Article

Natural history of the mammalian oocyte

Evelyn Telfer was awarded her PhD in ovarian development from the University of Edinburgh in 1987. From 1987 to 1989 she worked with Roger Gosden in the Department of Physiology where they developed one of the first culture systems to support murine follicle development. In 1989 she obtained a Rockefeller Reproductive Sciences award to undertake post-doctoral training with John Eppig at the Jackson Laboratory Bar Harbor Maine, USA. Evelyn returned to the University of Edinburgh as a lecturer in Animal Physiology in 1992 and is currently a Senior Lecturer in the Institute of Cell Biology, University of Edinburgh where she heads a research group working on ovarian development.

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Abstract

Combining cryopreservation of immature oocytes with in-vitro growth/maturation techniques is the ambition of many IVF clinics. Whilst these techniques have been demonstrated in rodents their application to humans and domestic species has been slow. There are many technical reasons for the lack of progress in these species, but the major problem is that we have very little knowledge of how the oocyte acquires developmental competence during its growth within the follicle. The life history of the mammalian oocyte involves a complex series of co-ordinated developmental processes that in the human take place over several months. This review will consider: (i) growth and development of the oocyte; (ii) the newly regenerated debate on the existence of germ-line stem cells in the mammalian ovary; and (iii) strategies for producing oocytes *in vitro*.

Keywords: *follicle development, germ-line stem cells, growth factors, in-vitro growth, in-vitro maturation, oocyte*

Introduction

'*Omne vivum ex ovo*' – 'all living things come from eggs' – was a conviction that William Harvey put forward very strongly in his publication on the generation of animals in 1651 (Harvey, 1651). This generalization has proven to be true, but at that time Harvey had no empirical evidence to support his conclusion (see Austin, 1961, for an historical overview). It was not until 1827 that the mammalian oocyte was first correctly identified (Von Baer, 1827). Since that time we have learned a great deal about oocyte development and its regulation but there is still much that remains a mystery. This paper will review our current understanding of oocyte development and utilization throughout life as well as considering the possibility of developing systems to support in-vitro growth of oocytes.

Life history of the oocyte

The oocyte is formed in the fetal ovary and suspended in diplotene of the first prophase of meiosis where it may remain for years before starting to grow. It is only when the growth process is complete that it can resume meiosis and undergo fertilization. During this time the oocyte has to acquire meiotic and developmental competence, i.e. become capable of being fertilized and directing early embryonic development.

Oocytes are derived from the primordial germ cells that segregate during embryonic development and from 5 to 6 weeks of gestation can be observed within the indifferent human gonad (Fujimoto *et al.*, 1977). Once at the gonadal ridge, the primordial germ cells become oogonia and divide for a limited time before entering meiosis. Meiosis begins in the human fetal ovary at about 11–12 weeks of gestation (Gondos *et al.*, 1971, 1986) and oocytes at the diplotene stage of prophase I are found at 16 weeks of gestation, reaching a peak at 19 weeks (Baker, 1963). At this stage oocytes are suspended in the diplotene phase until resumption occurs at ovulation. During this time the number of germ cells reaches a peak of around 7 million at 16–20 weeks (Baker, 1963), with a subsequent reduction to about 2 million at term.

Once oocytes have been formed they must make connections with somatic cells (pre-granulosa cells) in order for them to survive. In humans, the first primordial follicles are seen between 16 and 21 weeks of gestation (Baker and Neal, 1974;

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Konishi *et al*., 1986). The Factor in the Germline alpha (Fig-alpha), a germ-cell-specific basic helix–loop–helix transcription factor, has been implicated in the formation process since in Fig-alpha knockout mice no follicles are formed (Soyal *et al*., 2000). Fig-alpha protein has been found in fetal human ovaries (Bayne *et al*., 2004) and is up-regulated at the time of follicle formation (Bayne *et al*., 2004).

Oocyte/follicle development

Follicles begin their development as primordial structures that consist of an oocyte arrested at the diplotene stage of the first meiotic division, surrounded by a few flattened granulosa cells (Peters, 1969). Once the pool of primordial follicles has been established, and in response to either stimulatory or the release from inhibitory factors, follicles are gradually and continuously recruited to grow. The signals regulating the growth initiation of primordial follicles are still unknown but are likely to be an interaction of factors such as anti-Müllerian hormone that inhibit initiation (Durlinger *et al*., 1999) and a range of stimulatory factors such as c-kit and kit-ligand (Skinner, 2005). In mice, the forkhead transcription factor Foxo3a has been shown to be essential for regulating entry into the growth phase (Castrillon *et al*., 2003).

In the human fetal ovary, growing pre-antral follicles can be seen at around 24 weeks of gestation (Peters *et al*., 1978). During this early growth period, the oocyte grows and granulosa cells proliferate to form the multi-laminar pre-antral follicle (Sato *et al*., 2007). During pre-antral stages the oocyte increases rapidly in volume (Schultz and Wassarman, 1977; Eppig, 1994), granulosa cells multiply to form several layers, and thecal cells come to lie on the basement membrane surrounding the granulosa cells (Hirshfield, 1991b; Braw-Tal and Yossefi, 1997). These early stages of follicle growth are the most crucial for oocyte development as it is during this time that many of the proteins essential for further development, both before and after ovulation, are produced. The oocyte's diameter increases rapidly during pre-antral stages of follicle growth due to an increase in protein content (Schultz and Wassarman, 1977) and accumulation of resources essential for maturation, fertilization and preimplantation embryo development (Eppig, 1994; Borini *et al*., 2005) takes place. Oocytes from pre-antral follicles are unable to resume meiosis, but it is during this phase that they are synthesizing molecules essential for the resumption of meiosis (Bachvarova *et al*., 1985) (**Figure 1**).

Once the follicle reaches a species-specific size, granulosa cells start to secrete glycoproteins, which coalesce to form the fluid-filled antral cavity within the granulosa cell layers (Gosden and Telfer, 1987b; Lussier *et al*., 1987; Hirshfield, 1991a). It is at the antral stage that follicles become acutely dependent on gonadotrophins for further growth and development (Nayudu and Osborn, 1992) and the oocyte has completed most of its growth by the time of antral formation, taking up to 3 months in humans (Gougeon, 1994).

At the antral stage of development the granulosa cells separate from a common precursor population (Telfer *et al*., 1988) into two structurally and functionally distinct sub-types: mural granulosa cells, which are located on the inner side of the basal membrane, and cumulus granulosa cells that surround the oocyte. The maintenance of cumulus granulosa cell contact with the oocyte through gap junctions is essential, (Anderson and Albertini, 1976; Simon and Goodenough, 1998) as the oocyte is dependent on the contact for a supply of nutrients for further development. Oocytes in early antral follicles have become competent to resume the first meiotic division (Wickramasinghe *et al*., 1991) but granulosa cell contact maintains the oocyte in meiotic arrest until the follicle has reached the appropriate developmental stage, at which point ovulation occurs (Eppig and Downs, 1984; Wert and Larsen, 1989; Eppig, 1991). During the growth phase of oocyte development the maternal imprints are laid down on the genome. The imprints are not all established at the same time, with each imprinted gene having a specific time at which it will be methylated (Swales and Spears, 2005). At the end of this growth phase the oocyte within the pre-ovulatory follicle will have acquired meiotic and developmental competence (Ebner *et al*., 2006). The relationship between acquisition of developmental competence and oocyte development remains unclear; however, using a range of model systems we are gradually acquiring information on the array of factors regulating these processes.

Factors regulating oocyte/follicle development

The growth and development of the ovarian follicle involve tightly co-ordinated regulation by paracrine and endocrine factors (see **Figure 2** for a summary), and is dependent upon maintenance of cell communication between the component parts of the follicle at each stage of development.

Ever since the experiments of Pincus and Enzmann (1935) it has been established that the somatic cells of the follicle support oocyte development. Granulosa cells regulate meiosis by direct transfer of meiosis-arresting signals through gap junctions and the oocyte is dependent upon the surrounding granulosa cells for factors that will support growth and development (Eppig, 1979; Brower and Schultz, 1982; Buccione *et al*., 1990). Granulosa contact and differentiation are essential for the regulation of meiosis (Canipari *et al*., 1984; Buccione *et al*., 1990; Chesnel *et al*., 1994), modulation of oocyte transcriptional activity (De La Fuente and Eppig, 2001), inducing post-translational modifications of several oocyte proteins (Colonna *et al*. 1989; Cecconi *et al*., 1991) and improving preimplantation embryo development (Malekshah and Moghaddam, 2005).

It is now well established that a bi-directional communication exists between the oocyte and somatic cells, and that oocyte factors are essential for normal development of the somatic cells and subsequently of the oocyte itself (Eppig, 2001). Oocyte secreted factors have been shown to be important in differentiation of cumulus and mural granulosa cells (Vanderhyden *et al*., 1990), regulating cumulus cell expansion (Buccione *et al*., 1990; Ralph *et al*., 1995) and in regulating granulosa cell proliferation and maintaining structure during pre-antral follicle development follicles (Vanderhyden *et al*., 1990, 1992). It is now clear that the oocyte affects many processes at different stages of follicular development, and the influence of the oocyte on granulosa cells changes with the progression of their development (Latham *et al*., 2004). Subsequent studies have gone on to identify a whole range of factors known to be produced by the oocyte (Taft *et al*.,

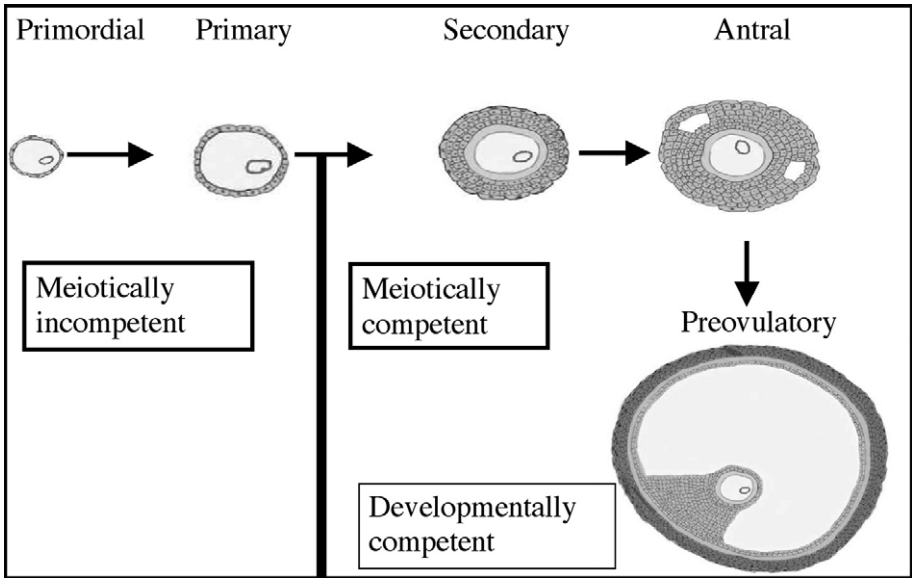

Figure 1. Stages of follicle development from primordial to pre-ovulatory. The whole period of development can take over 100 days in humans and around 24 days in mice. During the early growth phase the oocyte acquires meiotic competence with cytoplasmic and full developmental competence being achieved later in development.

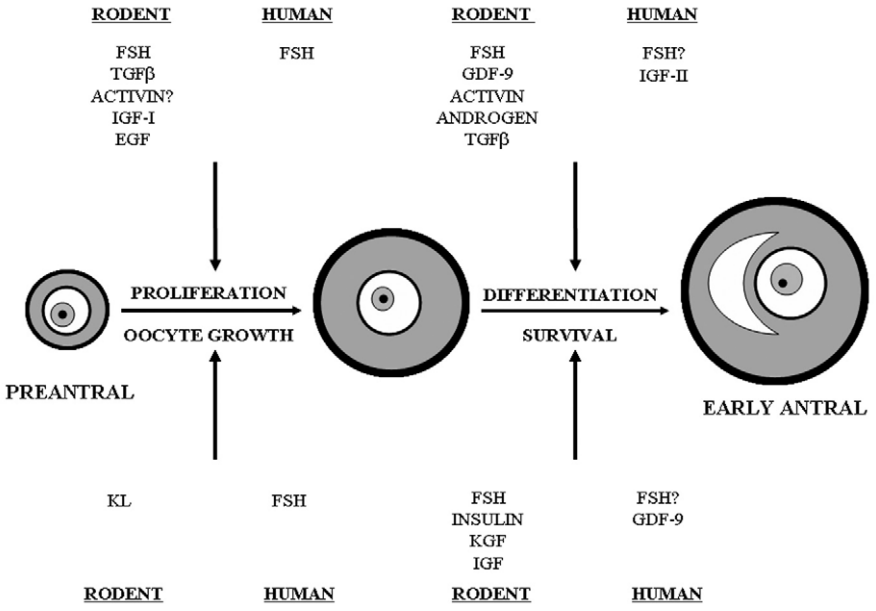

Figure 2. Summary of putative regulatory factors for somatic cell proliferation and differentiation (top) and oocyte growth and survival (bottom) during follicular development in rodents and humans. Most of these factors have been tested using follicle culture models (see Thomas *et al*., 2003). EGF = epidermal growth factor; GDF-9 = growth differentiation factor-9; IGF = insulin-like growth factor; KGF = keratinocyte growth factor; KL = kit-ligand; TGFβ = transforming growth factor β.

2002); however, the two most studied oocyte factors are growth differentiation factor-9 (McGrath *et al*., 1995; Dong *et al*., 1996) and bone morphogenetic protein-15 (Dube *et al*., 1998; Solloway *et al*., 1998).

Utilization of oocytes throughout life

The pool of primordial follicles is progressively reduced throughout life (Jones and Krohn, 1961; Gougeon *et al*., 1994) and in most species studied it has been shown that this loss is greatest during immature life, with almost no follicles present in the post-menopausal human ovary (Richardson *et al*., 1987; Faddy *et al*., 1992). The loss of follicles from the primordial pool must be as a result of both follicle growth and death, and an accurate measurement of both of these variables is important to determine rate of utilization during life. Predictions of how oocytes are utilized throughout life have been made using mathematical modelling under normal (Faddy *et al*., 1987) and manipulated conditions (Gosden *et al*., 1989; Telfer *et al*., 1991). These models all make the basic assumption that there is a fixed non-renewable population of primordial follicles that must last the female throughout her reproductive lifespan. However, this assumption has recently been challenged by the publication of two papers that suggest oocyte renewal/regeneration occurs in adult mice and possibly humans (Johnson *et al*., 2004, 2005).

Whether or not the numbers of germ cells are fixed early in life or capable of formation in adult life produced a lively scientific debate during the 1920s, with the general view being that the oocyte supply was fixed (Pearl and Schoppe, 1921) but this was challenged by Allen (1923). At this time it was proposed that cyclical proliferation of the germinal epithelium gave rise to oocytes at each oestrous/menstrual cycle (Allen, 1923; Allen and Creadick, 1937). This was a widely held view until the 1950s when Zuckerman (1951) ended the debate by an extensive study based on differential counts of follicles that concluded oocytes were not produced throughout life. Further studies using tritiated thymidine labelling of oocyte nuclei have confirmed that oocytes in juvenile and adult ovaries are direct descendants of the fetal germ cells and that there is no increase in number throughout life (Rudkin and Griech, 1962; Borum, 1966; Peters and Crone, 1967). The recent work from Tilly's laboratory (Johnson *et al*., 2004, 2005) has opened a new debate on the existence of germ-line stem cells in the mammalian ovary.

Johnson *et al*. (2004) have suggested that oocyte renewal must be occurring to maintain oocyte numbers given the level of observed degeneration of oocytes, and they claimed to have found germ-line stem cells in the ovarian surface epithelium of the adult mammalian ovary, but the evidence for this was open to interpretation and has been challenged by several ovarian biologists (Albertini, 2004; Gosden, 2004; Byskov *et al*., 2005). A further study by Johnson *et al*. (2005) suggested that the source of germ-line stem cells was not ovarian, but that bone marrow or peripheral blood acted as reservoirs of putative stem cells for germ-cell regeneration in the adult mouse ovary. Tilly's group showed that if oocytes were chemically destroyed and the mice subsequently injected with bone marrow from adult animals, the chemically ablated ovary was re-populated by small ovarian follicles (Johnson *et al*., 2005). The physiological significance of these findings was challenged (Telfer *et al*., 2005) and subsequent experimental evidence has cast doubt on the developmental potential of the follicle structures observed in the Johnson study (Eggan *et al*., 2006). The elegant study by Eggan *et al*. (2006) utilized a parabiotic animal model, where a pair of mice shares their circulatory systems, to determine if circulating germ-line stem cells could be transferred between animals. One animal of the pair ubiquitously expressed green fluorescent protein (GFP) while the other did not, therefore circulation of stem cells would be identified by mixing GFP and non-GFP cells in germ cells. It was demonstrated that no transfer of germ-line stem cells could be detected in ovulated oocytes and, furthermore, oocyte production was not rescued by bone marrow transfer after oocyte destruction by either chemical ablation or irradiation (Eggan *et al*., 2006). Whilst this latest piece of work would support the existence of a fixed population of oocytes, it does nothing to explain the observation of Johnson *et al*. (2005) of the development of small follicle-like structures after bone marrow transplantation.

These studies have raised two separate issues of: (i) whether pluripotent stem cells can develop into germ cell-like cells; and (ii) whether this is a mechanism for oocyte development in the normal functioning ovary. It has been shown that pluripotent stem cells can develop into oocyte-like structures (Hubner *et al*., 2003) and recent work has shown that bone marrow stem cells can develop into male germ cells (Nayernia *et al*., 2006); however, the scientific evidence goes against this being a mechanism for oocyte development under normal physiological conditions (Eggan *et al*., 2006).

In-vitro development of oocytes

The limitation in supply of mature, fertilizable oocytes (i.e. oocytes with full developmental competence) constitutes a major block to increasing the success of assisted reproduction techniques, developing strategies for fertility preservation after chemotherapy and stem cell derivation in humans. Improvements in assisted reproduction techniques are dependent on developing techniques that will improve the supply of developmentally competent oocytes. Recent work on derivation of germ cells from stem cells (Hubner *et al*., 2003) might be viewed as a potential source of oocytes; however, the normality of these structures has not been shown and their generation presents many practical and ethical problems.

It has long been recognized that one of the best means to boost the efficiency and efficacy of assisted reproduction programmes would be to use oocytes from immature follicles for in-vitro growth and subsequent in-vitro maturation. These techniques in combination with cryopreservation would offer new practical applications for fertility preservation. Complete growth in culture from the primordial stage with subsequent IVF of oocytes followed by embryo transfer and production of live offspring has, so far, only been achieved in the mouse (Eppig and O'Brien, 1996; O'Brien *et al*., 2003). Other rodent culture systems have produced developmentally competent oocytes and viable offspring, but they start with growing follicles and require a shorter period *in vitro* (Eppig and Schroeder, 1989; Roy and Greenwald, 1989; Spears *et al*., 1994; Cortvrindt *et al*., 1996). However, primordial follicles represent the most abundant population of oocytes in the ovary at any age (Gosden and Telfer, 1987a) and in-vitro culture of human primordial

and primary follicles within slices of ovarian tissue has shown follicles initiating, developing to secondary follicles and occasionally to early antral follicles (Hovatta *et al*., 1997, 1999; Wright *et al*., 1999; Louhio *et al*., 2000), but we are still a long way from being able to use follicle culture as a strategy for obtaining competent oocytes from human material (Thomas *et al*., 2003; Abir *et al*., 2006).

Development of culture systems for species such as cow and sheep provide good models to determine the feasibility of transferring these techniques for human application. The follicles of these domestic species are more closely matched to those of humans in terms of growth rates and size. Growth and differentiation of immature bovine (Gutierrez *et al*., 2000; McCaffery *et al*., 2000; Thomas *et al*., 2001; Walters, 2006) and ovine (Cecconi *et al*., 1999) follicles have been achieved *in vitro* and ovine oocyte–granulosa complexes from fresh and frozen–thawed tissue have been grown to the antral stage during 30 days of culture (Newton *et al*., 1999). However, none of these studies has produced oocytes capable of nuclear maturation. Most of these culture systems have resulted in accelerated somatic cell differentiation; however, this has to be co-ordinated with oocyte development (Thomas *et al*., 2003). Efficient delivery of factors to and from the oocyte at critical stages of development is essential for the co-ordination of oogenesis and folliculogenesis. As mentioned earlier, paracrine factors secreted by oocytes and somatic cells regulate many important aspects of follicular development, and there is substantial evidence that supports a model for bi-directional paracrine communication, based on the developmental regulation of the delivery and reception of paracrine factors at the oocyte–granulosa cell interface (Albertini *et al*., 2001).

The ability to mimic the full period of growth of a follicle is an ambitious task and in all species will involve a multi-step culture system (**Figure 3**). Each of the culture steps should be optimized for key transition stages, but ideally be accelerated since a protracted culture period is not desirable. Follicular development is a chain of many complex and precisely regulated events; therefore, gaining a better understanding of these processes is critical to the advancement of such technology (Thomas *et al*., 2003). Progress is being made in a step-wise manner, using different factors and follicle stages, allowing researchers to piece together the mechanisms governing follicular development. In human and domestic animal species, where follicular growth is a protracted process, it is important to focus on identification of cellular and molecular events that co-ordinate oocyte and somatic cell development. This, in turn, will allow adaptation of culture regimes for optimal oocyte growth and acquisition of developmental competence.

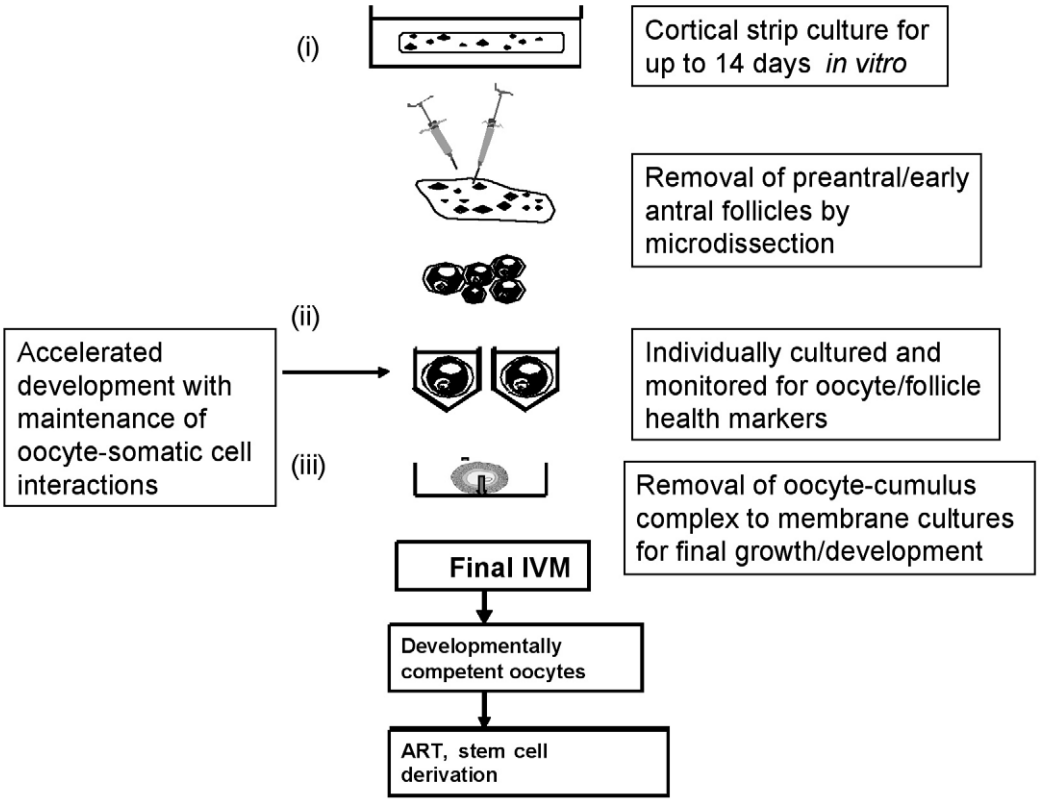

Figure 3. Three-step culture system for in-vitro growth of oocytes from primordial follicles: (i) Cortical strip culture of either fresh or frozen pieces for up to 14 days (Hovatta *et al*., 1997); followed by (ii) isolated follicle culture of dissected pre-antral/early antral follicles in individual wells to monitor markers of oocyte health (McCaffery *et al*., 2000; Telfer *et al*., 2000; Thomas *et al*.2001, 2007; Walters *et al*., 2006); followed by (iii) removal of oocyte–granulosa cell complexes for final growth and maturation on collagen membrane support (Hirao *et al*., 2004). ART = assisted reproduction technique; IVM = in-vitro maturation.

Finally, the development of non-invasive markers as a basis for monitoring follicular development during long-term culture and identifying good quality oocytes will be essential to the application of such techniques. Some markers such as the matrix metalloproteinases have been shown to be predictive of oocyte quality during culture of bovine pre-antral follicles (McCaffrey *et al*., 2000), but an array of markers at each transition point will be required to accurately predict the viability and/or fate of in-vitro-grown oocytes.

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