2008). Conditions to support development of isolated follicles have been defined in bovine systems (McCaffery *et al*., 2000; Thomas *et al*., 2001, 2003a, b; Walters *et al*., 2006; Thomas *et al*.,
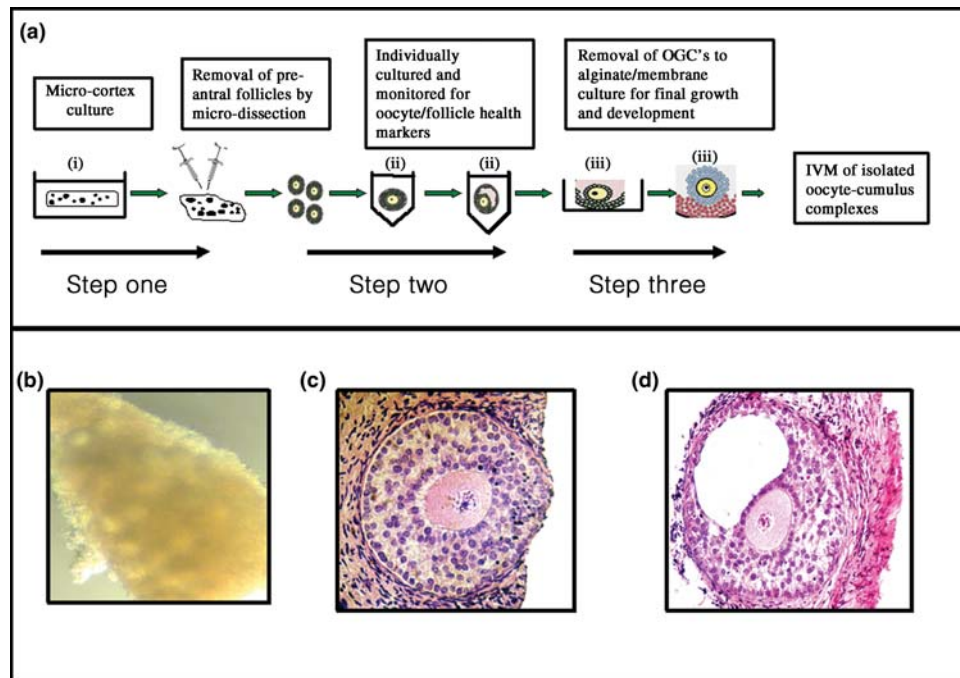
Figure 1 Multi-step culture system to support human follicle development *in vitro*. (**a**) Diagram showing steps involved in the culture of human primordial follicles to antral stages (i–ii) and removal of oocyte granulosa cell complexes (OGCs) for placement in alginate bead/membrane (iii) for further growth and development and subsequent IVM [as proposed by Telfer's lab (Telfer and McLaughlin, 2007)]. Human follicles can be developed from primordial (**b**) to pre-antral (**c**) and antral stages (**d**) in serum free medium (Telfer *et al.*, 2008).

2007) and this work formed the basis for optimising culture conditions for isolated human follicles (Telfer *et al.*, 2008). Key factors at this stage are activin A and FSH (Telfer *et al.*, 2008) and it will be important to determine the optimal timing and concentrations of their combined delivery during the culture period.

Further study is required to evaluate whether manipulating key signalling pathways such as PTEN and mTOR might be an original way to maximize *in vitro* yields of follicles from an ovarian tissue piece.

Essential hormones and growth factors for growing human follicles in vitro

Human ovarian follicle culture techniques have been developed since the early 1990s, with the ultimate goal of achieving mature oocytes from thawed tissue collected and frozen before initiation of fertility-threatening therapies, such as chemotherapy or radiation therapy. Research on the *in vitro* growth of follicles and maturation of oocytes has offered the additional opportunity to study the factors controlling human follicular development (for review, see Van Den Hurk and Zhao, 2005; Sadeu *et al.*, 2008). Effects of hormones and growth factors tested in *in vitro* follicle culture are shown in Table I.

Culture conditions. In the earliest studies, FBS or human serum-containing medium was used, but for safety reasons it was soon replaced by defined media substituted with human serum albumin (HSA) and a combination of insulin, selenium and transferrin (ITS). Many basal culture media were compared in the early studies, and since then, alpha minimal essential medium (MEM) has been used

predominantly. For clinical maturation cultures, a good manufacturing practices (GMP) grade culture medium is needed. A clinical grade keratinocyte medium was used by Sadeu and Smitz (2008). The human recombinant forms of hormones and growth factors should be used when available: a central repository of hormones and grow factors would facilitate the comparability of results from different laboratories. Factors that prevent apoptosis have also been added, such as ascorbic acid, cyclic GMP and cyclic AMP (Scott *et al.*, 2004a).

As the vast majority of oocytes exist within primordial follicles in ovarian cortical tissue, the culture setup should be designed to support growth initiation from this earliest follicle stage. As in animal experiments, the initiation of growth of follicles in cortical tissue is generally successful (Hovatta *et al.*, 1997). Isolation of the follicles after growth initiation is a possible strategy (Telfer *et al.*, 2008). Isolated follicle culture has been performed in small inserts with an extracellular cellular matrix (ECM) coating (human placental Matrigel) or without matrix, or isolated follicles may also be cultured on plastic in 24-well plates. For clinical purposes, clinical grade xeno-free, serum-free medium has to be used and follicle growth in this medium has been shown to be feasible (Scott *et al.*, 2004b).

Animal models, particularly the sheep and bovine models, have provided a basis for human follicle culture studies (Newton *et al.*, 1999; Gutierrez *et al.*, 2000). For setting up human ovarian tissue culture models, researchers have utilized various sources of tissue as listed in Table II. No difference in culture results has been observed between fresh or frozen-thawed tissue. Culture of ovarian tissue is a suitable way to evaluate the quality of the freezing and thawing

Table I Hormones and growth factors used in the culture of follicles from fresh and frozen-thawed human ovarian tissue.

Article	Hormone/growth factor	Fresh versus frozen	Stage in culture	Culture duration	Observed outcomes
Roy and Treacy (1993)	FSH, FCS	Fresh	Pre-antral to early antral	4 days	Increased growth, steroid production, antrum formation
Hovatta *et al.* (1997)	FSH, LH, insulin, human serum	Fresh and frozen	Primordial to early antral and secondary	15–21 days	Increased survival and growth
Abir *et al.* (1997)	FSH, LH, human serum	Fresh	Pre-antral to early antral	14 days	Improved survival
Roy and Kole (1998)	FSH, EGF, TGFβ, FCS	Fresh	Pre-antral	2 days	Growth increase by FSH and EGF
Wright *et al.* (1999)	FSH, ITS, HSA	Fresh	Primordial to pre-antral in cortex	10 days	Growth increase
Louhio *et al.* (2000)	Insulin, IGF-I, IGF-II, HSA	Fresh	Primordial to early pre-antral in cortex	21 days	Increased survival and growth
Hreinsson *et al.* (2002)	Rat recombinant GDF9, ITS, HSA	Fresh	Primordial to pre-antral in cortex	14 weeks	Increased development of secondary follicles and survival
Schmidt *et al.* (2005)	rhAMH, FSH, LH, testosterone, ITS, HSA	Frozen	Primordial to pre-antral within cortex	28 days	Increased initiation of growth and survival of the follicles
Carlsson *et al.* (2006a)	Kit ligand, FSH, ITS, anti c-kit antibody, HSA	Fresh	Primordial to early antral	28 days	Oocyte death after blocking Kit receptor
Carlsson *et al.* (2006b)	rhAMH, ITS, HSA	Fresh	Primordial in cortex	7 days	Inhibition of initiation of growth
Sadeu and Smitz (2008)	HSA	Frozen	Primordial to early antral in cortex	28 days	Expression of GDF9 and AMH
Telfer *et al.* (2008)	Activin A, ascorbic acid, HSA	Vitrified	Primordial in cortex, then isolated	Two-step culture: first 6 days in cortex, then 4 days as isolated follicles with activin A	Significant growth and maturation from primordial to antral stage
Aghajanova *et al.* (2009)	rhAMH, blocking antibody and receptor blocker, ITS, HSA	Fresh	Primordial to secondary	14 days	Increased development and survival with GDF9, blocking proved the role of endogenous GDF9
Garor and Abir (2009)	FGF2, FCS or HSA, FSH	Frozen	Primordial to early antral	28 days	Increased E2 production, no effect on follicle growth
Aghajanova *et al.* (2009)	HSA, ITS, TSH or T4	Fresh	Primordial and primary	3 days	No effect on follicles, cAMP or ERK phosphorylation

procedures. It remains unclear whether it is worthwhile to use ovarian tissue from women over 38 years of age and the same question remains as to the utility of ovarian tissue that had already been treated for 1–2 weeks by chemotherapy.

Evaluation of cultured follicle outcomes. Histological evaluation has always been the primary approach to assessing cultured follicles. Optimal fixation of the ovarian tissue can be achieved with Bouin's fixative, which fixes the oocytes without causing shrinkage and enables evaluation of the tissue and oocyte general morphology. Parameters of histological evaluation include: assessing follicular density per square mm of tissue; measuring the diameter of the oocyte and follicle and the thickness of the zona pellucida; counting the layers of granulosa cells; and noting the presence of theca cells. If immunohistochemistry (IHC) is intended, a portion of the tissue can be fixed in paraformaldehyde. Electron microscopy gives a more accurate evaluation of the tissue after freezing and culture, but is

extremely laborious. Functionality can be tested using cell proliferation stains by IHC. Hormone production, particularly estradiol (E2), can be measured in the culture medium. Follicle development can now be evaluated at the molecular level with gene expression analyses, quantitative real-time polymerase chain reaction (PCR) and microarrays.

Conclusions: growth factors and hormones for follicle culture. FSH, insulin, activin A and growth and differentiation factor 9 (GDF9) promote follicular development and survival. AMH inhibits the initiation of growth of primordial follicles. Multi-step culture followed by *in vitro* maturation (IVM) of the cumulus-enclosed oocyte will be needed to obtain fertilizable oocytes (Fig. 1).

The importance of extracellular matrix in in vitro *follicle culture*
Introduction to ECM composition and its regulation. In discussions of factors that drive ovarian follicle development, hormones and growth factors acting by endocrine, paracrine and autocrine

Table II Sources of human ovarian tissue for evaluation of growth factors.

Source	Fresh/frozen	Advantages	Disadvantages
Oophorectomy specimens, Abir et al. (1997)	Fresh and frozen	Large samples may be obtained	Advanced age of the donor, often few follicles
Part of the patient's frozen tissue, Xu et al. (2009c)	Fresh or frozen	Often good numbers of follicles obtained	Reduces the tissue left for clinical use
Transsexuals' tissue, Van den Broecke et al. (2001)	Fresh or frozen	Many follicles obtained	Androgen-treated
Donated biopsy specimens in tubal ligation or other laparoscopies, Scott et al. (2004a, b)	Fresh or frozen	Relatively easy to obtain	Varying numbers of follicles, infertile women, no ideal patient group
Tissue from donors who have died, Schmidt et al. (2005)	Frozen	Often good numbers of follicles obtained	Rare samples
Donated biopsy specimens from Caesarean sections, Carlsson et al. (2006a, b; 2009)	Fresh or frozen	Frequently performed operation, fertile women, good numbers of follicles obtained	Careful bloodless collection procedure required, small pieces

mechanisms are typically the first to be mentioned; however, the ECM within and around the follicle regulate numerous cellular processes associated with follicle development (Irving-Rodgers and Rodgers, 2006; Irving-Rodgers et al., 2009). The ECM is composed of a variety of molecules, which can include collagens, laminin, fibronectin, proteoglycans and polysaccharides, though the characterization of the ECM within the ovary is incomplete (for review Berkholtz et al., 2006a). In the mouse, collagen I is present throughout the ovary, with higher concentrations in the ovarian surface epithelium (OSE) and follicular compartments (for review Berkholtz et al., 2006b). Collagen IV is abundant in the theca cell compartment with low-level expression in the stroma and granulosa cells. Fibronectin is present in the stroma and theca cell compartment and increased throughout follicle development, although its presence in the granulosa cell compartment is decreased. Laminin is localized primarily to the theca cell compartment, with a defined ring at the exterior of the follicular granulosa cells marking the basement membrane. Low levels of laminin are also apparent in the stroma and granulosa cell compartment. The ECM influences a variety of cellular processes, such as cell morphology, aggregation and communication, proliferation, survival and steroidogenesis. Additionally, the turnover and remodelling of the ECM during folliculogenesis suggests that autocrine and paracrine signalling by secreted ECM molecules can regulate the transitions from one stage of development to another and ultimately the follicle fate (Kreeger et al., 2003, 2006).

Principles for choosing an ECM. The complete recapitulation of follicle growth and oocyte maturation *in vitro* is a goal that has profound implications both for discovery-based reproductive science and for patient health. The process of follicle development is remarkably similar between small and large animals having short and long reproductive cycles resulting in one or more mature oocytes. Follicles isolated from mouse, rat, sheep, cow, pig, non-human primate and human ovaries begin at a modest size, at roughly 35 µm in diameter for primordial follicles and 120 µm in diameter for two-layer secondary structures (for review: Hsueh et al., 2000; McGee and Hsueh, 2000). The final follicle diameter around ovulation is 700 µm in the mouse and around 15–22 mm in the human. The mature oocyte

from these mammalian species is also of similar size ranging from 82 to 110 µm. FSH-dependent follicle maturation from the secondary stage to a mature follicle takes 72 h in rodents (estrus cycle) and 10–12 days from the small antral stage to the pre-ovulatory stage in the human (follicular phase of the menstrual cycle) with remarkably similar patterns of steroid and peptide hormone regulation. The question is: what are the *in vitro* 'design principles' that are most important to the development of *in vitro* follicle maturation systems and how these principles might be altered depending on the species from which the immature follicle is isolated?

A major goal of *in vitro* follicle culture in follice culture systems centred around the use of ECM is to maintain the connections between the oocyte and the somatic cells. We know that the oocyte depends on the surrounding granulosa cells for metabolic regulation, pH balance and other small molecules. Thus, the first design principle for this type of approach is the use of a follicle culture matrix that provides sufficient support to ensure that the somatic cells do not 'abandon' the oocyte. A second design principle is that the material supporting the follicle should be permeable to the media, permitting hormones to access the follicle structure and factors secreted by the follicle to be released. A final design principle is that the surrounding material should be amenable to modification, including its rigidity and ECM characteristics. The material we elected to work with is alginate, a product of seaweed, which has each of these characteristics and has been used successfully to produce live, healthy offspring in mice born from *in vitro* matured follicles and to support growth of non-human primate and human follicles with the coordinate maturation of the oocyte (Xu et al., 2009a, b).

Alginate, a translational culture system. The development of culture systems for follicle development should account for the functions of the ECM, which are categorized as structural and biochemical (i.e. signalling). The ECM provides mechanical support to the follicle, which is essential for maintaining cell–cell contacts and paracrine signalling between the cellular compartments. Natural (e.g. collagen) and synthetic (e.g. alginate) hydrogels have been employed to maintain the 3D architecture, and have been referred to as 3D culture systems to differentiate them from traditional 2D culture on flat tissue

culture surface. Three-dimensional culture systems may have an advantage in overcoming the difficulty of maintaining follicular structure in early-stage follicles or in follicles from large species. In the case of human follicles, culture in a 3D collagen gel promoted follicle growth, whereas follicles on a 2D collagen coated surface maintained their original size (Abir et al., 2001). In the alginate system, however, secondary mouse, monkey and human follicles have been able to grow (Pangas et al., 2003), produce fluid-filled antral cavities (Xu et al., 2006a) and produce meiotically competent oocytes (Kreeger et al., 2005, 2006; Xu et al., 2009a, b), which were successfully fertilized and implanted to yield multiple live births of healthy mouse pups (Xu et al., 2006b). The mechanical properties of the 3D matrix have a significant role in supporting follicle development (Xu et al., 2006b). The hydrogel must have sufficient rigidity to maintain the 3D structure of the follicle, yet must also allow for expansion due to oocyte growth, granulosa cell proliferation and antrum formation (Xu et al., 2006a, b; West et al., 2007; West-Farrell et al., 2009). Forces are generated by follicles in 3D matrices because the growing follicle exerts an outward force on its surrounding matrix. The matrix also exerts a force on the follicle, which may affect actin organization, likely activating one or more of these mechanoresponsive pathways. In a 3D system, cell–cell and cell–matrix interactions are maintained; therefore, a mechanical force applied to the cells at the exterior of the follicle will be transmitted to all cells within the follicle to influence development and maturation.

The signals necessary for follicle development must be presented within the context of the biomaterial. Diffusible signals (i.e. hormones, growth factors) can be added to the culture media, and are generally able to transport through the hydrogel. The ECM regulates cellular function and differentiation within the follicle *in vivo*, processes which are disrupted during follicle isolation. Providing a particular ECM in an *in vitro* system allows for an analytical approach to cell and tissue culture and could therefore lead to a better understanding of factors determining the quality of the follicle in culture (Kong et al., 2003; West et al., 2007). Natural materials, such as collagen, are themselves composed of ECM proteins and provide innate interactions with encapsulated cells. Other matrices, such as alginate, do not possess these innate interactions and must be modified to better mimic the ECM *in vivo*. In the case of follicle development, the incorporation of ECM proteins (collagen I and IV, fibronectin and laminin) or the arginine–glycine–aspartic acid (RGD) peptide into an alginate matrix affected follicle growth and differentiation, as well as oocyte quality (Kreeger et al., 2006). The ECM in the 3D matrix may be useful in maintaining a gradient of paracrine growth factors essential to polarized growth. Note that the adhesivity of the hydrogel must be balanced to limit cell migration from the follicle and into the hydrogel, which can disrupt follicle structure and its growth potential. Taken together, determining the structural and biochemical design of the ECM for each stage of follicle environment will facilitate the growth of follicles during 3D culture.

Experience with alginate in mouse and human follicle culture. Secondary follicles have been isolated from mouse, rat (Heise et al., 2005), nonhuman primate (Xu et al., 2009a) and human (Amorim et al., 2009) ovaries and grown in alginate beads. The hypothesis was that the microvilli and transzonal projections between the oocyte and somatic cells would be maintained if follicles were cultured in alginate

(Albertini et al., 2001). Indeed, it has been shown that these membrane extensions are maintained in 3D alginate cultures (Pangas et al., 2003). The reason why high quality cultured oocytes from the mouse and healthy germinal vesicle-containing oocytes from the human are obtained is due to the maintenance of these vital corridors of communication between the two cellular compartments in the alginate matrix. Heise et al. (2005) demonstrated the presence of connexions between granulosa cells and oocytes in isolated pre-antral rat follicles cultured in alginate.

New horizons offered by 3D follicle culture systems: Since mouse follicles can be routinely cultured in this 3D *in vitro* system, it becomes possible to address some basic questions about follicle differentiation in relation to oocyte quality. First, what are the genes that control follicle selection and activation? What are the signalling pathways that exist between the oocyte and somatic cells during terminal follicle development? What signals antrum formation? What is the earliest follicle stage at which oocytes attain nuclear and cytoplasmic maturation? What is the role of the somatic cell in follicle development? What controls hormone secretion and what controls the rate of hormone production? What factors are essential for oocyte development? Very few tissues are able to mature independent of a vascular supply; indeed, the follicle is the only known example of autonomous tissue function. Many new and important discoveries in follicle biology are made possible by a follicle culture system that faithfully recapitulates the main feature of the ovary that contributes to follicle growth, its actual structure as an organ. Simultaneously, the alginate system is being applied for human follicle culture (Xu et al., 2009c).

Until recently the work has focused on the secondary follicles stage, which is in less abundance in adult ovaries but in sufficient prevalence to be useful to the cancer patient (Xu et al., 2009c). On the basis of these studies we now know that follicles can be grown autonomous of the surrounding stroma if sufficient physical support is provided to them. Whether primordial or primary follicles are independent of their cellular environment is a question that is currently being addressed. It is known that the oocyte and somatic cells need constant communication in order for the oocyte to mature (Albertini et al., 2001). Finally, these studies show that a rationally designed tissue engineered approach to follicle maturation is providing new insights into the physiology of the follicle which can be applied to the cancer patient setting. Current challenges are to understand better the role of the gonadotrophins, steroid and peptide hormones in follicle growth and the integration of hormones with the physical environment. These fundamental studies will be of great impact for the development of a robust system for human follicle development and oocyte maturation.

Experience with alginate in rhesus monkey follicle culture. Deficits in our understanding of the processes and factors controlling the onset, progression and maturation of the human follicle limit our ability to provide ovary-based options for preserving fertility, including for women experiencing cancer therapies. Various culture systems mentioned earlier in this review and, notably, recent advances that encapsulate individual follicles within a permeable matrix (Xu et al., 2006a, b), have been shown to support mouse follicle development *in vitro* and achieve oocyte maturation required for fertilization and production of live offspring. Whether such culture systems will support

similar development of primate follicles has received little attention. Pre-antral follicles from the marmoset, a New World monkey, developed to antral follicles and yielded mature oocytes during two dimensional culture (Nayudu et al., 2003). Old World macaques, such as the rhesus monkey (Macaca mulatta), are a valuable model for studying the primate ovary, as many of the characteristics and regulation of cyclic ovarian function are comparable to those in women. For example, rhesus macaques are monovular and display menstrual cycles of a month duration, with a 2-week follicular phase. Rhesus monkeys provide an opportunity to address issues specific to primate follicle development relative to rodent follicles, e.g. large differences in the duration of follicle growth to obtain mature ovulatory follicles and possible differences in hormonal and metabolic requirements. For obvious practical and ethical reasons, it is possible to address issues in macaques that can only be indirectly studied with human tissue or in an associative manner.

Therefore, studies were initiated (Zelinski et al., 2008; Xu et al., 2009a, b) to determine if secondary follicles isolated from adult female rhesus monkeys, encapsulated in alginate hydrogels and cultured individually in αMEM media supplemented with BSA, bovine fetuin, insulin, transferrin and selenium, would survive and grow in vitro. Initial studies addressed three questions: (i) does the stage of the ovarian cycle at follicle isolation impact follicle development in vitro? (ii) does the rigidity of the alginate hydrogel influence follicle survival and growth? and (iii) do follicles require in vitro exposure to pituitary gonadotropic hormones?

Our results indicate that secondary follicles isolated from prepubertal monkeys gave a better survival than from adult monkeys. Surprisingly the follicles from the early follicular phase of the menstrual cycle have a higher survival rate than those collected during the luteal phase. This difference correlated positively, and may be related to, the larger mean diameter of secondary follicles in the early follicular versus luteal phase (270 versus 191 μm). Follicles survived and grew in two hydrogel conditions (0.5 and 0.25% alginate). The alginate scaffold concentration affected follicle survival rates and the proliferation and differentiation of theca and granulosa cells. The higher tested alginate concentrations such as 1.5 and 1.0% hindered follicle development (Xu et al., 2006a, b). Follicles did not survive in culture in the absence of FSH (recombinant human FSH; Organon). However, follicle survival up to 30 days in vitro did not differ between follicles exposed to FSH alone or FSH plus recombinant human luteinizing hormone (LH; Merck Serono). Follicles exposed to either FSH alone or FSH plus LH demonstrated continuous growth (increased follicular diameter), and approached 1 mm diameter with an extracellular compartment reminiscent of the antrum within 30 days of culture (Fig. 2). Unexpectedly, follicles cultured with FSH alone had a greater mean diameter than those cultured with FSH and LH (750 versus 550 μm; $P < 0.05$). To consider the steroidogenic function of these encapsulated follicles during growth in vitro, media samples were assayed for androstenedione (A), E2 and progesterone (P) content. Steroid levels were low during the first week of culture, but increased 5–10-fold ($P < 0.05$) by 30 days of culture in the presence of FSH alone or FSH plus LH. Addition of LH tended to increase E and A
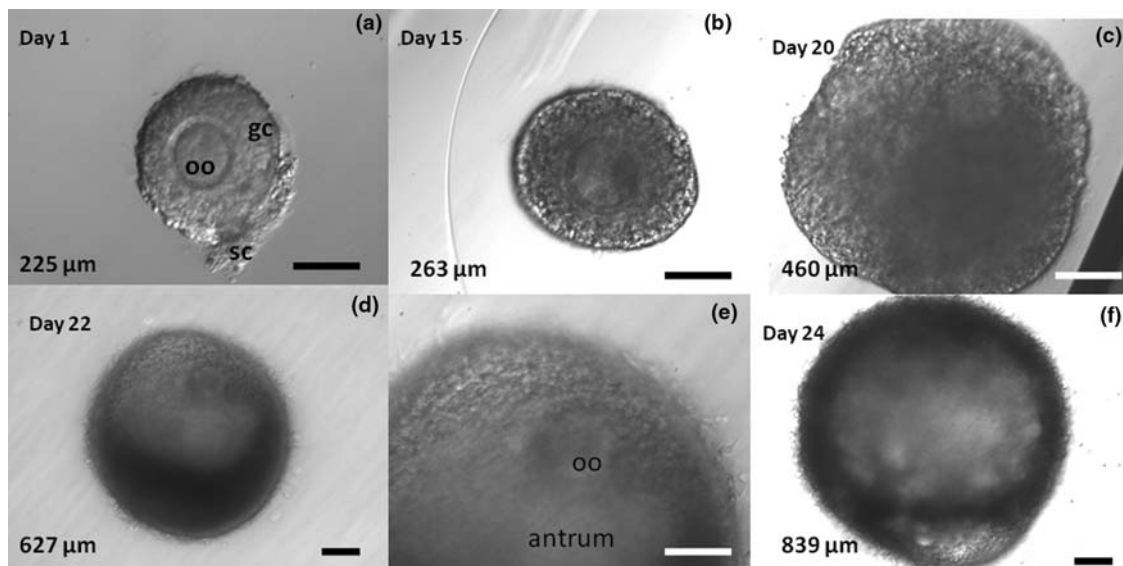
Figure 2 Rhesus monkey secondary follicles grown in alginate hydrogel over 3 weeks. Photomicrographs illustrate the morphological and size changes in follicles during the 24-day culture period. (**a**) Upon initial isolation and encapsulation in alginate, the oocyte (oo) is centrally located within a single layer of granulosa cells (gc). A few stromal cells (sc) remain with the follicle. (**b**) After 15 days in culture, the follicle has increased almost 20% in diameter. (**c**) By Day 20, the follicle has more than doubled its original size and a developing antrum can be observed. The oocyte is no longer centrally located within the follicle. (**d, e**) By Day 22, the follicle has further increased in diameter and a well-defined antrum can be seen. (**f**) On Day 24, the follicle has increased in diameter almost 4-fold, yet retains its spherical morphology and antral cavity, both of which are supported by the alginate hydrogel. Some follicles (≈30–50% of those surviving depending upon the culture conditions) achieve diameters typical of a small antral follicle in the macaque by the fourth week in vitro. Upper left = day of culture; lower left = follicle diameter; lower right bars = 100 μm. Images on Days 1, 15, and 20 (top row) and of the antrum and oocyte on Day 22 (middle, bottom row) were taken at 20×. Images on Days 22 and 24 were taken at 10×. Images from M. Zelinski.

levels at Day 30 ($P = 0.07$), and decrease P levels ($P < 0.05$). Thus, an alginate hydrogel matrix supports the 3D structure of individual secondary follicles from macaques, and permits follicle growth to the small antral stage within 30 days of culture. FSH, but not LH, is required for follicle survival and growth, and increased steroidogenesis is associated with antral development between Days 20 and 30 of culture. LH increased E and A and reduced P production in the follicle. The effects of LH activity observed were compatible with data addressing the needs for LH activity in human *in vitro* fertilization (IVF) showing a similar effect upon A/E balance and reduced pre-ovulatory progesterone production (Smitz et al., 2007).

Ongoing studies are addressing additional questions: (i) will this 3D follicle culture system support the growth of follicles from young (prepubertal, ≈4–5 years of age), and older (≥12 years of age) adult macaques; (ii) does sequential exposure to FSH, followed by FSH plus LH, promote further follicular growth; (iii) are paracrine factors, such as AMH, produced by macaque follicles during 3D culture; (iv) what is the effect of the FSH/LH balance upon oocyte developmental capacity?; (v) what is the role of theca during *in vitro* follicle growth on antrum formation and on steroid production; (vi) what oxygen tension should be applied taking into account the fast effects of oxidative stress and (vii) how could one evaluate oocyte maturity in a non-invasive manner?

Initial evidence suggests that a greater percentage of secondary follicles from prepubertal animals survive for 30 days *in vitro* compared with those from adult monkeys (77 versus 59%; $P < 0.05$). At collection, follicle diameters did not differ between prepubertal and adult animals, but by Day 30, the diameters of growing follicles were larger if obtained from adults compared with prepubertal animals. However, an antrum developed by Day 25 regardless of age. Notably, compared with FSH alone, addition of LH after Day 30 culture increased the diameter of follicles from prepubertal animals ($P < 0.05$), but did not affect those from adults (Zelinski et al., 2008). Further analyses indicated that surviving follicles could be assigned to three categories according to diameters achieved by Day 40: no growth (<300 μm); slow growth (301–600 μm) and fast growth (>600 μm). Although follicle diameters in these groups did not differ during early culture (Days 1–15), AMH levels in the media after 1 week were higher for fast growth follicles compared with no growth follicles ($P < 0.05$), and after 2 weeks were higher in fast growth versus slow growth follicles ($P < 0.05$; Bernuci et al., 2008). AMH levels peaked at 2 weeks in the presence of FSH and declined to low levels by 4 weeks of culture ($P < 0.05$). Thus, AMH is produced by macaque pre-antral follicles and is associated with more rapid follicle growth *in vitro*.

This model could become valuable for understanding the role of gonadotrophins, steroids and peptide hormones in follicle development and its link to oocyte developmental capacity. Once achieved, this knowledge might be of benefit to human follicle culture and could be combined with other assisted reproductive technologies to restore fertility to women, including cancer survivors.

Transplantation of ovarian tissue

Follicle isolation and selection

For patients at risk of ovarian metastatic involvement (e.g. leukemia, neuroblastoma, breast cancer), alternatives to ovarian tissue fragment reimplantation need to be considered. However, ovarian tissue fragments can be reimplanted when the risk of cancerous involvement of the ovary is absent or minimal, and when autografting would thus present little or no danger. Indeed, studies evaluating the safety of cryopreserved human ovarian tissue reimplantation have suggested that ovarian tissue grafting in Hodgkin's disease may be considered safe (Meirow et al., 1998; Kim et al., 2001; Seshadri et al., 2006). However, in leukemia, malignant cells are present in the bloodstream and are at risk of being transferred along with the cryopreserved tissue (Jahnukainen et al., 2001). For this reason, some alternatives have been under development. Ovarian tissue could be *in vitro* cultured for subsequent *in vitro* follicle culture or used for isolation of pre-antral follicles from the surrounding stroma that may potentially contain cancerous cells. These healthy follicles could then be safely retransplanted to patients (Dolmans et al., 2007). Obtaining individual isolated follicles has been studied in mice (Gosden, 1990; Eppig, 1994), sheep (Amorim et al., 2006), goats (Lucci et al., 1999), cows (Figueiredo et al., 1993), pigs (Greenwald and Moor, 1989) and humans (Oktay et al., 1997; Abir et al., 1999; Dolmans et al., 2006), but only one study, in mice, demonstrated fertility restoration after transplantation of isolated follicles (Carroll and Gosden, 1993).

Since human ovarian cortex is extremely tough to handle, this rules out mechanical isolation to separate intact primordial follicles. Enzymatic tissue digestion using collagenase is currently used for human ovarian follicle isolation (Oktay et al., 1997; Abir et al., 1999). However, since collagenase may contain high levels of endotoxins, which could severely impair culture and grafting outcomes, a protocol to digest human ovarian cortex using a purified endotoxin-poor enzyme (Liberase) has been described to isolate primordial and primary follicles from ovarian cortical tissue (Dolmans et al., 2006). Recovery of the isolated follicles is then facilitated by passing the suspension through a Ficoll gradient prior to microscopic examination (Martinez-Madrid et al., 2004). This technique allows isolation of large numbers of primordial follicles, with high viability, unaltered morphology and a well preserved ultrastructure (Dolmans et al., 2006). The viability of these isolated human follicles has been tested by vital fluorescent dyes (live/dead assays) and *in vivo* xenografting in a fibrin clot to the nude mouse ovarian bursa. Their survival and growth after transplantation was evidenced by their morphologically normal structure, their progression to more advanced follicular stages, and their positive staining for human Ki-67 1 week post-transplantation (Dolmans et al., 2007). Furthermore, recent data indicate that human isolated follicles grafted for 6 months can reach antral stages, demonstrating the capacity of oocytes to survive and grow further (Dolmans et al., 2008). Isolation of human follicles could be done directly after tissue harvesting on fresh ovarian tissue, as it has been done in experimental studies so far. These isolated follicles could then be cryopreserved and banked until use, as described previously with sheep follicles (Amorim et al., 2007). Although the easiest and most logical way to select is probably to cryopreserve the ovarian biopsy, in strips or fragments, and thaw them the day of isolation and grafting, it needs to be investigated whether there could be an advantage in cryopreserving follicles over tissue. The cryoprotectant permeation might be more efficacious through a cell suspension than through tissue blocks.

As this approach has been shown to successfully restore fertility in mice (Carroll and Gosden, 1993), optimization of follicle isolation and recovery protocols will allow us to consider its development for

humans, in cases where the risk of reintroducing malignant cells into cured cancer patients by ovarian tissue autografting cannot be excluded. Further focus should be given to the development of sensitive and rapid detection methods for cancer detection in ovarian stroma. Reseeding purified follicle fractions, free of cancer cells, requires new surgical approaches to increase efficiency of graft recovery. New artificial matrices might play a role in this.

Transplantation of ovarian tissue to orthotopic sites: case studies
Cryopreservation of ovarian tissue has proved to be a valuable option for fertility preservation in young patients at risk of premature ovarian failure. It is the only option available for prepubertal girls, as well as women who cannot delay the start of chemotherapy and/or undergo ovarian stimulation with embryo cryopreservation (Gosden et al., 1994a; Donnez and Bassil, 1998; Dolmans et al., 2005; Donnez et al., 2006a).

Approximately 30 cases of autotransplantation of cryopreserved ovarian tissue to orthotopic sites have been reported worldwide and eight live births have been published to date (Donnez et al., 2004; Meirow et al., 2005; Demeestere et al., 2007; Andersen et al., 2008; Silber et al., 2008b; Piver et al., 2009). Donnez et al. reported the first successful transplantation of frozen-thawed ovarian tissue resulting in a pregnancy and live birth in 2004. From his series of seven published autotransplantations of cryopreserved ovarian tissue, all patients recovered ovarian function within a period of 4–5 months post-grafting (Donnez et al., 2008). Two techniques were successfully used to reimplant frozen-thawed ovarian tissue in an orthotopic site: either in a specially created window on the peritoneum (two steps technique, Donnez et al., 2004) or on the remaining ovary (Donnez et al., 2006a). When using large tissue strips (10 × 4 mm), the ovarian fragments can be sutured onto the remaining ovary after removal of the native cortex. In case of small cubes (2 × 2 mm), they can be placed on the decorticated medulla and held in place with an absorbable adhesion barrier, itself sutured to the remaining cortex of the native ovary (Donnez et al., 2008).

Although several live births have been reported after cryopreserved ovarian tissue transplantation, some important questions remain. Experimental studies show a considerable decrease in the number of primordial follicles in grafted tissue. This may be due to hypoxia and the delay that occurs before reimplanted cortical tissue becomes revascularized. The loss of primordial follicles in frozen-thawed ovarian tissue after transplantation is estimated to be 50–65% in some studies (Baird et al., 1999; Nisolle et al., 2000) and >90% in one study (Aubard, 1999; Nottola et al., 2008). On the other hand, the primordial follicle pool also appears to be depleted by follicular activation after grafting. Experimental studies demonstrated a significant increase in the proportion of growing follicles, from <20% in ovaries before grafting to >70% after grafting in sheep (Baird et al., 2004), monkeys (Gougeon and Busso, 2000) and humans (Dolmans et al., 2007). According to Baird et al. (2004), this massive recruitment of primordial follicles, which also occurs in cultured fragments, suggests the removal of some inhibitory mechanisms regulating FSH. Following autotransplantation, the number of antral follicles and inhibin A secretion are reduced, resulting in raised basal levels of FSH that may account for the massive recruitment, although the early stage of folliculogenesis can occur in the absence of FSH and LH. Other factors such as significantly reduced

circulating AMH levels, might contribute to the underlying rapid depletion similar to that described in the AMH knock-out model (Lie Fong et al., 2008, 2009).

A crucial question is how to prevent this premature activation of the transplanted follicular pool and whether active angiogenesis can be induced to accelerate the process of neovascularization in grafted tissue. Research is actually conducted to evaluate, by oximetry and electro-paramagnetic resonance, the reoxygenation and revascularization of human ovarian xenografts in order to have objective parameters to monitor the revascularization process (Van Eyck et al., 2009a, b). Indeed, these studies have shown that revascularization of avascular grafts are only complete on Day 10 post-grafting, and that the follicle pool remains anoxic for ∼5 days.

Another important question is how to improve oocyte quality in frozen-thawed transplanted tissue. A study recently presented by Dolmans et al. (2009) reported a higher risk of empty follicles, abnormal or immature oocytes and low embryo transfer rates in patients with cryopreserved autotransplanted ovarian tissue. In this IVF series, 21 oocyte retrievals yielded 16 oocytes, 10 of which were in metaphase II (MII). Five MII oocytes successfully fertilized with normal subsequent embryo development, but no pregnancy occurred. Women who have undergone grafting of frozen-thawed ovarian tissue have reduced ovarian reserves and elevated FSH concentrations, and there is frequently a failure to recover oocytes in the aspirates from follicles from these patients. This might arise from the inappropriate activation of granulosa cells and oocytes by increased FSH concentrations, which could provoke an asynchronous maturation. Another hypothesis to explain the prevalence of empty follicles is that the oocytes have been damaged by the cryopreservation, thawing and transplantation procedures.

Finally, the standard method for human ovarian tissue cryopreservation is slow programmed freezing, using HSA-containing medium with either propanediol, dimethylsulphoxide or ethylene glycol as a cryoprotectant, combined or not with sucrose. Vitrification is still at the experimental stage for human tissue, but appears to yield promising results (Kagawa et al., 2009; Keros et al., 2009).

In conclusion, live births obtained after transplantation of frozen-thawed ovarian tissue in humans give hope to young cancer patients, but the techniques can be improved at several levels. Research programmes need to determine the ideal fragment size for cryopreservation and transplantation, whether active angiogenesis can be induced to accelerate the process of neovascularization in grafted tissue, and whether oocyte quality is affected by the freezing and grafting procedures applied.

Techniques for safeguarding fertility in rare animal species

All felid species in the wild are near extinction
Aside from their application to fertility preservation in humans, the studies described above raise the hope that advances in culture and cryopreservation of ovarian tissue may provide a valuable tool for assisted reproduction in rare and endangered species.

Of the 37 felid species, all but the domestic cat are threatened worldwide and face extinction in all or part of their native habitats (CITES-appendices, www.cites.org). In some species, a greater number of individuals are present in zoo facilities than in their

natural habitats. Advanced reproductive techniques (e.g. IVF and embryo transfer) are undoubtedly powerful tools for safeguarding rare and endangered felid species (Wildt *et al.*, 1992; Jewgenow *et al.*, 1997; Pope *et al.*, 2006), but the most critically limiting factor to their application is the lack of mature and fertilizable female germ cells (Jewgenow and Paris, 2006). This problem could be addressed by accessing the large source of oocytes available within the pre-antral and primordial follicles in the ovarian cortex and culturing them to maturity *in vitro*. In this respect, ovarian tissue from wild cats that must be spayed or that either die suddenly or be euthanized for medical reasons, may serve as an oocyte pool for rescuing the valuable genetic potential of females and subsequently aid in the propagation of these species.

The domestic cat as a model for in vitro *culture and transplantation*
The full-term culture of primordial follicles to a mature stage and the birth of viable offspring after IVF and embryo transfer have not yet been achieved in a felid species (Jewgenow and Pitra, 1993; Jewgenow, 1996, 1998; Jewgenow and Paris, 2006). Therefore, transplantation of ovarian tissue in a xenogeneic host is discussed as an long-term alternative method for follicle growth and development (Jewgenow and Paris, 2006). Gosden *et al.* (1994b) first reported the xenotransplantation of fresh ovarian tissue of domestic cat in SCID mice. Transplant survival as well as the development of antral follicles was documented up to 9 months after grafting at autopsy. More recently, Bosch *et al.* (2004) showed antral follicle stages following xenografting of cortex fragments from frozen/thawed domestic cat ovaries in SCID mice by post-mortem histology 2 months after grafting. Fassbender *et al.* (2007) demonstrated that fresh ovarian cortex from domestic cats survived xenotransplantation in athymic nude rats and that high resolution ultrasonography provided a reliable method to follow xenograft survival and follicular development within the grafts. It turned out that this technique is suitable to assess the efficiency of hormonal treatments and to narrow the optimal time frame for oocyte retrieval in living recipients. The success of all ovarian tissue transplantation, independent of the tissue status (fresh or frozen) is highly dependent on the time point of oocyte retrieval to ensure maturation and subsequent IVF trails, particularly if the donor ovary originated from a rare and valuable animal. Unfortunately, the existing protocols for ovarian cryopreservation and grafting are species-specific and most protocols only apply to just a few species (humans and laboratory animals). A network of research in a broad diversity of species is required to develop and apply cryobanking technology to felid conservation. Development of cryopreservation protocols specifically for felids will impact a number of other threatened cat species worldwide, and a concerted, integrated effort to conserve critically endangered European carnivores is urgently needed.

Discussion

Surveys of cancer survivors have identified an increased risk of emotional distress in those who become infertile because of their treatment (Berkowitz K., 2003; Partridge *et al.*, 2004; Carter *et al.*, 2005). Loss of fertility potential is something difficult to understand for young children, but is potentially traumatic to them as adults. It is considered good clinical practise that all cancer patients of reproductive age be informed about the possibility of treatment-related infertility.

Professional societies recommend that oocyte and ovarian tissue cryopreservation be performed only in centres with demonstrated expertise using only IRB-approved protocols [British Fertility Society (BFS): http://www.britishfertilitysociety.org.uk; European Society of Human Reproduction and Embryology (ESHRE): http://www.eshre.com; American Society for Reproductive Medicine (ASRM): http://www.asrm.org; American Society for Clinical Oncologists (ASCO): http://www.asco.org). Guidance documents stress that this field of medicine is still considered to be experimental. Although the practice of oocyte and ovarian tissue cryopreservation has been spreading rapidly, there has been almost no research effort directed towards future use of this tissue.

In view of the rapid introduction of fertility preservation in clinical practice, organization of the field in a more professional way has become a high priority.

As cancer treatments evolve rapidly, so should current understanding on their impact on female fertility, so that clinicians can provide their patients with the most accurate information about their risk of infertility related to cancer treatment (Jeruss and Woodruff, 2009). Most of the available literature documenting cancer treatment-related infertility risks reports rates of azoospermia and amenorrhea, which are surrogate markers of limited practical value. There are better biochemical markers of fertility, such as AMH and non-invasive imaging techniques (ultrasound, Doppler, magnetic resonance) that allow a more precise evaluation of the damage to the reproductive organs (ovary, testicle, uterus; van Beek *et al.*, 2007). A follow-up of all cancer patients that receive potentially gonadotoxic drugs with these investigative tools must be recommended in order to gather precise information on the following questions: what cancers directly affect fertility? Which cancer drugs should be avoided in girls and premenopausal women because of their gonadal toxicity? Which combination of drugs should be banned for negative effects on the ovary and uterus?

In most countries, patients are followed up by oncology but receive no expert advice from a fertility specialist as part of the patient management protocol. Newer techniques, such as AMH measurements, would allow a better appreciation of post-treatment fertility potential (Lie Fong *et al.*, 2008, 2009). Providing adequate support and funding for regional or national 'fertility preservation banks' would allow better organization and coordination of patient follow-up.

Restoration of fertility after cancer by transplantation techniques

It was estimated that ∼20% of grafted ovarian tissue cases led to childbirth in the first reported series worldwide, which is an encouraging figure. However, there is still a lot to do towards improving the techniques for grafting of ovarian tissue or isolated primordial follicles. More specifically, new approaches to the revascularization of the grafted tissue are needed, with the goal of reducing the number of follicles that die within the first hours after transplantation by ischemia. As the clinical benefit of transplanted ovarian cortical pieces is directly proportional to their longevity, useful information can be gained from recent work by Silber and Gosden (2007) and by Silber *et al.* (2008a, b). Ovarian cortex transplantation between monozygotic twin sisters, where ovarian tissue transplants had not been frozen before grafting of the thinned cortex using a microsurgical technique provide evidence

that it is now technically possible to restore ovarian function within 4 months post-surgery, to obtain pregnancies within a median period of 15 months and for healthy babies to be born. Two to four years post-transplantation of on average 30% of cortical tissue from the donated ovary, the acceptors were still menstruating and some patients had even had a second child from the graft.

Pioneering work on transplantation of previously cryopreserved human ovarian tissue in a heterotopic site such as a subcutaneous location (Oktay et al., 2001; Callejo et al., 2001; Oktay et al., 2003) or in the space between the rectus sheath and rectus muscle (Kim et al., 2009) has proven that follicular function can be recovered and this research has led to the development of several technical improvements. However, until now only a few intact oocytes could be recovered over several 'cycles' from the developed follicles in the transplant (Oktay et al., 2004; Kim et al., 2009). These oocytes fertilized normally and a few embryos developed in vitro. Of the few patients that had consented to undergo heterotopic grafting, none have become pregnant.

Many other challenges are remaining in the field of transplantation, to determine the optimal site for grafting ovarian tissue or its isolated follicles (Donnez et al., 2006b; Demeestere et al., 2009), and to restore ovarian function after transplantation of frozen-thawed whole ovary (Jeremias et al., 2002; Salle et al., 2002; Arav et al., 2005; Courbiere et al., 2005; Imhof et al., 2006; Martinez-Madrid et al., 2007).

Until today, only a few patients ($\pm 1\%$) who have been declared cancer-free have come back with a request for reimplantation of ovarian tissue, so our experience is still very limited. The amount of data on the quality of oocytes after the combined procedures of cryo-preservation and transplantation is lacking. Basic knowledge regarding neovascularization, reinnervation and paracrine and autocrine regulation is still very scarce, and basic research efforts in these areas requires support in order to overcome some of the challenges that limit the success of the transplantation.

Advances and challenges with tissue and follicle culture techniques

Significant progress has been made in improving the methods for in vitro growth and maturation of ovarian follicles from a range of species including rodents, ruminants, primates and humans (Xu et al., 2006b; Nogueira et al., 2008; Picton et al., 2008; Sadeu et al., 2008; Xu et al., 2009a, b). A strategy to maximize the reproductive potential of fresh and cryopreserved human ovarian tissue used in follicle culture strategies must be orientated around the activation and sustained in vitro growth of primordial, primary or secondary follicles, as these follicles are all in the human ovarian cortex. In this context, although long-term ruminant and primate follicle culture systems are a better model for human follicle culture than shorter term rodent follicle culture systems, ultimately the best model for the therapeutic derivation of fertile human oocytes is a human follicle culture system. Human follicle culture systems must work equally effectively in both fresh and cryopreserved ovarian tissues (Newton et al., 1999; Muruvi et al., 2005, 2009; Xu et al., 2009b).

Progress in the development of systems for the derivation of mature human oocytes from immature follicles in vitro is hampered by the very limited availability of accurate biological information on the genes and growth factors that initiatal follicle growth and support follicle and oocyte development to maturity in vivo in humans (Picton, 2001; Xu et al., 2006a; West et al., 2007). A non-limitative list of current knowledge gaps on basic biological mechanisms related to in vitro oocyte growth is shown in Table III. Making significant progress in

Table III Current gaps and opportunities in follicle and oocyte *in vitro* culture.

Component	Controversy
Follicle harvest strategy	Isolated follicle culture versus in-situ or *in ovo* culture systems
Follicle attachment system	Use of adherent versus non-adherent culture systems
Follicle growth strategy	Use of linear (2D) versus spherical (3D) culture systems
Culture matrix	Inclusion versus exclusion of matrix and/or scaffolds to support 3D culture and antrum formation
Culture system continuity	Use of consistent system for all stages of follicle growth development versus sequential, follicle phase-dependent systems
Base media	Optimization of base media and buffering systems to suit species and culture strategy
Oxygen tension	Use of high (20%) versus low (5–6%) oxygen tensions
Media supplementation	Use of serum-based versus defined serum-free media
Developmental timeframes	Use of accelerated versus protracted growth systems
Growth additives	Supplementation of media with pharmacological versus physiological doses of growth additives
Hormonal additives	Supplementation of growth media with FSH and/or LH and estrogen and/or androgens
Metabolic requirements	Use of continuous versus sequential systems that modify the culture environment to meet the changing metabolic requirements of follicles and oocytes during *in vitro* growth and maturation
Culture starting and end points	Optimal initial and terminal size for oocytes and follicles at the start and end of follicle culture, species dependent
End-point measures	Agreement on best end-point measures, including morphology, viability assays, follicle and oocyte dimensions, growth rates, hormone secretion, cell signalling, molecular markers, IVM, fertilization, embryo production and live births
Normality testing	Agreement on how and what markers to measure to definitively test the normality, methylation and epigenetic programming, fertility and developmental competence of *in vitro* cultured oocytes and the embryos they produce

understanding which factors regulate the early stages of follicle development requires the availability of costly high-tech infrastructure, including laser capture microdissection, quantitative PCR, confocal microscopy and DNA microarrays. It is important that the consequences of extended follicle culture and the use of assisted reproductive technologies on key programming events during oogenesis and embryogenesis can be apprehended (Pesty *et al.*, 2007; Huntriss and Picton, 2008; Anckaert *et al.*, 2009a, b). Research also needs to evaluate the metabolic requirements that support acceptable rates of somatic and oocyte growth *in vitro* and how these demands change as oocyte and follicle development progress (Harris *et al.*, 2007, 2009; Harris and Picton, 2007). Ultimately, the information gained from this fundamental research must be applied to the biological and practical problems associated with human follicle culture.

Typical biopsies obtained for research are often from women over 35 years with gynecological pathologies and these tissues are inhomogeneous due to age-related follicle depletion. It is difficult to obtain cortical ovarian tissue from young patients for experimentation. Active recruitment of young and fertile tissue donors (e.g. at the time of Caesarean section) should be encouraged and the spare tissues and cells from IVF laboratories should be collected and properly stored for use in research (Schubert *et al.*, 2008). Other possible sources of research material for culture are human fetal ovarian tissue (therapeutic abortion material obtained from prenatal diagnostic units) and ovarian tissue from consenting cancer patients (Sadeu *et al.*, 2006). This material can be obtained via collaboration with a prenatal diagnosis unit and through operative laparoscopists.

Today, a number of different culture systems have been developed that have been partially or fully optimized for a range of different animal models (Picton *et al.*, 2008). Although some systems are more advanced than others, no one system is fully optimized for the complete *in vitro* growth and maturation of human follicles and oocytes. The variety in system development is vital at this stage in the evolution of human follicle culture as each of the different published systems have strengths and weaknesses that may be usefully exploited to meet the challenges of human follicle culture. A good example of this is the recent development of the alginate systems detailed in this review that have been shown to be an effective vehicle to support the three dimensional growth of pre-antral to antral staged murine follicles with the production of live offspring (Xu *et al.*, 2006b; West *et al.*, 2007). In contrast, a physiological, serum-free culture system has also been shown to be highly effective in supporting antral cavity formation and the production of mature, fertile ovine follicles and oocytes in the absence of any ECM support (Newton *et al.*, 1999). It is likely that the great majority of the advances made in follicle culture with small animals will not prove to be robust enough to be scaled-up to meet the challenges of extended human follicle growth *in vitro*. At the time of writing, many fundamental biological approaches (Table III) are being scrutinized. For example, there is no clear agreement between follicle culture practitioners within or across species on the efficacy and safety of serum-based culture systems supplemented with pharmacological levels of growth additives (Picton *et al.*, 2008) or of the more challenging, but consistent, serum-free culture systems supplemented with low doses of additives (Newton *et al.*, 1999), based on the physiological requirements to support somatic cell interactions and differentiation (Campbell *et al.*, 1996; Picton *et al.*, 1999a, b) and gamete maturation *in vitro* (Wynn *et al.*, 1998; Nogueira *et al.*, 2003, 2006; Sutton *et al.*, 2003; Gilchrist and Thompson, 2007; Romero and Smitz, 2008, 2009) in a number of species.

Challenges to fertility preservation research funding in Europe

Studies on early embryogenesis in humans should be allowed as this is the only way to reliably evaluate current fertility preservation techniques. Spare oocytes from human IVF/ICSI practises should be canalized to a dedicated research laboratory to make optimal use of this rare material. In light of the few human oocytes available and the associated ethical considerations, difficult molecular techniques may be used to evaluate genetic and epigenetic damage on well-validated animal models, including mice and larger animals such as the cow, sheep, goat or pig.

A major hurdle which remains to be tackled in the context of human follicle culture is the need for comprehensive testing of human oocytes derived by *in vitro* growth and maturation. The molecular and epigenetic programming and chromosomal health of *in vitro* derived human MII oocytes, as well as the fertility of these gametes and evaluation of all aspects of the health, molecular and genetic normality and developmental competence of the embryos they produce, must be tested to quantify the risks of long-term culture. The testing of the health of human oocytes and the embryos they produce is fraught with technical and ethical challenges that must be overcome before *in vitro* derived human gametes can be used safely to treat patients. Until further extensive research has been conducted, the potential of *in vitro* oocyte growth and maturation technologies remains to be realized.

A template for the European program is the U.S.-based Oncofertility Consortium, which includes experts from Europe on its advisory board and is involved in all aspects of fertility preservation research from the fundamentals of follicle maturation to the ethics, legal and economic complexities facing the patients and providers. The development of a sister-EU based Oncofertility Consortium will provide worldwide strength by bringing experts together to solve common problems and reduce the time to development of strong technologies. Indeed, the first step in this process has occurred with the ESHRE and ASRM liaison board agreeing to support the first ever meeting on the topic of Oncofertility at the World Health Organization (WHO) to be held September 2010 at Northwestern University in Chicago. This unification of purpose will advance the pace and quality of research in a significant manner and ensure that the patients who are storing their tissues today will have an opportunity to use them in the future.

Development of a central European database on 'assisted reproduction after cancer' and a Web site with the addresses of the Centres with expertise in the field should be accessible to all professional bodies and patients from the European Community. A European equivalent to the US Web site 'www.myoncofertility.com', which is sponsored by the NIH, should be organized on a multinational level. ESHRE, as the largest European Society fostering advances in the field assisted reproduction, will need to take the initiative in driving a multidisciplinary multinational collaboration via its Task Forces in 'Basic Science in Reproduction' and 'Fertility Preservation' and involve most of its Special Interest Groups (www.eshre.com).

Conclusion

In short, we have convened to discuss a matter of urgency for young women facing a life-preserving but fertility-threatening cancer treatment. We have identified the opportunities and the gaps in our field both at the basic and translational levels. We have clear ways to tackle the problems and take advantage of the opportunities. Central to this program is the strong collaboration of a global team of investigators and clinicians working on the maturation of the ovarian follicle. This unprecedented team has convened at an unprecedented time—more and more young cancer patients are surviving their disease. Our work will ensure that not only do we learn more about follicle development, but we provide appropriate options to these people who are banking their ovaries on the notion that we will succeed in our work.

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