Focus on Fertility Preservation

The *in vitro* growth and maturation of follicles

H M Picton, S E Harris, W Muruvi and E L Chambers

Reproduction and Early Development Research Group, The Light Laboratories, Leeds Institute of Genetics, Health and Therapeutics, University of Leeds, Clarendon Way, Leeds LS2 9JT, UK

Correspondence should be addressed to H M Picton; Email: h.m.picton@leeds.ac.uk

Abstract

The development of technologies to grow oocytes from the most abundant primordial follicles to maturity *in vitro* holds many attractions for clinical practice, animal production technology and research. The production of fertile oocytes and live offspring has been achieved in mice following the long-term culture of oocytes in primordial follicles from both fresh and cryopreserved ovarian tissue. In contrast, in non-rodent species advances in follicle culture are centred on the growth of isolated preantral follicles. As a functional unit, mammalian preantral follicles are well-suited to culture but primordial and primary follicles do not grow well after isolation from the ovarian stroma. The current challenges for follicle culture are numerous and include: optimisation of culture media and the tailoring of culture environments to match the physiological needs of the cell *in vivo*; the maintenance of cell–cell communication and signalling during culture; and the evaluation of the epigenetic status, genetic health and fertility of *in vitro* derived mature oocytes. In large animals and humans, the complete *in vitro* growth and maturation of oocytes is only likely to be achieved following the development of a multistage strategy that closely mimics the ovary *in vivo*. In this approach, primordial follicle growth will be initiated *in situ* by the culture of ovarian cortex. Isolated preantral follicles will then be grown to antral stages before steroidogenic function is induced in the somatic cells. Finally, cytoplasmic and nuclear maturation will be induced in the *in vitro* derived oocytes with the production of fertile metaphase II gametes.

Reproduction (2008) 136 703–715

Introduction

The development of technologies to grow and mature oocytes from the most abundant primordial follicles hold many attractions for assisted conception, in humans and in animal production technology. The relatively small size, undifferentiated status and large numbers of primordial follicles present in mammalian ovaries make them prime candidates for use in association with ovarian cryopreservation for the preservation of endangered species and the genetic manipulation of domestic species. The combined technologies of ovarian tissue cryopreservation and *in vitro* growth (IVG) and *in vitro* maturation (IVM) of oocytes could therefore be used to help young patients to preserve their fertility through the storage of their own germ cells before abdominal surgery or aggressive chemo- and/or radiotherapy partially or completely destroys their follicular reserve (Picton *et al.* 2000). Finally, the development of whole follicle culture systems can be used as a research tool to investigate the biology and toxicology of oogenesis in both animals and humans. Research in rodents has provided proof of the potential value of follicle culture as a source of fertile oocytes; however, the advances made using murine IVG/IVM systems have proved difficult to replicate in large animals and humans. In these species, the normal follicular growth span from the primordial to

Graafian follicle stages is very protracted and the changes in the trophic requirements of the cells and their cellular interactions as well as the sheer increase in bulk as the follicular antrum forms present major challenges to follicle culture practitioners. This review will therefore provide insight into the biological landmarks that occur during oocyte and follicle growth *in vivo* and apply this knowledge to the development of strategies for the IVG and maturation of oocytes in animals and humans.

Overview of follicle and oocyte development *in vivo*

In all mammalian species, follicle and oocyte development follow a characteristic sequence of events that commences with the establishment of the ovary shortly after conception and terminates with the ovulation of a fertile, metaphase II oocyte. Throughout the lengthy preantral phase of mammalian folliculogenesis, the development of the oocyte is dependent on, and is concurrent with that of the follicular granulosa cell (GC) layers (Diaz *et al.* 2007). Effective communication between the different follicular cell types is achieved via homologous and heterologous gap junctional contacts (Picton *et al.* 2007). Indeed, the cross talk between the somatic GCs and the oocyte is vital to sustain oocyte growth because the major

source of nutrients for the gamete is the somatic compartment (Harris & Picton 2007). During follicle growth, oocyte volume and diameter increases as a result of the accumulation of water, ions, carbohydrates and lipids (Fair *et al.* 1997) such that, in mice for example, there is a 150 fold increase in oocyte volume between primordial and preovulatory oocytes (Pan *et al.* 2005). The morphological changes and developmental time frames associated with follicle and oocyte development are well characterised across the species (Peters *et al.* 1975, Gougeon 1996, McNatty *et al.* 1999) and antral cavity formation commonly occurs when follicular diameters of between 180–250 µm are reached. By this stage, the majority of oocyte growth has been completed. In contrast, species specific differences have been recorded in a number of parameters including: i) the overall timeframe for completion of folliculogenesis and oogenesis; ii) the size of the ovulatory follicles and mature oocytes; and iii) differences in the nature, concentrations and effects of the putative growth factors that moderate follicle and oocyte production *in vivo*. These differences are highly relevant when developing systems that support the complete IVG and IVM of follicles and oocytes.

Recent research has revealed that oocyte secreted factors regulate the initiation of primordial follicle growth (see Picton 2001 for review) and moderate the known trophic actions of the gonadotrophins FSH and LH on preantral and antral follicle growth (Knight & Glister 2006, McNatty *et al.* 2007, Webb & Campbell 2007). A plethora of putative regulators of folliculogenesis *in vivo* have been identified, some examples of these factors are shown in Fig. 1. The tyrosine kinase receptor Kit and the two different isoforms of its ligand, kit ligand (KL), have been localised to oocytes and GCs and have been shown to promote oocyte

growth and maintenance of meiotic arrest in response to FSH receptor (FSHR) levels. While low concentrations of FSH promote oocyte growth by increasing KL-2 expression and by reducing the ratio of KL-1/KL-2, high concentrations of FSH enhance follicle development but impair oocyte growth (Thomas & Vanderhyden 2007). Other regulators of follicle growth include epidermal growth factor (EGF; Qu *et al.* 2000) and its receptor; activin (Hulshof *et al.* 1997, Telfer *et al.* 2008); basic fibroblastic growth factor (bFGF; Shikone *et al.* 1992); members of the insulin like growth factor (IGF) family and their binding proteins (Thomas *et al.* 2007), transforming growth factor-β (TGFB) superfamily members (Knight & Glister 2006) including somatic derived anti-Müllerian hormone (AMH), oocyte derived growth differentiation factor-9 (GDF9; McGrath *et al.* 1995, Dong *et al.* 1996); and the bone morphogenetic proteins (BMPs) especially BMP4, BMP7 and BMP15 (Shimasaki *et al.* 1999, Otsuka *et al.* 2001). Other factors such as retinoblastoma protein (RB1) may also be important (Bukovsky *et al.* 1995). Changes in follicle morphology and cell number, together with the stage specific follicular responsiveness to the growth factors detailed here and the development of steroidogenic capacity can be used as functional markers to confirm the normality of follicle development *in vitro*.

Oocyte developmental competence is progressively acquired following a coordinated series of structural and functional changes in the gamete and surrounding cumulus cells. However, it is not until the final days of follicular development that oocytes acquire the capacity to undergo meiotic progression to metaphase II. The redistribution of the cortical granules and changes in mitochondrial number, activity and distribution occur during the terminal stages of oocyte maturation *in vivo*

Figure 1 Diagrammatic representation of the regulators and developmental time frames of mammalian follicle and oocyte development *in vivo*. Examples of key growth regulators of somatic and oocyte origin are shown. Follicle sizes and growth rates are approximations for each species. + indicates putative growth stimulators; – indicates growth inhibitors; all abbreviations are defined in the text. The figures were collated from data published in a number of different sources as detailed in the text including Gosden & Telfer (1987), Gougeon (1996), McNatty *et al.* (1999, 2007), Van den Hurk *et al.* (2000) and Thomas *et al.* (2003).

(Wilding *et al.* 2001, Van Blerkom *et al.* 2002). Cytoplasmic and nuclear maturation of the oocyte is further characterised by a cascade of molecular events and check points which include changes in the transcription and translation of RNA, DNA replication and repair, chromosome condensation, spindle formation and development of the mechanisms for sperm head penetration (Swain & Smith 2007). Oocyte maturation *in vivo* is triggered by the preovulatory surge of gonadotrophins and particularly by high levels of LH. The LH driven follicular cascade results in a shift in steroid production by GCs from predominately oestrogen to a progestogenic environment and a decrease in intracellular cAMP in the oocyte, which is induced at least in part by the loss of junctional contacts between the cumulus cells and oocyte (Mehlmann 2005, Sela-Abramovich *et al.* 2006). Activation of the molecular pathways leading to the production of hyaluronic acid by the cumulus cells occurs in response to the LH surge leads to the mucification and expansion of the cumulus (Schoenfelder & Einspanier 2003). The loss of junctional contacts between the oocyte and somatic cells in turn triggers the resumption of meiosis in mature oocytes as it further reduces cAMP concentrations (Vaccari *et al.* 2008), leads to deactivation of cAMP dependent protein kinase A and reduces the inhibitory influences of purines on the maintenance of meiotic arrest (Swain & Smith 2007). All the molecular and cellular events which occur during oocyte maturation *in vivo* must be mimicked *in vitro* to produce fertile gametes.

Follicle and oocyte growth and development *in vitro*

The avascular follicular unit is relatively well-suited to culture environments. With the exception of oocytes from antral follicles that typically only need a short period of manipulation and maturation in culture, oocytes from small antral follicles and all preantral stages of follicle development require extended periods of growth *in vitro* to acquire developmental competence. It is vital therefore, that a detailed knowledge of biology of follicle and oocyte development *in vivo* is used to inform the development of strategies for the growth of follicles and oocytes *in vitro*. The success of IVG and IVM systems will be influenced by the nature of the tissue, the culture environment and the choice of endpoint(s) selected. The latter can be the production of antral or Graafian follicles, metaphase II oocytes, cleavage stage embryos or blastocysts (Fig. 2).

The follicle culture environment *in vitro*

A number of different culture systems have been developed to grow follicles from different species. There is some commonality among these systems but there are also many species-specific differences. In all IVG protocols, it is vital to optimise: i) the supply of nutrients, electrolytes, antioxidants, amino acids, energy substrates, vitamins

Figure 2 Summary of the critical determinants of the success of *in vitro* growth and maturation systems for mammalian oocytes. The success of the culture strategy will be influenced by the endpoint selected. These end points can be the production of antral or Graafian follicles, metaphase II oocytes, cleavage stage embryos or blastocysts.

and growth factors as these components of the culture environment are essential to sustain viability and growth; and ii) the removal of all waste products such as ammonia that may accumulate, as these waste secretions may be detrimental to growth. A variety of base media have been used for IVG of follicles from different species, these include: minimum essential medium (Eppig & Schroeder 1989, Spears *et al.* 1994, Cortvrindt *et al.* 1996, Jewgenow 1998, Newton *et al.* 1999, Picton *et al.* 1999b); Waymouth medium (Eppig & O'Brien 1996, Muruvi *et al.* 2005); and McCoy's 5a medium (Telfer *et al.* 2008). The base media used for follicle culture is supplemented with different culture additives including: antibiotics/antimycotics; commercial preparations of insulin, transferrin and selenium (Wright *et al.* 1999); physiologically relevant levels of insulin, IGF1 and IGF2 (Newton *et al.* 1999, Picton *et al.* 1999b, Louhio *et al.* 2000); GDF9 (Hreinsson *et al.* 2002); activin (Telfer *et al.* 2008); and 8-bromo cAMP and guanosine monophosphate as apoptosis inhibitors (Scott *et al.* 2004b, Zhang *et al.* 2004). The efficacy of these additives in supporting follicle growth and survival depends on the culture system itself, the presence or absence of other factors including serum, growth factor concentration and the duration of exposure.

An important consideration when developing a culture medium for the IVG and maturation of follicles is the type and concentration of energy substrate included in the base medium. Basic research has shown that glucose is a common energy source for almost all types of animal cells; however, over the last few decades, it has become clear that mature oocytes of many mammalian species, including mice (Biggers *et al.* 1967, Leese & Barton 1984, Harris *et al.* 2007, 2008), cows (Gandolfi *et al.* 1998)

and humans (Roberts *et al.* 2002, Harris & Picton 2007) preferentially metabolise pyruvate and have little capacity for the metabolism of glucose. Laboratory research has also shown that mouse oocytes have a high capacity for the consumption of pyruvate and oxygen at all stages of follicle development (Harris *et al.* 2008) while glucose consumption is below detection levels (Biggers *et al.* 1967, Eppig 1976, Harris *et al.* 2007). As a consequence of this evidence, glucose would be preferentially utilised by the somatic cells as the primary energy substrate in IVG media supporting follicle growth, whereas IVM media should be supplemented with pyruvate to support the energy needs of the maturing oocyte.

The inclusion of serum from homologous or heterologous sources in IVG and IVM media has ignited considerable debate. Serum contains proteins, amino acids, carbohydrates, trace elements, hormones, growth factors and extracellular matrix components which promote cell adhesion and proliferation. Extracellular matrix has been shown to moderate follicle survival in organ culture (Hovatta *et al.* 1999, Scott *et al.* 2004a). Serum acts as a source of albumin, which balances the osmolality and scavenges potentially harmful molecules and metal ions that can act as a source of free oxygen radicals. Serum also acts as a source of precursors for steroid biosynthesis (Hulshof *et al.* 1995). However, the inclusion of serum frequently blunts the response of proliferating cells to physiological doses of growth factors and it can act as a source of transmissible pathogens and viruses such as hepatitis B and C and bovine viral diarrhoea virus. Finally, the addition of serum in long-term cultures has been shown to lead to epigenetic modifications and foetal overgrowth syndromes (for example see Young *et al.* 1998, van Wagtenonk-de Leeuw *et al.* 2000). If serum is omitted from culture media, it is necessary to supplement the media with purified serum albumen or synthetic serum substitutes as a carrier molecule. Comparisons of the efficacy of IVG serum-based versus serum-free culture systems have been conducted in mice. Eppig *et al.* (1998) compared oocyte-GC complex development in culture medium supplemented with either foetal calf serum (FCS) or bovine serum albumin (BSA). More oocytes cultured with FCS cleaved after fertilisation and were competent to develop to the blastocyst stage than those cultured in BSA alone. In contrast, we and other authors have shown that FCS increased oocyte extrusion and follicle degeneration in isolated ovine follicles (for example Newton *et al.* 1999). Similarly, the replacement of FCS with BSA increased the percentage of morphologically intact preantral cat follicles by up to 30% (Jewgenow 1998).

There is a clear need to optimise the pH, temperature and oxygen tension of the culture environment in order to maximise the potential of *in vitro* derived oocytes (Ye *et al.* 2007). For example, although murine ovarian tissue is exposed to plasma oxygen levels of about 5% *in vivo*, mouse follicles grown *in vitro* in 5% oxygen had a higher frequency of mature oocytes with incorrectly aligned

chromosomes and many died prematurely when compared with follicles cultured in 20% oxygen (Hu *et al.* 2001). Similarly, sheep follicles grown in 5% oxygen had reduced antral cavity formation and increased lactate and glucose consumption when grown under hypoxic conditions of 5% oxygen relative to sized matched counterparts that were grown in 20% oxygen (Jin *et al.* 2007). Additionally, the temperature of the media used for tissue transportation and holding prior to follicle harvest must be optimised to minimise apoptosis (Schmidt *et al.* 2003, Lucci *et al.* 2004).

Follicle culture practitioners need to consider the direct effects of long-term culture and sub-optimal culture environments on the epigenetic information and methylation status of key imprinted genes in oocytes (Huntriss & Picton 2008). This issue is of particular relevance to IVG and maturation strategies as epigenetic information that is essential for normal development is acquired during gametogenesis and a growing body of evidence suggests that assisted reproductive technologies, and especially those involving extended culture and invasive micromanipulations may affect these complex processes and lead to disorders of genomic imprinting (Huntriss & Picton 2008). Imprinted genes including the maternally expressed *H19* gene, *IGF2* and also epigenetic regulators such as members of the DNA methyltransferases family *DNMT1* and *DNMT3A* have been shown to be aberrantly expressed following assisted reproduction technologies (Fernandez-Gonzalez *et al.* 2004, Sagirkaya *et al.* 2007). Analysis of the DNA methylation status of differentially methylated regions of the imprinted genes *H19*, paternally expressed gene 1/mesoderm-specific transcript (*Peg1/Mest*) and *Igf2r* in fully grown murine, germinal vesicle- staged oocytes derived *in vitro* from preantral follicles compared with *in vivo* grown counterparts indicated a loss of methylation at the *Igf2r* and *Mest/Peg1* loci, as well as a gain of methylation of *H19* (Kerjean *et al.* 2003). Furthermore, demethylation of the *Peg1/Mest* gene differentially methylated region has been observed in murine oocytes after extended culture (Imamura *et al.* 2005) suggesting that maternal imprints may be susceptible to demethylation during follicle culture. Further confirmation of this observation is provided following the detection of abnormal hypermethylation at the *H19* locus in human oocytes matured *in vitro* (Borghol *et al.* 2006). Culture induced epigenetics disruption has been associated with the additions to FCS to different media (Khosla *et al.* 2001, Fernandez-Gonzalez *et al.* 2004). Although the exact mechanisms of imprinting disruption following extended culture are unclear, it is likely that cellular stress induced by suboptimal culture environments is highly relevant with the latter being manifest by the accumulation of NH_3 in spent culture media (Lane & Gardner 2003). It is, however, important to note that there is a high degree of discordance between the imprinting status of genes in humans and other species (Morison *et al.* 2005) and there is variation in the regulation of epigenetic information including DNA

methylation during preimplantation development between mammalian species (Beaujean *et al.* 2004, Fulka *et al.* 2004, Vassena *et al.* 2005). Consequently, it is vital that epigenetic health checks are conducted on all oocytes derived following IVG and IVM before these tissues can be used in a clinical setting.

Methods for harvesting follicles for culture

Different follicle isolation methods have developed for preantral follicle culture. Follicle yield is dependent on the species of interest and on the reproductive age of the ovarian tissue as follicles are more numerous and the isolation method tends to be easier in neonatal and prepubertal ovaries than in adult ovaries (Figueiredo *et al.* 1993, Carámbula *et al.* 1999). Mechanical isolation methods using fine gauge needles or forceps have been used to isolate follicles from mice (Boland *et al.* 1993, Spears *et al.* 1994, Cortvrindt *et al.* 1996), rats (Zhao *et al.* 2000), pigs (Wu *et al.* 2001), cattle (Figueiredo *et al.* 1994, Gutierrez *et al.* 2000), sheep (Cecconi *et al.* 1999) and humans. Some workers have advocated combining ovarian dissociation methods such as grating or mincing with sieving methods (Jewgenow 1998). Follicles have also been successfully dissected out of thin slices of cortex that has been prepared using a skin-grafting knife and/or small scalpel blades (Gutierrez *et al.* 2000, Picton *et al.* 2003). The main advantage of these mechanical isolation methods is that they recover intact follicles surrounded by both a basement membrane and theca cell layer(s). However, all of these techniques are slow and labour intensive, and typically they only permit the recovery of a small number of follicles (Telfer *et al.* 2000). To combat these technical problems many researchers routinely used enzymes to aid follicle recovery. Enzymatic follicle isolation typically involves incubating tissue in collagenase and/or DNase (for example Eppig & Schroeder 1989, Eppig & O'Brien 1996, Newton *et al.* 1999) to soften and disaggregate the tissue matrix, followed by detachment of the follicles from stromal tissue using needles. The most common adverse consequence of enzymatic isolation of follicles is that the basement membrane is degraded and the theca cell layer(s) are removed (Nayudu *et al.* 2001). Although, this damage can be minimised by limiting enzyme exposure (Shuttleworth *et al.* 2002), the destruction of the basement membrane and theca layers can result in the spontaneous migration of GCs away from the oocyte, once the follicles are placed into culture.

Culture systems for the IVG and maturation of mammalian follicles and oocytes

A series of culture systems have been developed to support the IVG and maturation of follicles and oocytes from a range of different species. Each system is tailored to the stage of follicle development of interest.

Primordial and primary follicle culture

To maximise the reproductive potential of fresh and cryopreserved ovarian tissue it is necessary to develop culture strategies that support the activation and sustained IVG of primordial follicles as this is the most abundant stage of follicles development present in mammalian ovaries. Initial attempts at primordial follicle growth *in vitro* focussed on the growth of isolated follicles. While the data clearly show that it is possible to harvest rodent primordial follicles by mechanical means, in the tougher cortex of large animals and humans primordial follicles have to be isolated using enzymatic methods (Greenwald & Moor 1989; and human Oktay *et al.* 1997, Abir *et al.* 1999, Huntriss *et al.* 2002, Rice *et al.* 2008). Consequently, although a high percentage of primordial follicles are viable following extraction (~71%; Oktay *et al.* 1997), and growth can be supported for 24 h within a collagen gel (Abir *et al.* 1999), beyond this time follicles rapidly lose their three dimensional structure (Spears *et al.* 1994, Cortvrindt *et al.* 1996), the follicles collapse, the pregranulosa cells migrate away from the oocyte and/or oocyte extrusion and degeneration occurs. It is possible that the lack of influence from the extra-follicular cortical cells contributes to the demise of isolated primordial follicles *in vitro* as the presence of stromal cells around primordial follicles has been shown to improve initial culture success (Osborn *et al.* 1997). In contrast to the majority of publications, a recent report demonstrated that lectin-aggregated, serum-free cultures supported the long-term survival and growth of isolated ovine primordial follicles from both fresh and cryopreserved neonatal lamb ovary ovarian cortex (Muruvi *et al.* 2005). Oocyte growth and GC differentiation were further enhanced in lectin-aggregated cultures following exposure to KL *in vitro* and the oocytes reached diameters equivalent to those of oocytes from primary follicles derived *in vivo* (Muruvi *et al.* 2005). While these recent results are encouraging, they are by no means definitive as the optimal methodology for primordial follicle growth *in vitro*.

An alternative approach that has consistently proved successful for primordial follicle culture in a number of species is to grow these early stages *in situ* within fragments of the ovarian cortex. In early *in situ* culture experiments, large numbers of oocytes underwent atresia (Blandau *et al.* 1965), and no follicular growth was observed (Baker & Neal 1974). However, Eppig & O'Brien (1996) reported the birth of live pups from oocytes derived from primordial follicles grown *in situ* following the culture of foetal mouse ovaries. Two subsequent studies have also produced live offspring following the growth of oocytes present within cryopreserved ovaries (Liu *et al.* 2001) and foetal ovaries obtained at E12.5 (Obata *et al.* 2002). *In situ* cultures of primordial and primary follicles can be conducted by culturing fragments of ovarian cortex directly or by

grafting the cortical fragments beneath the highly vascularised chorioallantoic membrane of chick embryos (Fortune *et al.* 2000, Cushman *et al.* 2002, Qureshi *et al.* 2008). Whichever route is adopted, culture of thin cortical strips: i) avoids the damage caused by the follicle harvesting procedure; ii) minimises the chances of necrosis by maximising the surface area of the tissue for gaseous exchange and fulfilment of the nutrient requirements; and iii) provides a complex support system that more closely resembles the ovary *in vivo* as the follicles remain both intact and in contact with the surrounding stromal cells, which provide the local biochemical control pathways that trigger the initiation of follicle growth.

In situ cortical cultures have been conducted for a number of species over varying time frames with or without extracellular matrix and high levels of serum (Hovatta *et al.* 1999, Wright *et al.* 1999) or in serum-free medium (Picton *et al.* 1999b, Webber *et al.* 2007, Telfer *et al.* 2008). During the *in situ* culture pieces of ovarian cortex are placed onto a physical support, such as a porous membrane insert, in culture wells and the tissue is then covered with a meniscus of culture media (Devine *et al.* 2002). Using this approach, follicles may remain viable and are morphologically normal after 1–3 weeks of culture. Using this method, recruitment of primordial follicle growth has been demonstrated in a number of species (cows: Wandji *et al.* 1996, 1997, Braw-Tal & Yossefi 1997, Fortune *et al.* 1998; baboons: Fortune *et al.* 1998, hamsters: Yu & Roy 1999, humans: Hovatta *et al.* 1999, Picton *et al.* 1999b, Wright *et al.* 1999). Furthermore, multilayer preantral follicles have been isolated following the culture of fragments of ovarian cortex (Telfer *et al.* 2008) or after culture of whole mouse ovaries (Eppig & O'Brien 1996). Despite these successes, not all cortical strip culture systems are equally effective and further optimisation of the oxygen tension and growth additives are required to sustain high levels of primordial and primary follicle growth *in vitro*.

The problems encountered during the culture of isolated primordial follicles are replicated during primary follicle IVG. However, recent data have shown that it is possible to use an attachment based culture system to drive secondary follicles *in vitro* from isolated primary follicles harvested from fresh or cryopreserved neonatal lamb ovaries (Muruvi *et al.* 2008). Here, the oocytes from isolated primary follicles were shown to be able to reassemble functional 'follicle units' *in vitro* and to support the sustained growth of these reorganised follicles to secondary stages over more than 42 days *in vitro*. After 7, 11 and 30 days of culture increases in follicle diameter of 7 µm (Nuttinck *et al.* 1993), 19 µm (Itoh & Hoshi 2000) and 35 µm (Saha *et al.* 2000) respectively, have been reported for small bovine follicles. In this cohort of studies, follicle survival and growth depended on the use of feeder layers of somatic cells.

Preantral-antral follicle culture

Unlike primordial and primary follicle culture, preantral-antral cultures are rarely conducted *in situ* but rather follicle isolation appears to be crucial for future development *in vitro*. The growth characteristics of preantral follicles *in vitro* and the duration of culture are highly dependent on the culture system and the species of interest. Preantral follicles have been cultured with serum on a plastic substratum (Figueiredo *et al.* 1994, Jewgenow 1998), grown on somatic cell monolayers (Itoh & Hoshi 2000), or in serum-free environments (Newton *et al.* 1999, Gutierrez *et al.* 2000). Preantral follicle culture systems broadly fall into two categories: i) culture systems that allow follicle attachment and loss of follicle integrity as illustrated in Fig. 3 (mouse: Eppig & Schroeder 1989, Cortvrindt *et al.* 1996); or ii) three dimensional (3-D) culture systems that maintain follicle integrity. Three dimensional systems have been achieved through the use of physiological serum-free media approaches as shown in Fig. 4 (sheep: Newton *et al.* 1999; human: Picton *et al.* 1999b, Telfer *et al.* 2008), through the use of hydrophobic membranes that prevent attachment (mouse: Nayudu & Osborn 1992); or via encapsulation in collagen (mouse: Carroll *et al.* 1991a; pig: Hirao *et al.* 1994) or alginate gels (Xu *et al.* 2006, West *et al.* 2007).

Preantral follicle culture in rodents

Preantral follicle culture has been pioneered in rodents. The advantage of using rodents is that their ovaries are small and contain a large number of follicles embedded within a soft stromal matrix (Hartshorne 1997). Enzymatic follicle harvest has been used extensively in this species and the problems associated with the loss of follicle integrity following isolation have been addressed by placing granulosa-oocyte complexes onto collagen-treated porous membranes or poly-L-lysine coated dishes (Eppig & Schroeder 1989, Cain *et al.* 1995). Here, the outer GCs attach to the collagen or lysine, which limits migration away from the oocyte (Eppig 1994). Alternatively, intact follicles can be placed into microdrops of culture media under oil in uncoated dishes. In the latter case, the theca cells migrate onto the surface of the dish to form a monolayer that then anchors the follicle and prevents further GC migration (Nayudu *et al.* 2001). The latter approach has permitted the successful development of follicles isolated from both fresh (Cortvrindt *et al.* 1996) and cryopreserved (Smitz & Cortvrindt 1998, Newton & Illingworth 2001) murine tissue (see examples in Fig. 3). Recent data have also confirmed that for murine oocytes derived using an attachment IVG system, criteria such as oocyte diameter, chromatin configuration, transcriptional activity, intracytoplasmic calcium signalling and ability to undergo meiosis were similar in the *in vitro* derived oocytes compared with *in vivo* grown counterparts (Pesty *et al.* 2007).

Figure 3 Morphology and growth characteristics *in vitro* of isolated murine follicles at different stages of development after growth *in vitro* in an attachment based culture system. Images (a)–(d), and (g)–(h), are of *in vivo* grown follicles and oocytes for comparison: (a) primordial follicle; (b) primary follicle with 1–2 layers of granulosa cells; (c) secondary follicle with 2–3 layers of granulosa cells and 1 layer of theca cells; (d) immature oocyte from a secondary follicle: the germinal vesicle is visible (arrow); (e) antral follicle; (f) pre-ovulatory follicle; (g) *in vivo* grown cumulus-oocyte complex; (h) denuded metaphase II oocyte grown and matured *in vivo*. Images (i)–(l) are of *in vitro* grown follicles and oocytes derived from the attachment culture system of Cortvriendt *et al.* (1996): (i) *in vitro* grown two-dimensional follicle with antral-like cavity; (j) *in vitro* grown two-dimensional pre-ovulatory follicle with antral-like cavity; (k) *in vitro* grown follicle after ovulation of the cumulus oocyte complex (arrow); (l) denuded metaphase II oocyte derived from a pre-antral follicle grown to maturity *in vitro*. Scale bars = 100 µm, except in (a) where bar = 20 µm. Reproduced from Picton *et al.* (2004), page 198, Figure 1, with permission from Taylor & Francis.

Three dimensional IVG systems originated with the culture of murine preantral follicles (for example: Eppig & Schroeder 1989, Carroll *et al.* 1991b, Nayudu & Osborn 1992, Hartshorne *et al.* 1994, Spears *et al.* 1994, Cortvriendt *et al.* 1996). Using this approach, intact follicles are cultured on hydrophobic membranes that inhibit cellular attachment and migration (Nayudu & Osborn 1992). Alternatively, mouse follicles are cultured in microdrops under mineral oil and grown from mid-preantral sized follicles to the Graafian stage in about 6 days. The daily transfer of follicles to a new culture well prevents follicular attachment (Boland *et al.* 1993). The antral follicles derived using the three-dimensional system have normal morphology and steroid production (Nayudu & Osborn 1992, Hartshorne *et al.* 1994, Spears *et al.* 1994); furthermore, up to 80% of these follicles 'ovulate' *in vitro* in response to an exogenous ovulatory stimulus (Rose *et al.* 1999) and the oocytes are healthy and can be fertilised with the production of live young (Spears *et al.* 1994).

A number of different approaches for three-dimensional culture have been tested in mice; these methods may be more compatible with the IVG requirements of

the larger follicles collected from domestic ruminants and humans. Anchorage independent cultures of whole murine follicles have utilised inverted drop cultures of preantral follicles of 180–240 µm diameter (Wycherley *et al.* 2004). Rotating wall suspension cultures have also been tried (Rowghani *et al.* 2004). Alternatively, encapsulation of follicles in inert alginate hydrogels has been shown to be a good option for the sustained growth of larger follicles. Alginate is a linear polysaccharide copolymer of β -D-mannuronic acid and α -L-guluronic acid, which is produced by brown algae (Haug *et al.* 1967). It is thought that the alginate matrix mimics the stromal microenvironment of the ovary and as such will better support the growth and maturation of multilayered secondary follicles in large animals, primates and humans (Kreeger *et al.* 2007). Early reports demonstrated that murine follicles grown in alginate capsules reached the sizes typically observed *in vivo* (Pangas *et al.* 2003, Kreeger *et al.* 2007). Extended growth, coordinated differentiation of follicular cell types, antral cavity formation, theca cell differentiation, and oocyte maturation and hormone production were achieved by

Figure 4 *In vitro* growth sequence of isolated preantral (180–220 µm diameter) sheep follicles grown for 30 days in serum-free culture media. Theca-free follicles harvested by enzyme digestion (a) develop antral cavities *in vitro* (b, c) and show no evidence of the presence of the basement membrane at the end of culture as indicated by the lack of blue staining for the presence of collagen (d). In contrast, theca-enclosed follicles collected by mechanical isolation (e) show evidence of antral cavity formation by outgrowth from the dense theca and stromal cells (f, g; arrows) which remain concentrated within one area as indicated by blue staining for collagen (h) after extended culture. Scale bars = 50 µm. Reproduced from Picton *et al.* (2003) with permission from Nottingham University Press.

decreasing matrix stiffness and by altering the alginate scaffold concentration (Xu *et al.* 2006, West *et al.* 2007). This method of *in vitro* folliculogenesis from preantral stages has also resulted in the production of live young (Xu *et al.* 2006). While these data are very encouraging, it is important to note that the follicle and oocyte growth rates in all of these reports are increased over *in vivo*

grown counterparts. The health of oocytes derived from follicles with accelerated growth rates needs to be fully verified before these systems can be considered suitable for the clinical use of *in vitro* derived oocytes.

Preantral follicle culture in large domestic animals and humans

Despite the many advances in rodent follicle culture detailed above, these achievements have proved difficult to replicate in large animals and humans. One of the main reasons for the lack of progress is that it is technically more difficult to recover follicles from these species, as the cortex has a more dense and fibrous consistency (Telfer 1996) and follicle isolation frequently requires the use of aggressive enzyme-based protocols. Nevertheless, in recent years, it has proved possible to maintain the three-dimensional integrity of ruminant (Newton *et al.* 1999, Gutierrez *et al.* 2000, Picton *et al.* 2003, Thomas *et al.* 2007) and human follicles (Picton *et al.* 1999b, Telfer *et al.* 2008). The philosophy that underpins this system is that cultured GCs form distinct clumps of rounded cells that closely resemble the morphology (Chang *et al.* 1977) and steroidogenic phenotype of GCs *in vivo* (Campbell *et al.* 1996, Picton *et al.* 1999a) rather than acquiring a fibroblastic character associated with attachment to the plastic culture dish. This species-specific system maintains follicle integrity (Fig. 4) and follicle cells retain their normal ultrastructure (Jin *et al.* 2004). Antral cavity formation occurs in 50% of isolated ovine secondary follicles with a start size of 180–220 µm after 12–14 days of culture, with growth being supported up to diameters of 1.2 mm over 30 days in both fresh and cryopreserved tissues (Newton *et al.* 1999). Using this system, follicle growth rates can be manipulated by the presence/absence of the theca cell layer, as well as the presence/absence of basement membrane components in the culture media. These data support the concept first observed in murine follicles by Boland & Gosden (1994), that the theca cells have biochemical effects on oocyte growth and development *in vitro*, which are mediated via the GCs (Kotsuji *et al.* 1994, Richard & Sirard 1996). Additionally, on the provision of appropriate concentrations of androgen substrate, sheep follicles grown over 30 days resulted in the biosynthesis of limited, but physiological levels of oestradiol *in vitro* (Newton *et al.* 1999). Although the follicular growth rates recorded in this system *in vitro* are slightly accelerated compared with growth rates in adult animals, they closely mimic the growth rates of similar staged follicles in foetal ovaries *in vivo*. This extended growth phase is important as it facilitates the sequential accumulation of the payload of RNAs and proteins required to support oocyte developmental competence.

The potential of three-dimensional pre-antral follicle culture has been demonstrated in other domestic species including pigs (Wu *et al.* 2001); cattle (Gutierrez *et al.* 2000, McCaffery *et al.* 2000, Thomas *et al.* 2001, 2007)

and humans (Picton *et al.* 1999b, Telfer *et al.* 2008). The IVG of porcine follicles has produced a small number of meiotically competent oocytes following growth in both collagen gels (Hirao *et al.* 1994), and in uncoated culture wells (Telfer *et al.* 2000). *In vitro* fertilisation of IVG-derived porcine metaphase II oocytes resulted in limited blastocyst production (Wu *et al.* 2001). The IVG of human pre-antral graafosa-oocyte-complexes and whole follicles have resulted in development up to antral sizes following enzymatic isolation and culture within agar (Roy & Treacy 1993) or collagen (Abir *et al.* 1999) gels. Mechanical isolation and IVG have also been demonstrated (Abir *et al.* 1997), although levels of atresia following culture were found to be high. In the most recent report (Telfer *et al.* 2008), pre-antral follicles have been isolated mechanically from cortical tissue after 6 days of culture and antral cavity formation occurred in the isolated follicles very rapidly, within 2 days of isolation. The meiotic competence and developmental capacity of human oocytes grown from pre-antral stages *in vitro* has yet to be confirmed.

The IVM of oocytes

The final phase in a strategy for the complete IVG of follicles is the IVM of the oocytes derived following *in vitro* folliculogenesis. There are already reports in the literature that clearly demonstrate the utility of IVM as a stand alone technology for assisted reproduction in many species including humans (for example, for review see van Wagtenonk-de Leeuw *et al.* 2000, Picton 2002, Banwell & Thompson 2008). Oocyte maturational competence is linked to follicle size and the follicular microenvironment immediately before nuclear and cytoplasmic maturation. Just as has been recorded for IVG, there is a clear need to optimise IVM culture media (Filali *et al.* 2008) and to test the efficacy of physiologically relevant additives such as human chorionic gonadotrophin (Ge *et al.* 2008); umbilical cord blood (Zhang *et al.* 2007); and FCS (van Wagtenonk-de Leeuw *et al.* 2000); as well as to test the

efficacy of physiologically relevant additives such as follicular fluid meiosis activating sterol (Gröndahl *et al.* 2000a, 2000b) and fetuin (Schroeder *et al.* 1990, Dell'Aquila *et al.* 1999); and to standardise factors such as priming with FSH, time of aspiration and the time interval to maturation *in vitro* (Mikkelsen *et al.* 2000). There is evidence that meiotic maturation and fertilisation rates and blastocyst production are compromised when compared with *in vivo* matured counterparts (Banwell & Thompson 2008). Furthermore, there are concerns about the safety of IVM and the genetic health of IVM oocytes (Smitz *et al.* 2007) and the long-term health of embryos, fetuses and neonates after IVM. The efficacy of stand alone IVM systems also appears to depend on cumulus-GC association, GC differentiation and follicle size and atresia at the time of oocyte harvest. It is likely that the requirements for IVM will become even more rigorous when applied to the maturation of the limited numbers of mature oocytes harvested following the IVG of follicles.

Future prospects for the IVG and maturation of follicles

Overall, the evidence reviewed here supports the concept that the complete IVG of follicles from large animals and human will only be achieved through the development and use of a multistage culture strategy (Fig. 5) as has previously been proposed by a number of follicle culture practitioners (for example: Eppig & O'Brien 1996, Picton *et al.* 1999b). Four steps are required to facilitate the complete IVG of follicles with the production of healthy, fertile oocytes:

- Phase 1 – *in situ* culture of primordial and primary follicles. This step will support the initiation and maintenance of primordial and primary follicle growth to the secondary stage of development.
- Phase 2 – isolated pre-antral–antral follicle culture. This stage will support antral cavity formation and early antral growth as well as oocyte growth to full size.

Figure 5 Multi-step strategy for the complete *in vitro* growth and maturation of follicles from large animals and humans. The images shown are of human follicles and oocytes.

- Phase 3 – induction of differentiated function in follicular somatic cells. Phase 3 will support the induction of steroidogenic function, especially aromatase activity and oestradiol synthesis by the GCs that are required to facilitate the acquisition of oocyte cytoplasmic competence.
- Phase 4 – IVM of oocytes. The final phase of the IVG system enables the harvest and maturation of the fully-grown, cumulus enclosed, germinal vesicle staged oocytes from *in vitro* grown follicles and drives meiotic progression of oocytes to metaphase II before harvest and insemination.

Conclusion

Significant progress has been made in the development of improved methods for the IVG and maturation of follicles and oocytes across a range of mammalian species. The potential for the complete IVG from primordial follicles is realistic but this has only been achieved in mouse with limited efficiency and the full maturation of oocytes from species with longer developmental times for completion of oogenesis is a more formidable task. Further technological advances and optimisation of culture environments are required to improve and extend IVG and IVM culture methodologies before they can be used effectively in large animals and humans. Finally, before these strategies can be utilised therapeutically, extensive testing is required to confirm the epigenetic health and genetic normality of IVG-derived oocytes before the fertility of the oocytes and developmental competence of the embryos they produce can be confirmed. Until further extensive research has been conducted the therapeutic potential of IVG and IVM technologies will not be fully realised.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

The authors gratefully acknowledge that Dr SE Harris is supported by a Research Grant (Reference Number BB/C007395/1) from Biotechnology and Biological Sciences Research Council. Dr EL Chambers is supported by a grant from the Candlelighters, Childrens Cancer Charity, UK.

References

Abir R, Franks S, Mobberley MA, Moore PA, Margara RA & Winston RML 1997 Mechanical isolation and *in vitro* growth of preantral and small antral human follicles. *Fertility and Sterility* **68** 682–688.

Abir R, Roizman P, Fisch B, Nitke S, Okon E, Orvieto R & Ben Rafael Z 1999 Pilot study of isolated early human follicles cultured in collagen gels for 24 hours. *Human Reproduction* **14** 1299–1301.

Baker TG & Neal P 1974 Organ culture of cortical fragments and Graafian follicles from human ovaries. *Journal of Anatomy* **117** 361–371.

Banwell KM & Thompson JG 2008 *In vitro* maturation of mammalian oocytes: outcomes and consequences. *Seminars in Reproductive Medicine* **26** 162–174.

Beaujean N, Hartshorne G, Cavilla J, Taylor J, Gardner J, Wilmot I, Meehan R & Young L 2004 Non-conservation of mammalian preimplantation methylation dynamics. *Current Biology* **14** 266–267.

Biggers J, Whittingham D & Donahue R 1967 The pattern of energy metabolism in the mouse oocyte and zygote. *PNAS* **58** 567.

Blandau RJ, Warrick E & Rumery RE 1965 *In vitro* cultivation of fetal mouse ovaries. *Fertility and Sterility* **16** 705–715.

Van Blerkom J, Davis P, Mathwig V & Alexander S 2002 Domains of high-polarised and low-polarised mitochondrial may occur in mouse and human oocytes and early embryos. *Human Reproduction* **17** 393–406.

Boland NI & Gosden RG 1994 Effects of epidermal growth factor on the growth and differentiation of cultured mouse ovarian follicles. *Journal of Reproduction and Fertility* **101** 369–374.

Boland NI, Humpherson PG, Leese HJ & Gosden RG 1993 Pattern of lactate production and steroidogenesis during growth and maturation of mouse ovarian follicles *in vitro*. *Biology of Reproduction* **48** 798–806.

Borghol N, Lornage J, Blachere T, Sophie Garret A & Lefevre A 2006 Epigenetic status of the H10 locus in human oocytes following *in vitro* maturation. *Genomics* **87** 417–426.

Braw-Tal R & Yossefi S 1997 Studies *in vivo* and *in vitro* on the initiation of follicle growth in the bovine ovary. *Journal of Reproduction and Fertility* **109** 165–171.

Bukovsky A, Caudle MR, Keenan JA, Wimalasena J, Foster JS & Van Meter SE 1995 Quantitative evaluation of the cell cycle-related retinoblastoma protein and localization of Thy-1 differentiation protein macrophages during follicular development and atresia, and in human corpora lutea. *Biology of Reproduction* **52** 776–792.

Cain L, Chatterjee S & Collins TJ 1995 *In vitro* folliculogenesis of rat preantral follicles. *Endocrinology* **136** 3369–3377.

Campbell BK, Scaramuzzi RJ & Webb R 1996 Induction and maintenance of oestradiol and immunoreactive inhibin production with FSH by ovine granulosa cells in serum-free media. *Journal of Reproduction and Fertility* **106** 7–16.

Carámbula SF, Figueiredo JR, Wheeler MB, Neves JP & Mondadori RG 1999 Effect of fetal age and method of recovery on isolation of preantral follicles from bovine ovaries. *Theriogenology* **52** 563–571.

Carroll J, Whittingham DG & Wood MJ 1991a Effect of gonadotrophin environment on growth and development of isolated mouse primary ovarian follicles. *Journal of Reproduction and Fertility* **93** 71–79.

Carroll J, Whittingham DG & Wood MJ 1991b Growth *in vitro* and acquisition of meiotic competence after the cryopreservation of isolated mouse primary ovarian follicles. *Reproduction, Fertility, and Development* **3** 593–599.

Cecconi S, Barboni B, Coccia M & Mattioli M 1999 *In vitro* development of sheep preantral follicles. *Biology of Reproduction* **60** 594–601.

Chang SCS, Anderson W, Lewis JC, Ryan RJ & Kang YH 1977 The porcine ovarian follicle. II. Electron microscopic study of surface features of granulosa cells at different stages of development. *Biology of Reproduction* **16** 349–357.

Cortvrindt R, Smits J & Van Steirteghem AC 1996 *In vitro* maturation, fertilization and embryo development of immature oocytes from early preantral follicles from prepubertal mice in a simplified culture system. *Human Reproduction* **11** 2656–2666.

Cushman RA, Wahl CM & Fortune JE 2002 Bovine cortical pieces grafted to chick embryonic membranes: a model for studies on the activation of primordial follicles. *Human Reproduction* **17** 48–54.

Dell'Aquila ME, Felici MD, Massari S, Maritato F & Minoia P 1999 Effect of fetuin on zona pellucida hardening and fertilisability of equine oocytes matured *in vitro*. *Biology of Reproduction* **61** 533–540.

Devine PJ, Rajapaksa KS & Hoyer PB 2002 *In vitro* ovarian tissue and organ culture: a review. *Frontiers in Bioscience* **7** d1979–d1989.

Diaz FJ, Wigglesworth K & Eppig JJ 2007 Oocytes are required for the preantral granulosa cell to cumulus cell transition in mice. *Developmental Biology* **305** 300–311.

- Dong J, Albertini D, Nishimori K, Kumar T, Lu N & Matzuk M 1996 Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature* **383** 531–535.
- Eppig JJ 1976 Analysis of mouse oogenesis *in vitro*. Oocyte isolation and the utilization of exogenous energy sources by growing oocytes. *Journal of Experimental Zoology* **198** 375–382.
- Eppig JJ 1994 Further reflections on culture systems for the growth of oocytes *in vitro*. *Human Reproduction* **9** 974–976.
- Eppig JJ & O'Brien MJ 1996 Development *in vitro* of mouse oocytes from primordial follicles. *Biology of Reproduction* **54** 197–207.
- Eppig JJ & Schroeder AC 1989 Capacity of mouse oocytes from preantral follicles to undergo embryogenesis and development to live young after growth, maturation, and fertilization *in vitro*. *Biology of Reproduction* **41** 268–276.
- Eppig JJ, O'Brien MJ, Pendola FL & Watanabe S 1998 Factors affecting the developmental competence of mouse oocytes grown *in vitro*: follicle-stimulating hormone and insulin. *Biology of Reproduction* **59** 1445–1453.
- Fair T, Hulshof SCJ, Hyttel P & Greve T 1997 Oocyte ultrastructure in bovine primordial to early tertiary follicles. *Anatomy and Embryology* **195** 327–336.
- Fernandez-Gonzalez R, Moreira P, Bilbao A, Jimenez A, Perez-Crespo M, Ramirez MA, Rodriguez De Fonseca F, Pintado B & Gutierrez-Adan A 2004 Long-term effect of *in vitro* culture of mouse embryos with serum on mRNA expression of imprinting genes, development, and behaviour. *PNAS* **101** 5880–5885.
- Figueiredo JR, Hulshof SC, Van den Hurk R, Bevers MM, Nussgens B & Beckers JF 1993 Development of a new mechanical method for the isolation of intact preantral follicles from fetal, calf and adult bovine ovaries. *Theriogenology* **40** 789–799.
- Figueiredo JR, Hulshof SCJ, Vandenhurk R, Nussgens B, Bevers MM, Ectors FJ & Beckers JF 1994 Preservation of oocyte and granulosa-cell morphology in bovine preantral follicles cultured *in vitro*. *Theriogenology* **41** 1333–1346.
- Filali M, Hesters L, Franchin R, Tachdjian G, Frydman R & Frydman N 2008 Retrospective comparison of two media for *in vitro* maturation of oocytes. *Reproductive Biomedicine Online* **16** 250–256.
- Fortune JE, Kito S, Wandji SA & Srsen V 1998 Activation of bovine and baboon primordial follicles *in vitro*. *Theriogenology* **49** 441–449.
- Fortune JE, Cushman RA, Wahl CM & Kito S 2000 The primordial to primary follicle transition. *Molecular and Cellular Endocrinology* **163** 53–60.
- Fulka H, Mrazek M, Tepla O & Fulka J Jr 2004 DNA methylation pattern in human zygotes and developing embryos. *Reproduction* **128** 703–708.
- Gandolfi F, Milanese E, Pocar P, Luciano AM, Brevini TA, Acocella F, Lauria A & Armstrong DT 1998 Comparative analysis of calf and cow oocytes during *in vitro* maturation. *Molecular Reproduction and Development* **49** 168–175.
- Ge HS, Huang XF, Zhang W, Zhao JZ, Lin JJ & Zhou W 2008 Exposure to human chorionic gonadotrophin during *in vitro* maturation does not improve the maturation rate and developmental potential of immature oocytes from patients with polycystic ovary syndrome. *Fertility and Sterility* **89** 98–103.
- Gosden RG & Telfer E 1987 Scaling of follicular sizes in mammalian ovaries. *Journal of Zoology* **211** 157–168.
- Gougeon A 1996 Regulation of ovarian follicular development in primates – facts and hypotheses. *Endocrine Reviews* **17** 121–155.
- Greenwald GS & Moor RM 1989 Isolation and preliminary characterization of pig primordial follicles. *Journal of Reproduction and Fertility* **87** 561–571.
- Grøndahl C, Hansen TH, Marky-Nielsen K, Ottesen JL & Hyttel P 2000a Human oocyte maturation *in vitro* is stimulated by meiosis-activating sterol. *Human Reproduction* **15** 3–10.
- Grøndahl C, Lessl M, Farge I, Hefe-Huarterung C, Wassermann K & Ottesen JL 2000b Meiosis activating sterol mediated resumption of meiosis in mouse oocytes *in vitro* is influenced by protein synthesis inhibition and cholera toxin. *Biology of Reproduction* **62** 775–780.
- Gutierrez CG, Ralph JH, Telfer EE, Wilmut I & Webb R 2000 Growth and antrum formation of bovine preantral follicles in long-term culture *in vitro*. *Biology of Reproduction* **62** 1322–1328.
- Harris SE & Picton HM 2007 Metabolism of follicles and oocytes during growth and maturation. In *In-Vitro Maturation of Human Oocytes – Basic Science to Clinical Application*, pp 15–36. Eds SL Tan, RC Chian & WM Buckett. Oxon, UK: Informa Health.
- Harris SE, Adriaens I, Leese HJ, Gosden RG & Picton HM 2007 Carbohydrate metabolism by murine ovarian follicles and oocytes grown *in vitro*. *Reproduction* **134** 415–424.
- Harris SE, Leese HJ, Gosden RG & Picton HM 2008 Pyruvate and oxygen consumption throughout the growth and development of murine oocytes. *Molecular Reproduction and Development* [in press] DOI:10.1002/mrd.20945.
- Hartshorne GM 1997 *In vitro* culture of ovarian follicles. *Reviews of Reproduction* **2** 94–104.
- Hartshorne GM, Sargent IL & Barlow DH 1994 Meiotic progression of mouse oocytes throughout follicle growth and ovulation *in vitro*. *Human Reproduction* **9** 352–359.
- Haug A, Larsen B & Smidsrod O 1967 Studies of the sequence of uronic acid residues in alginic acid. *Acta Chemica Scandinavica* **21** 691–704.
- Hirao Y, Nagai T, Kubo M, Miyano T, Miyake M & Kato S 1994 *In vitro* growth and maturation of pig oocytes. *Journal of Reproduction and Fertility* **100** 333–339.
- Hovatta O, Wright C, Krausz T, Hardy K & Winston RML 1999 Human primordial, primary and secondary ovarian follicles in long-term culture: effect of partial isolation. *Human Reproduction* **14** 2519–2524.
- Hreinsson JG, Scott JE, Rasmussen C, Swahn ML, Hsueh AJ & Hovatta O 2002 Growth differentiation factor-9 promotes the growth, development and survival of human ovarian follicles in organ culture. *Journal of Clinical Endocrinology and Metabolism* **87** 316–321.
- Hu Y, Betzendahl I, Cortvrindt R, Smits J & Eichenlaub-Ritter U 2001 Effects of low O₂ and ageing on spindles and chromosomes in mouse oocytes from pre-antral follicle culture. *Human Reproduction* **16** 737–748.
- Hulshof SCJ, Figueiredo JR, Beckers JF, Bevers MM, Vanderdonk JA & Vandenhurk R 1995 Effects of fetal bovine serum, FSH and 17- β -estradiol on the culture of bovine preantral follicles. *Theriogenology* **44** 217–226.
- Hulshof SCJ, Figueiredo JR, Beckers JF, Bevers MM, Vanderstichele H & van den Hurk R 1997 Bovine preantral follicles and activin: immunohistochemistry for activin and activin receptor and the effect of bovine activin A *in vitro*. *Theriogenology* **48** 133–142.
- Huntriss J & Picton HM 2008 Epigenetic consequences of assisted reproduction and infertility on the human preimplantation embryo. *Human Fertility* **11** 85–94.
- Huntriss J, Gosden R, Hinkins M, Oliver B, Miller D, Rutherford A & Picton H 2002 Isolation, characterisation, and expression of the human factor in the germline alpha (FIGLA) in ovarian follicles and oocytes. *Molecular Human Reproduction* **8** 1087–1095.
- Van den Hurk R, Abir R, Telfer EE & Bevers MM 2000 Primate and bovine immature oocytes and follicles as sources of fertilizable oocytes. *Human Reproduction Update* **6** 457–474.
- Imamura T, Kerjean A, Heams T, Kupiec JJ, Thenevin C & Paldi A 2005 Dynamic CpG and non-CpG methylation of the Peg1/Mest gene in the mouse oocyte and preimplantation embryo. *Journal of Biological Chemistry* **280** 20171–20175.
- Itoh T & Hoshi H 2000 Efficient isolation and long-term viability of bovine small preantral follicles *in vitro*. *In Vitro Cellular & Developmental Biology. Animal* **36** 235–240.
- Jewgenow K 1998 Role of media, protein and energy supplements on maintenance of morphology and DNA-synthesis of small preantral domestic cat follicles during short-term culture. *Theriogenology* **49** 1567–1577.
- Jin P, Harris SE & Picton HM 2004 The effect of ascorbic acid on ovine preantral follicle development *in vitro*. *Proceedings of the Society for Reproduction and Fertility*, 2004 Annual Conference, Ghent, Belgium. P22.
- Jin P, Harris SE & Picton HM 2007 Effect of oxygen tension on ovine follicle growth *in vitro*. *Fertility* 2007 P35.
- Kerjean A, Couvert P, Heams T, Chalas C, Poirier K, Chelly J, Jouannet P, Paldi A & Poirot C 2003 *In vitro* follicular growth affects oocyte imprinting establishment in mice. *European Journal of Human Genetics* **11** 493–496.
- Khosla S, Dean W, Brown D, Reik W & Feil R 2001 Culture of preimplantation mouse embryos affects fetal development and the expression of imprinted genes. *Biology of Reproduction* **64** 918–926.
- Knight PG & Glister C 2006 TGF- β superfamily members and ovarian follicle development. *Reproduction* **132** 191–206.

- Kotsuji F, Kubo M & Tominaga A 1994 Effect of interactions between granulosa and theca cells on meiotic arrest in bovine oocytes. *Journal of Reproduction and Fertility* **100** 151–156.
- Kreeger PK, Deck JW, Woodruff TK & Shea LD 2007 The *in vitro* regulation of ovarian follicle development using alginate-extracellular matrix gels. *Biomaterials* **27** 714–723.
- Lane M & Gardner DK 2003 Ammonium induces aberrant blastocyst differentiation, metabolism, pH regulation, gene expression and subsequently alters fetal development in the mouse. *Biology of Reproduction* **69** 1109–1117.
- Leese HJ & Barton AM 1984 Pyruvate and glucose uptake by mouse ova and preimplantation embryos. *Journal of Reproduction and Fertility* **72** 9–13.
- Liu J, van der Elst J, van den Broecke R & Dhont M 2001 Live offspring by *in vitro* fertilization of oocytes from cryopreserved primordial mouse follicles after sequential *in vivo* transplantation and *in vivo* maturation. *Biology of Reproduction* **64** 171–178.
- Louhio H, Hovatta O, Sjöberg J & Tuuri T 2000 The effects of insulin, and insulin-like growth factors I and II on human ovarian follicles in long-term culture. *Molecular Human Reproduction* **6** 694–698.
- Lucci CM, Kacinskis MA, Rumpf R & Bao SN 2004 Effects of lowered temperatures and media on short-term preservation of zebu (*Bos indicus*) preantral ovarian follicles. *Theriogenology* **61** 461–472.
- McCaffery FH, Leask R, Riley SC & Telfer EE 2000 Culture of bovine preantral follicles in a serum-free system: markers for assessment of growth and development. *Biology of Reproduction* **63** 267–273.
- McGrath SA, Esqueda AF & Lee S-J 1995 Oocyte-specific expression of growth/differentiation factor-9. *Molecular Endocrinology* **9** 131–136.
- McNatty KP, Heath DA, Lundy T, Fidler AE, Quirke L, O'Connell A, Smith P, Groome N & Tisdall DJ 1999 Control of early ovarian follicular development. *Journal of Reproduction and Fertility* **49** 123–135.
- McNatty KP, Reader K, Smith P, Heath DA & Juengel JL 2007 Control of ovarian follicular development to the gonadotrophin-dependent phase: a 2006 perspective. *Society of Reproduction and Fertility* **64** 55–68.
- Mehlmann LM 2005 Oocyte-specific expression of Gpr3 is required for the maintenance of meiotic arrest in mouse oocytes. *Developmental Biology* **288** 397–404.
- Mikkelsen AL, Smith S & Lindenberg S 2000 Possible factors affecting the development of oocytes in *in vitro* maturation. *Human Reproduction* **15** 11–17.
- Morison IM, Ramsay JP & Spencer HG 2005 A census of mammalian imprinting. *Trends in Genetics* **21** 457–465.
- Muruvi W, Picton HM, Rodway RG & Joyce IM 2005 *In vitro* growth of oocytes from primordial follicles isolated from frozen-thawed lamb ovaries. *Theriogenology* **64** 1357–1370.
- Muruvi W, Picton HM, Rodway RG & Joyce IM 2008 *In vitro* growth and differentiation of primary follicles isolated from cryopreserved sheep ovarian tissue. *Animal Reproduction Science* [in press] DOI:10.1016/j.anireprosci.2008.04.001.
- Nayudu PL & Osborn SM 1992 Factors influencing the rate of preantral and antral growth of mouse ovarian follicles *in vitro*. *Journal of Reproduction and Fertility* **95** 349–362.
- Nayudu PL, Fehrenbach A, Kiesel P, Vitt UA, Pancharatna K & Osborn SM 2001 Progress towards understanding follicle development *in vitro*: appearances are not deceiving. *Archives of Medical Research* **32** 587–594.
- Newton H & Illingworth P 2001 *In vitro* growth of murine preantral follicles after isolation from cryopreserved ovarian tissue. *Human Reproduction* **16** 423–429.
- Newton H, Picton HM & Gosden RG 1999 *In vitro* growth of oocyte-granulosa cell complexes isolated from cryopreserved ovine tissue. *Journal of Reproduction and Fertility* **115** 141–150.
- Nuttinck F, Mermillod P, Massip A & Dessy F 1993 Characterization of *in vitro* growth of bovine preantral ovarian follicles – a preliminary study. *Theriogenology* **39** 811–821.
- Obata Y, Kono T & Hatada I 2002 Maturation of mouse fetal germ cells *in vitro*. *Nature* **418** 497–498.
- Oktay K, Briggs D & Gosden RG 1997 Ontogeny of follicle stimulating hormone receptor gene expression in isolated human ovarian follicles. *Journal of Clinical Endocrinology and Metabolism* **82** 3748–3751.
- Osborn SM, Gook DA, Stern K & Speirs AL 1997 The isolation and culture of human primordial follicles from fresh ovarian tissue. *Human Reproduction* **12** (Supplement 1) 153.
- Otsuka F, Moore RK, Iemura S, Ueno N & Shimasaki S 2001 Follistatin inhibits the function of the oocyte-derived factor BMP-15. *Biochemical and Biophysical Research Communications* **289** 961–966.
- Pan H, O'Brien MJ, Wigglesworth K, Eppig JJ & Schultz RM 2005 Transcript profiling during mouse oocyte development and the effect of gonadotrophin priming and development *in vitro*. *Developmental Biology* **286** 493–506.
- Pangas SA, Saudye H, Shea LD & Woodruff TH 2003 Novel approaches for three-dimensional culture of granulosa cell–oocyte complexes. *Tissue Engineering* **9** 1013–1021.
- Pesty A, Miyara F, Debey P, Lefevre B & Poirot C 2007 Multiparameter assessment of mouse oogenesis during follicular growth *in vitro*. *Molecular Human Reproduction* **13** 3–9.
- Peters H, Byskov AG, Himmelstein-Braw R & Faber M 1975 Follicular growth: the basic event in the mouse and human ovary. *Journal of Reproduction and Fertility* **45** 559–566.
- Picton HM 2001 Activation of follicle development: the primordial follicle. *Theriogenology* **55** 1193–1210.
- Picton HM 2002 Oocyte maturation *in vitro*. *Current Opinion in Obstetrics and Gynecology* **14** 295–302.
- Picton HM, Campbell BK & Hunter MG 1999a Maintenance of oestradiol production and cytochrome P450 aromatase enzyme messenger ribonucleic acid expression in long-term serum-free cultures of porcine granulosa cells. *Journal of Reproduction and Fertility* **115** 67–77.
- Picton HM, Mkandla A, Salha O, Wynn P & Gosden RG 1999b Initiation of human primordial follicle growth *in vitro* in ultra-thin slices of ovarian cortex. *Human Reproduction* **14** (Abstract book 1) 11 (abstract 0-020).
- Picton HM, Kim S & Gosden RG 2000 Cryopreservation of gonadal tissue and cells. *British Medical Bulletin* **56** 603–615.
- Picton HM, Danfour M, Harris SE, Chambers E & Huntriss J 2003 *In vitro* growth and maturation of oocyte. *Reproduction Supplement* **61** 445–462.
- Picton HM, Harris SE & Chambers EL 2004 Prospects for follicle culture. In *Preservation of Fertility*, pp 191–207. Eds T Tulandi & RG Gosden. London: Taylor and Francis Group.
- Picton HM, Muruvi W & Jin P 2007 Interaction of oocyte and somatic cells. In *In-Vitro Maturation of Human Oocytes – Basic Science to Clinical Application*, pp 37–48. Eds SL Tan, RC Chian & WM Buckett. Oxon, UK: Informa Health.
- Qu J, Godin PA, Nisolle M & Donnez J 2000 Distribution and epidermal growth factor receptor expression of primordial follicles in human ovarian tissue before and after cryopreservation. *Human Reproduction* **15** 302–310.
- Qureshi A, Nussey S, Bano G, Musonda P, Whitehead S & Mason H 2008 Testosterone selectively increases primary follicles in ovarian cortex grafted onto embryonic chick membranes: relevance to polycystic ovaries. *Reproduction* **136** 187–194.
- Rice S, Ojha K & Mason H 2008 Human ovarian biopsies as a viable source of pre-antral follicles. *Human Reproduction* **23** 600–605.
- Richard FJ & Sirard MA 1996 Effects of follicular cells on oocyte maturation. II. Theca cell inhibition of bovine oocyte maturation *in vitro*. *Biology of Reproduction* **54** 22–28.
- Roberts R, Franks S & Hardy K 2002 Culture environment modulates maturation and metabolism of human oocytes. *Human Reproduction* **17** 2950–2956.
- Rose UM, Hanssen RG & Kloosterboer HJ 1999 Development and characterisation of an *in vitro* ovulation model using mouse ovarian follicles. *Biology of Reproduction* **61** 503–511.
- Rowghani NM, Heise MK, McKeel D, McGee EA, Koepsel RR & Russel AJ 2004 Maintenance of morphology and growth of ovarian follicles in suspension culture. *Tissue Engineering* **10** 545–552.
- Roy SK & Treacy BJ 1993 Isolation and long-term culture of human pre-antral follicles. *Fertility and Sterility* **59** 783–790.
- Sagirkaya H, Misirlioglu M, Kaya A, First NL, Parrish JJ & Memili E 2007 Developmental potential of bovine oocytes cultured in different maturation and culture conditions. *Animal Reproduction Science* **101** 225–240.
- Saha S, Shimizu M, Geshi M & Izaike Y 2000 *In vitro* culture of bovine preantral follicles. *Animal Reproduction Science* **63** 27–39.

- Schmidt KL, Ernst E, Byskov AG, Nyboe Anderson A & Yding Anderson C 2003 Survival of primordial follicles following prolonged transportation of ovarian tissue prior to cryopreservation. *Human Reproduction* **18** 2654–2659.
- Schoenfelder M & Einspanier R 2003 Expression of hyaluronan synthases and corresponding hyaluronan receptors is differentially regulated during oocyte maturation in cattle. *Biology of Reproduction* **69** 269–277.
- Schroeder AC, Schultz RM, Kopf GS, Tayler FR, Becker RB & Eppig JJ 1990 Fetuin inhibits zona pellucida and conversion of ZP2 to ZP2f during spontaneous mouse oocyte maturation *in vitro* in the absence of serum. *Biology of Reproduction* **43** 891–897.
- Scott JE, Carlsson IB, Bavister BD & Hovatta O 2004a Human ovarian tissue cultures: extracellular matrix composition, coating density and tissue dimensions. *Reproductive Biomedicine Online* **9** 287–293.
- Scott JE, Zhang P & Hovatta O 2004b Benefits of 8-bromo-guanosine 3',5'-cyclic monophosphate (8-br-cGMP) in human ovarian cortical tissue culture. *Reproductive Biomedicine Online* **8** 319–324.
- Sela-Abramovich S, Edry I, Galiani D, Nevo N & Dekel N 2006 Disruption of gap junctional communication within the ovarian follicle induces oocyte maturation. *Endocrinology* **147** 2280–2286.
- Shikone T, Yamoto M & Nakano R 1992 Follicle-stimulating hormone induces functional receptors for basic fibroblast growth factor in rat granulosa cells. *Endocrinology* **131** 1063–1068.
- Shimasaki S, Zachow RJ, Li D, Kim H, Iemura S-I, Ueno N, Sampath K, Chang RJ & Erickson GF 1999 A functional bone morphogenic protein system in the ovary. *PNAS* **96** 7282–7287.
- Shuttleworth G, Broughton Pipkin F & Hunter MG 2002 *In vitro* development of pig preantral follicles cultured in a serum-free medium and the effect of angiotensin II. *Reproduction* **123** 807–818.
- Smitz J & Cortvrindt R 1998 Follicle culture after ovarian cryostorage. *Maturitas* **30** 171–179.
- Smitz J, Picton HM, Platteau P, Rutherford A, Cortvrindt R, Clyde J, Nogueira D, Devroey P, Lyby K & Gröndahl C 2007 Principal findings from a multicenter trial investigating the safety of follicular-fluid meiosis-activating sterol for *in vitro* maturation of human cumulus-enclosed oocytes. *Fertility and Sterility* **87** 949–964.
- Spears N, Boland NI, Murray AA & Gosden RG 1994 Mouse oocytes derived from *in vitro* grown primary ovarian follicles are fertile. *Human Reproduction* **9** 527–532.
- Swain JE & Smith GD 2007 Mechanism of oocyte maturation. In *In-Vitro Maturation of Human Oocytes – Basic Science to Clinical Application*, pp 83–101. Eds SL Tan, RC Chian & WM Buckett. Oxon, UK: Informa Health.
- Telfer EE 1996 The development of methods for isolation and culture of preantral follicles from bovine and porcine ovaries. *Theriogenology* **45** 101–110.
- Telfer EE, Binnie JP, McCaffery FH & Campbell BK 2000 *In vitro* development of oocytes from porcine and bovine primary follicles. *Molecular and Cellular Endocrinology* **163** 117–123.
- Telfer EE, McLaughlin M, Ding C & Thong KJ 2008 A two-step serum-free culture system supports development of human oocytes from primordial follicles in the presence of activin. *Human Reproduction* **23** 1151–1158.
- Thomas FH & Vanderhyden BC 2007 Oocyte growth and developmental competence. In *In-Vitro Maturation of Human Oocytes – Basic Science to Clinical Application*, pp 1–15. Eds SL Tan, RC Chian & WM Buckett. Oxon, UK: Informa Health.
- Thomas FH, Leask R, Srsen V, Riley SC, Spears N & Telfer EE 2001 Effect of ascorbic acid on health and morphology of bovine preantral follicles during long-term culture. *Reproduction* **122** 487–495.
- Thomas FH, Walters KA & Telfer EE 2003 How to make a good oocytes: an update on *in vitro* models to study follicle regulation. *Human Reproduction Update* **9** 541–555.
- Thomas FH, Campbell BK, Armstrong DG & Telfer EE 2007 Effects of IGF-I bioavailability on bovine preantral follicular development *in vitro*. *Reproduction* **133** 1121–1128.
- Vaccari S, Horner K, Mehlmann LM & Conti M 2008 Generation of mouse oocytes defective in cAMP synthesis and degradation: endogenous cyclic AMP is essential for meiotic arrest. *Developmental Biology* **316** 124–134.
- Vassena R, Dee Schramm R & Latham KE 2005 Species-dependent expression patterns of DNA methyltransferase genes in mammalian oocytes and preimplantation embryos. *Molecular Reproduction and Development* **72** 430–436.
- van Wagtenonck-de Leeuw AM, Mullaart E, de Roos APW, Merton JS, den Daas JHG, Kemp B & de Ruigh L 2000 Effects of different reproductive techniques: AI, MOET or IVP on health and welfare of bovine offspring. *Theriogenology* **53** 575–597.
- Wandji S-A, Srsen V, Voss AK, Eppig JJ & Fortune JE 1996 Initiation *in vitro* of growth of bovine primordial follicles. *Biology of Reproduction* **55** 942–948.
- Wandji S-A, Srsen V, Nathanielsz PW, Eppig JJ & Fortune JE 1997 Initiation of growth of baboon primordial follicles *in vitro*. *Human Reproduction* **12** 1993–2001.
- Webb R & Campbell BK 2007 Development of the dominant follicle: mechanisms of selection and maintenance of oocyte quality. *Society of Reproduction and Fertility* **64** 141–163.
- Webber LJ, Stubbs SA, Stark J, Margara RA, Trew GH, Lavery SA, Hardy K & Franks S 2007 Prolonged survival in culture of preantral follicles from polycystic ovaries. *Journal of Clinical Endocrinology and Metabolism* **92** 1975–1978.
- West ER, Xu M, Woodruff TK & Shea LD 2007 Physical properties of alginate hydrogels and their effects on *in vitro* follicle development. *Biomaterials* **28** 4439–4448.
- Wilding M, Dale B, Marino M, di Matteo L, Alviggi C, Pisaturo ML, Lombardi L & De Placido G 2001 Mitochondrial aggregation patterns and activity in human oocytes and preimplantation embryos. *Human Reproduction* **16** 909–917.
- Wright C, Hovatta O, Margara R, Trowe G, Winston RML, Franks S & Hardy K 1999 Effect of follicle stimulating hormone and serum substitution on the development and growth of early human follicles. *Human Reproduction* **14** 1555–1562.
- Wu J, Emery BR & Carrell DT 2001 *In vitro* growth, maturation, fertilization, and embryonic development of oocytes from porcine preantral follicles. *Biology of Reproduction* **64** 375–381.
- Wycherley G, Downey D, Kane MT & Hynes AC 2004 A novel follicle culture system markedly increases follicle volume and growth of ovarian follicles in suspension culture. *Reproduction* **127** 669–677.
- Xu M, West E, Shea LD & Woodruff TK 2006 Identification of a stage specific permissive *in vitro* culture environment for follicle growth and oocyte development. *Biology of Reproduction* **75** 916–923.
- Ye J, Coleman J, Hunter MG, Craigon J, Campbell KH & Luck MR 2007 Physiological temperature variants and culture media modify meiotic progression and developmental potential of pig oocytes *in vitro*. *Reproduction* **133** 877–886.
- Young LE, Sinclair KD & Wilmut I 1998 Large offspring syndrome in cattle and sheep. *Reviews of Reproduction* **3** 155–163.
- Yu N & Roy SK 1999 Development of primordial and prenatal follicles from undifferentiated somatic cells and oocytes in the hamster prenatal ovary *in vitro*: effect of insulin. *Biology of Reproduction* **61** 1558–1567.
- Zhang P, Louhio H, Tuuri T, Sjöberg J, Hreinsson J, Telfer EE & Hovatta O 2004 *In vitro* effect of cyclic adenosine 3',5'-monophosphate (cAMP) on early human ovarian follicles. *Journal of Assisted Reproduction and Genetics* **21** 310–306.
- Zhang ZG, Zhao JH, Wei ZL, Cong L, Zhou P & Cao YX 2007 Human umbilical cord blood serum in culture medium on oocyte maturation *in vitro*. *Archives of Andrology* **53** 303–307.
- Zhao J, Dorland M, Taverne MA, Van Der Weijden GC, Bevers MM & Van Den Hurk R 2000 *In vitro* culture of rat pre-antral follicles with emphasis on follicular interactions. *Molecular Reproduction and Development* **55** 65–74.

Received 5 July 2008

First decision 4 August 2008

Revised manuscript received 15 September 2008

Accepted 7 October 2008