ABSTRACT: Prenatal oogenesis produces hundreds of thousands of oocytes, most of which are discarded through apoptosis before birth. Despite this large-scale selection, the survivors do not constitute a perfect population, and the factors at the cellular level that result in apoptosis or survival of any individual oocyte are largely unknown. What then are the selection criteria that determine the size and quality of the ovarian reserve in women? This review focuses on new data at the cellular level, on human prenatal oogenesis, offering clues about the importance of the timing of entry to meiotic prophase I by linking the stages and progress through MPI with the presence or absence of apoptotic markers. The characteristics and responsiveness of cultured human fetal ovarian tissue at different gestational ages to growth factor supplementation and the impact of meiotic abnormalities upon apoptotic markers are discussed. Future work will require the use of a tissue culture model of prenatal oogenesis in order to investigate the fate of individual live oocytes at different stages of development.

Key words: oogenesis / fetal / oocyte / apoptosis / meiosis

Introduction

The quality of mature oocytes is frequently suboptimal in humans. Despite only a small proportion of a large prenatal population being selected to continue development, many surviving oocytes carry anomalies that may impair their ultimate function as gametes (Hunt and Hassold, 2008). There have been many attempts to explain the biological rationale for such apparently wasteful cellular attrition in the germline; for example, sub-lethal anomalies could contribute to the potential for variation and evolutionary success, and non-surviving cells could donate crucial components to be recycled by survivors (Baum et al., 2005). However, none of the ideas proposed can explain the massive scale and apparent lack of efficiency of the selection process. A large majority of oocytes are lost before birth, and most of the rest before puberty. The remaining oocytes are not a perfectly viable population and so the criteria upon which they have been selected remain unclear. In this review, we focus on what is known in this area and add some additional insights from our ongoing research.

Hypothesis

The purpose of our work is to understand the bases upon which oocytes are selected over the prolonged period from their prenatal formation, when oogonia enter meiosis, to their eventual demise, or exceptionally, their ovulation during reproductive adulthood. Our current hypothesis is that prenatal loss of oocytes reflects selection at the cellular level, for or against cell death, on the basis of certain measurable criteria. Accordingly, we hypothesize that events at the cellular level must be coordinated to result in the quantitatively reliable changes that eventually result in a relatively consistent population of oocytes in the ovarian reserve. At present, we are studying how prenatal oocytes are selected for further development or apoptosis at an individual oocyte level.

Our concept of selection also infers a deliberate process rather than random chance. Our idea is that a cellular decision is made, to survive or die, in association with or in response to the prevailing circumstances (a bottom-up approach). The alternative approach might be that a set of controlling measures originates from a higher level, for...
example, a requirement for an ovarian reserve of a particular size (a top-down approach). Biomarkers that reflect the ovarian reserve, such as the concentration of anti-Mullerian hormone, or response to gonadotrophin-releasing hormone challenge, are sometimes measured clinically in order to estimate the ovarian reserve of a woman at risk of infertility (Coccia and Rizzello, 2008). Such methods are particularly relevant where there is a known likelihood of reduced numbers of oocytes, for example, owing to advanced age, or chemotherapy. However, definitions by which to diagnose ‘normal’ or ‘abnormal’ on the basis of these markers have not been achieved and no clinically feasible means of slowing the age-related decline of the ovarian reserve has yet emerged. We have taken the contrasting bottom-up approach because the size of the ovarian reserve seems remarkably resistant to alteration owing to adverse external circumstances arising from the maternal environment, such as undernutrition (Lumey and Stein, 1997), which are capable of affecting many other developmental processes (Barker and Clark, 1997). Therefore, we are looking for factors local to the fetal ovary, as a first step towards understanding oocyte selection for both quality and quantity.

**Overview of oocyte selection**

Selection for survival or cell death within the ovary can occur at several stages of oocyte development. Selection during germ cell migration to the ovary is not considered here. This article mainly addresses the first two stages listed below, when selected oogonia enter meiosis and progress through meiotic prophase I (MPI) to arrest at the diplotene stage. However, an overview is provided to place these in context. This article refers to humans throughout, unless otherwise stated.

We have focused on humans since important differences from model animals have been noted. A timeline for human prenatal oogenesis is presented in Fig. 1 showing the progressive but often overlapping stages. The following are important stages in oogenesis:

(i) oogonia to oocyte transition (entry to MPI);
(ii) MPI;
(iii) breakdown of oocyte nests;
(iv) enclosure by somatic cells to form primordial follicles;
(v) timing of initiation of early follicle growth (or failure of timely arrest of follicle growth at the primordial stage);
(vi) follicular growth and competition leading towards ovulation in adulthood.

(i) Cell death is quantitatively a major activity of prenatal oocytes, particularly in the mid-trimester for humans (Vaskivuo et al., 2001) and from around 13 dpc in mice (Lobascio et al., 2007). This contrasts with the preceding stage of active expansion of the germline, where primordial germ cells (PGCs) migrate to the ovary and replicate to form diploid oogonia, which continue to undergo mitosis (Kurilo, 1981). Apoptotic cell death does occur in PGCs and oogonia, notably via mechanisms that are responsive to the presence of survival factors such as stem cell factor (SCF) (also known as kit ligand) (Krysко et al., 2008) and possibly oxidative stress (Murdoch et al., 2003, sheep), but the overall result in vivo at this time is the enlargement of the precursor cell population. This stage has been reviewed previously (De Felici et al., 2005; Oktem and Oktay, 2008).

(ii) Oogonia enter MPI and are now termed ‘oocytes’. Oocytes progress through MPI, via the leptotene, zygotene and pachytene stages towards the diplotene stage, to enter meiotic arrest at that point. Extensive apoptosis occurs as oocytes progress through MPI, leading to the loss of many of them. In humans, meiosis is not synchronous (Gondos, 1987), and even in species such as the mouse, where more synchrony is evident, there is still variation between neighbouring oocytes in terms of meiotic progression. The local signals controlling the survival of oogonia and oocytes in early MPI are not clear, although much progress has been made towards understanding the female germline in mice (e.g. Farini et al., 2005).

(iii) Oocytes entering meiosis are normally found in so-called ‘nests’. These clustered oocytes, generally lacking closely associated pre-granulosa cells, are believed to arise from a group of daughter cells derived from the same dividing oogonium. The oocytes within a nest communicate via intercellular bridges and also trend to maintain more synchrony within, compared with between, nests (Gondos, 1987; Pepling, 2006, mouse). However, inevitably, not all of the oocytes of a nest survive to form follicles, and it has been proposed that some of the oocytes may die altruistically to ensure that one or two from a particular nest can survive (Pepling and Spradling, 2001, mouse). The factors that determine which oocytes from a nest are selected for survival are unclear, but may represent one level of coordination that could eventually result in a suitably sized ovarian reserve. Estrogen may be a mediator in this process (Zachos et al., 2002).

(iv) Oocytes continuing through meiosis to complete the diplotene stage (or perhaps earlier in MPI, Ohno et al., 1962; Yang and Fortune, 2008, cattle) can recruit somatic pre-granulosa cells to surround them, resulting in primordial follicles. The formation of primordial follicles can be influenced by genetic manipulation, for example, knockout mice illustrate that several genes influence oocyte survival or apoptosis, or nest breakdown, thereby affecting the numbers of follicles formed (e.g. bcl-2, Ratts et al., 1995, mice; caspase 2, Bergeron et al., 1998, mice; bax, Perez et al., 1999, mice). Bax and bcl-2 are active in human fetal ovaries (Albamonte et al., 2008), but many of the potential effectors identified in mice have not yet been studied in humans. Primordial follicles have been reported from as early as 13 weeks of gestation in humans (Forabosco and Sforza, 2007), but are generally considered to become abundant from around 16–20 weeks of gestation (Rabinovici and Jafe, 1990; Albamonte et al., 2008). Changes in gene expression in human fetal ovaries around the time of primordial follicle assembly are now providing clues about the pathways controlling early folliculogenesis (Fowler et al., 2009). For recent reviews of the timing and control of primordial follicle assembly, see Skinner (2005), or Maheshwari and Fowler (2008).

(v) If the resulting primordial follicles are to participate in reproductive life, they must remain in the primordial stage for several years, at least until puberty. In this state, the oocytes remain arrested in the diplotene stage of MPI, and the surrounding granulosa cells support the oocyte’s metabolic and signalling requirements (for example, see Matzuk et al., 2002; Gilchri$t et al., 2008). However, a proportion of oocytes and follicles either do not become, or do not stay, arrested (probably by escaping local inhibitors, Wandji et al., 1997, baboon; Yang and Fortune, 2008, cattle), or they initiate growth during prenatal or prepubertal life. These growing follicles are a consistent feature of ovaries during childhood (Peters et al., 1976). They always become
atretic due to the prevailing immaturity of the hypothalamo-pituitary axis (Djahanbakhch et al., 2007). Their function is unclear; however, a significant consequence is to reduce the ovarian reserve from around 700,000 at birth to 300,000 at puberty (Faddy et al., 1999).

In mice, follicle growth begins only after birth (in contrast to humans). In mice, specific deletion of Pten in oocytes caused the premature activation of the primordial follicle pool, first evident at postnatal day 8 and resulting in complete elimination of all follicles by about 3 months (Reddy et al., 2008). The possible role of Pten in human fetal ovaries has not yet been studied.

Also in mice, there is an interesting shift in the predominant mechanism of oocyte loss from apoptosis before folliculogenesis, to multiple pathways including non-apoptotic mechanisms, causing loss of primordial follicles (Rodrigues et al., 2009; Tingen et al., 2009). The critical role of the somatic cell fraction is barely understood at this early stage, but must have major impacts, most obviously as pre-granulosa cells engage intimately with the oocyte during folliculogenesis, their cytoplasmic extensions forming junctions with the oolemma.

(vi) Follicles that initiate growth during adulthood may be capable of yielding a mature fertilizable oocyte, depending on the endocrine environment at the time of their growth. The process of selection of the ovulating follicle is competitive, and apoptotic processes in the adult ovary have been studied extensively (Hussein, 2005).

This article will highlight some emerging concepts on oocyte selection during prenatal oogenesis, shedding light on the interactions between apoptosis and timing of meiosis, using data acquired from human tissues, where possible.

### Oogonia to oocyte transition: timing of entry into MPI

This transition is marked by entry into meiosis of diploid oogonia derived from the PGCs. In humans, estimates of the proportion of oogonia that enter meiosis vary widely (50%, Baker, 1963; 90%, Kurilo, 1981). Using data from McClellan et al. (2003) in CD-1...
mice, we estimate that at most 80% of oogonia make this transition. Genetic mutant mice that lack \textit{bax}, a pro-apoptotic gene, have excessive numbers of oocytes entering meiosis, suggesting this gene’s involvement in oogonial selection (Alton and Taketo, 2007). Oogonia that do not enter meiosis are removed either by apoptosis (Albamonte et al., 2008) or by arrest in a metaphase-like conformation associated with an unusual form of cell death (Wartenberg et al., 2001). Retinoic acid (RA) may be the critical factor whose presence induces meiosis in females, but whose relative absence protects against premature meiosis in males (Bowles and Koopman, 2007, mice). Oogonia located towards the medulla in humans are the first to enter MPI (Bendsen et al., 2006) and the most advanced during fetal folliculogenesis, initiating follicle formation and growth first (Byskov and Lintern Moore, 1973, mice). In mice and other species studied, meiosis begins in the part of the ovary that is in closest proximity to the mesonephros or the rete ovarii, derived from mesonephros cells migrating into the gonad (Bowles and Koopman, 2007). It is likely that the timing and geographical organization of entry into meiosis reflect the location and origins of RA, which originates from the mesonephros or rete ovarii, and which is a small diffusible molecule whose gradient may have a vital role in the organization and timing of the onset of MPI at the ovarian level (Bowles and Koopman, 2007, mice). RA has not yet been studied in human fetal ovaries, where a similar role may be anticipated; however, additional local control will be necessary to account for the variability in entry to meiosis among adjacent oocytes, which would not be expected if RA gradient were the sole mediator.

A possible influence of the timing of meiotic entry upon later oocyte quality has long been suspected (Jones, 2008). The timing of entry to meiosis has been proposed as one possible mechanism by which the maternal age-associated increase in chromosomal abnormalities in children could arise. The so-called ‘first-in, first-out’ hypothesis links the sequence of oocyte formation with utilization, proposing that those formed later are utilized later in life, resulting in increasing abnormalities as the age of the mother increases (Henderson and Edwards, 1968; Polani and Crolla, 1991). Other hypotheses are based upon ideas on accumulated oxidative damage (Harman, 1956), telomere function (Keefe et al., 2006) and, most recently, ovarian mosaicism (Hultén et al., 2008); however, the origin of the maternal age effect is an enduring enigma that may only be elucidated by a greater understanding of prenatal events in oogenesis.

We are interested in how human oogonia may be selected to enter MPI. We have therefore investigated the timing of entry to MPI, with a view to understanding whether the timing is important for subsequent utilization or apoptosis of oocytes.

Entry into meiosis requires the availability of precursor cells. We checked this in primary explants of fetal human ovaries by alkaline phosphatase (AP) staining of cells manually spread on glass slides. AP is present in PGCs and oogonia, but is reduced and eventually lost as oocytes enter meiosis. In humans, strongly AP-positive precursor cells were evident at least as late as 19 weeks of gestation (Fig. 2), long after MPI has begun in many cells. The arrest of the remaining oogonia in a metaphase-like state, which probably originates as an aborted entry into meiotic prophase, appears to occur in humans at around 23–26 weeks (Kurilo, 1981), so some of the persistent oogonia may be capable of making a late but successful transition into MPI. Using a meiosis-specific marker for synaptonemal complex (SC) proteins, we found that zygote-stage oocytes were evident from 14 weeks, and pachyten-stage oocytes from 15 weeks of gestation (Hartshorne et al., 1999). However, many oocytes in these early stages of MPI were present until at least 19 weeks of gestation (as shown in Fig. 1 and in agreement with Kurilo, 1981; Speed, 1985; Roig et al., 2005a, b).

In order to test whether oocytes entering MPI earlier or later have an increased likelihood of being selected for cell death, we used terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) as a marker of DNA breaks in oocytes at different stages of MPI at known gestational ages. TUNEL highlights breaks in DNA that occur during the later stages of apoptosis. It is not a specific marker of apoptosis, but may offer insights into the state of DNA. TUNEL was used successfully as a correlate of cell death in human oocytes by Modi et al. (2003), who found that 3–7% of oocytes were apoptotic between weeks 13–23 in normal ovaries, rising to >50% in Turner’s syndrome 45XO ovaries, where extensive prenatal loss of oocytes occurs. Like Modi et al. (2003), Albamonte et al. (2008) observed low levels (<10%) of TUNEL-positive germ cells throughout the early second trimester, but they found a higher incidence (~20%) at 18–20 weeks, which in their study coincided with the switch from a bcl-2/bax balance to bax dominance, as bcl-2, which inhibits apoptosis, became undetectable at this stage. However, Abir et al. (2002) reported conflicting results, noting overexpression of bcl-2 in all samples at all ages, with TUNEL positivity undetectable before 23 weeks.

Unfortunately, tissue sections do not allow reliable identification of MPI stages. For this, oocyte spreads are essential. We therefore applied TUNEL on oocytes that had been spread on slides, enabling accurate cytogenetic determination of their stage of MPI. Spreads confer increased accessibility of reagents because the cytoplasm has been removed, in contrast with sections of tissues embedded in wax or other media. Consequently, the extent of TUNEL staining in our data and that of others using this method (DeFelici et al., 2008) is higher than that reported for tissue sections. Figure 3 shows that pre-leptotene oocytes are predominantly TUNEL-positive, as expected owing to dsDNA breaks during reorganization in preparation for meiosis (Mahadevaiah et al., 2001). dsDNA breaks are also prevalent in the leptotene stage. However, we found that leptotene, and particularly zygote-stage oocytes that persisted at 18 and 19 weeks of gestation, were more likely to be TUNEL-positive than those at 17, 16 and 15 weeks of gestation. TUNEL-positive oocytes comprised at most half of the total of leptotene- and zygote-stage oocytes until 17 weeks. These novel data therefore tend to support the idea that oocytes entering late into meiosis may be more likely to succumb to DNA damage and possible demise. If the observed TUNEL coincides with the DNA fragmentation of cell death, it is possible that oocyte elimination in human fetal ovaries may take place at the zygote stage rather than at the diplotene stage (as in mice). Further investigation of human oocytes using specific markers of apoptosis is necessary to test this idea.

Our data also confirm that oocytes are found in the pre-leptotene and leptotene stages of MPI across a wide range of gestations, supporting the idea of gradual non-synchronous entry of human oogonia into MPI. This lack of synchrony adds complexity to the interpretation of human data from fixed preparations. Clarity will only be achieved when individual live oocytes can be followed through MPI in vitro. Too few pachytene- and diplotene-stage oocytes were analysed to draw conclusions regarding the completion of MPI.
Interestingly, a more extensive study using the same method in mice found different results (Fig. 4, details in Ghafari et al., 2007). In mice, oocytes reaching the pachytene stage early (16 dpc) were more likely to be positive for TUNEL, whereas those in pachytene on Day 18 were more likely to be TUNEL-negative. These significant results contrasted with our data in humans (Fig. 3) but showed some similarities to dsDNA break detection in spermatogenesis (Qin et al., 2004). Co-staining for cleaved poly-(ADP-ribose) polymerase-1 (PARP-1), a marker of apoptosis, showed that mouse oocytes in the pachytene stage, either early (15.5 dpc) or late (18 dpc), were less likely to be apoptotic than those on Days 16 or 17 (Fig. 4). From this, we conclude that, in mice, there may be an optimal schedule for oocyte progression, deviation from which may increase the chances of apoptosis. We also note that the apoptotic marker, cleaved PARP-1, and TUNEL staining were not coincident, demanding cautious interpretation. It is also highly likely that different pathways of apoptosis are active and their interaction and relative prevalence at different times are not yet fully understood (DeFelici et al., 2008).

Thus, we offer some additional evidence that the fate of oocytes may be affected by the timing of their entry into meiosis. The transition time through the stages of MPI may reflect, to some extent, oocyte developmental competence (Roig et al., 2005a, b), and further studies are needed to track individual oocytes in vitro and gain detail on apoptotic decision-making at the cellular level. Although the question of apoptotic mechanisms in vitro has not yet been addressed for human fetal oocytes, progress has been made in mice by DeFelici et al. (2008) using short-term cultures. Their results clearly show that several pathways of apoptosis are active in mouse fetal oocytes, and they also present evidence of autophagy and necrosis (DeFelici et al., 2008). Importantly, they note that inhibitors of caspases, enzymes that mediate the cascade leading to DNA destruction, are only partially effective in inhibiting oocyte apoptosis, indicating that caspase-independent mechanisms must also be operational. They also confirm our observation in human oocytes that DNA fragmentation identified by TUNEL is not always associated with apoptosis (Ghafari et al., 2007). Currently, the interactions of the many possible pathways of cell death involved in prenatal oocyte selection are not understood. Their further study will prove vital in understanding the different selection criteria applied to oocytes at a cellular level.

**Mechanisms affecting oocyte survival decisions: the changing ovarian environment**

It is unclear to what extent the balance for individual oocyte decisions to continue development or to initiate cell death might relate to local effects of the developing ovarian somatic cell compartment upon oocyte survival (e.g. endocrine milieu, intercellular signalling, extracellular matrix), or to the inherent qualities of oocytes in MPI (e.g. errors in synapsis which may delay meiotic progression or cause arrest). We are interested in comparing these intrinsic and extrinsic possibilities at different stages of development, thereby exploring tolerance for deviation from the normal programme of development.

A range of paracrine factors, identified in the human fetal ovary, may affect oocyte survival, at one stage or another (e.g. Bennett et al., 1996; Schilling and Yeh, 1999; Martins da Silva et al., 2004; Coutts et al., 2008). For some paracrine factors, progress has been made in identifying the originating cell type and the locations of receptors; however, the local ovarian environment is complex and constantly changing both through fetal life and subsequently. There are a number of so-called ‘survival factors’, without which germine cells will die, for example, SCF, which is essential during PGC migration and for the survival of early oocytes (Moniruzzaman et al., 2007, mice; Gu et al., 2009, mice). SCF, insulin-like growth factor 1 (IGF-1) and leukocyte inhibitory factor support the survival of post-migratory germine cells (Morita et al., 1999, mice). Superimposed upon these are the pro- and anti-apoptotic mechanisms and other cell death pathways that are functional at particular stages (see, e.g. Quenby et al., 1999; Vaskivuo et al., 2001; Fulton et al., 2005). So, for example, in mice, an absence of kit signalling activates the Fas cell death pathway within pre-follicular oocytes (Sakata et al., 2003, mice), and
tumour necrosis factor alpha promotes apoptosis around the time of follicle formation, detected by TUNEL, in oocytes, interstitial and granulosa cells (Morrison and Marcinkiewicz, 2002, rat).

Within nests, inter-oocyte communication is mediated via cytoplasmic bridges, but after nest breakdown, inter-follicular communication might continue, for example, via molecules produced by granulosa cells (DaSilva-Buttkus et al., 2009). The developing ovary is also influenced by the developing fetal endocrine system, and transcripts of some steroidogenic enzymes are present in the ovary from at least 15 weeks of gestation (Voutilainen and Miller, 1986), whereas the capacity to metabolize androgen to estrogen is present from around 12 weeks of gestation (George and Wilson, 1978). Estrogen and particularly progesterone inhibit follicular assembly in rats, possibly via inhibiting apoptosis in oocytes (Kezele and Skinner, 2003). However, the progesterone may be at least partially endocrine rather than local, because an accelerated transition from primordial to primary follicles, owing to a lack of progesterone, was noted when ovaries had been removed from the in vivo environment.

**Figure 3** The proportions of TUNEL-positive and TUNEL-negative oocytes at different stages of MPI, as identified by SCP3 staining, in human fetal ovaries at different gestational ages. Notice, at 18 and 19 weeks gestation, TUNEL-positive oocytes are more prevalent, in contrast to earlier ages. The numbers of fetuses examined from weeks 13 to 19 of gestation were 1, 2, 2, 2, 2, 2, 1, respectively. Continuous lines represent TUNEL-positive. Broken lines represent TUNEL-negative.
Figure 4 TUNEL and cleaved PARP-1 staining for apoptosis in mouse oocytes. TUNEL indicates DNA breaks, and cleaved PARP-1 is a marker of apoptosis. (A) The proportions of TUNEL-positive and TUNEL-negative B6CBf2 mouse oocytes at different stages of MPI between 14.5 and 21 days post-coitum. (B) The proportions of cleaved PARP-1-positive and cleaved PARP-1-negative B6CBf2 mouse oocytes at different stages of MPI between 14.5 and 21 days post-coitum. a versus b, c versus d, P < 0.05. Each oocyte was classified as being in a particular stage of MPI by core protein (COR-1) staining and was also categorized as staining for either TUNEL or cleaved PARP-1, or both or neither (as described in Ghafari et al., 2007). In the graphs presented, TUNEL-positive oocytes may be either positive or negative for cleaved PARP-1, and PARP-1-positive oocytes may be either positive or negative for TUNEL. A total of 1960 oocytes from 24 fetal mice (3 per time point) were analysed.
Possible extra-gonadal sources of such steroids have been identified in human fetal tissues (Pezzi et al., 2003). Experiments where ovaries of a particular genotype are transplanted into severe combined immune-deficient mice can be applied to demonstrate whether local action of a gene is needed, so, for example, local presence of the Fas gene within the ovary is
We found that cultures of ovarian fragments from fetuses at 14 weeks of gestation were prone to expansion in vitro, with cells moving away from the initial fragment and eventually covering much of the membrane with clusters and aggregates (Hartshorne et al., 1999). AP staining suggested that germ cells were mainly concentrated in the initial tissue fragment and in clusters that formed in the outgrowth (data not shown). The curious ability of oocytes to migrate during fetal life, even during MPI, has already been noted by previous authors (Blandau et al., 1963, mice; Motta et al., 1997). Similar but smaller aggregates were observed by Zhang et al., (1995), whose methods were similar; however, their tissue donors were slightly older, aged 16–20 weeks. Since the pioneering studies on culture of human fetal ovarian tissue (Baker and Neal, 1974), interest has renewed recently and other methods have been reported. For example, Wu et al. (2002) used minced tissue in dishes, whereas Biron-Shental et al. (2004) used organ culture techniques, as applied for adult tissue. Both of these groups observed follicle survival and growth in vitro from tissue of 16–23 and 22–23 weeks of gestation, respectively. Roig et al. (2006) and Sadeu et al. (2006), respectively, cultured ovarian samples of 17–22 weeks of gestation and 20–22 weeks of gestation in tubes. Roig et al. (2006) confirmed meiotic initiation and progression similar to that observed in our culture system (Hartshorne et al., 1999). Interestingly, Sadeu et al. (2006) showed clear ultrastructural evidence of relatively slow follicle growth in vitro to primary follicles in their tubal culture system, which compared with faster growth to a more advanced follicular stage observed with adult ovaries cultured similarly (Sadeu and Smitz, 2008). However, these authors did not describe the cellular outgrowth that we observed, and which may be a feature of the type of culture system employed. Optimization of culture methods for fetal ovaries will provide a valuable tool with which to study hormonal and paracrine influences upon key aspects of human ovarian development.

The mechanism of movement of the germline cells in our system is currently under study. This capacity for expansion was significantly enhanced by the inclusion of growth factors in the culture medium (SCF 10 ng/ml and IGF-I 5 ng/ml) (Fig. 5), and qualitative observations suggested more AP-positive cells in cultures with SCF than those without. In contrast, ovarian tissue of 15 weeks of gestation expanded far less in vitro, and the stimulatory effect of growth factors was no longer significant (Fig. 5). We have also noted that later gestational ages (up to 23 weeks), cultured under similar conditions, tend to round up in vitro and develop a dense surface epithelium, with little or no attachment to the substrate (data not shown).

It is well known that mouse PGCs can be cultured successfully and their numbers increased in cultures supplemented with growth factors including SCF (kit ligand) (De Felici et al., 2005). It is also known that c-kit is present on human oogonia and oocytes from 14–21 weeks of gestation (Hoyer et al., 2005). For example, Hoyer et al. (2005), using immunohistochemistry, found that PGCs and oogonia stain positive for SCF and c-kit, whereas unenclosed oocytes or those enclosed in primordial follicles stained only faintly or not at all for SCF and c-kit. c-kit became detectable again at the surface of oocytes in growing follicles (Hoyer et al., 2005). However, Abir et al. (2004) found c-kit expression to be evident in oocytes from the primordial follicle stage only. We interpret the growth of 14 week gestation samples...
in culture as probably due to the proliferation of PGCs. However, the dramatic difference between the behaviour of 14 weeks and 15 weeks of gestation tissue in vitro is surprising. We know that precursor cells remain in fetal ovaries, long after this time (Fig. 2), so perhaps they proliferate less, or undergo apoptosis more at later stages.

We then assessed the progression of MPI in the same tissues cultured under different conditions using SC proteins as a specific marker of MPI (as described in Hartshorne et al., 1999). The results are shown in Fig. 6. Oocyte numbers markedly declined during the first week in culture, probably owing to the shock of environmental change, as noted by others (Byskov et al., 1997, mice; Roig et al., 2006). Thereafter, oogenesis recovered with numbers of leptotene and zygotene oocytes increasing during the following week of culture. Recovery was more pronounced in 14 week than 15 week samples, and was evident with or without growth factor supplementation, until the second week in vitro. Thereafter, cultures with growth factors were more likely to maintain recovery, whereas those without tended to slow or decline in oocyte numbers. It is not yet clear whether the growth factors support the precursor cells, the oocytes directly, or have an indirect effect via the somatic environment. Further study will require the application of specific markers to distinguish between oocytes and precursor cells at different stages, and the use of tissue samples from a wider range of gestational ages.

**Factors affecting apoptosis during MPI**

In humans, MPI starts from around 13 weeks, and entry to meiosis continues until at least 19 weeks of gestation, with different stages of MPI, oogonia and degenerating oocytes present concurrently (Fig. 1). Many researchers have proposed that oocyte cell death during MPI is stage-specific, affecting the pachytene stage in particular, and linked to the theme that oocytes that cannot complete the pachytene stage consequently arrest and undergo cell death (Modi et al., 2003). Pachytene arrest is certainly functional in males (Guichaoua et al., 2005); however, the situation in females seems more complex and the controls of genetic integrity less stringent (Roig et al., 2004).

In addition, histological methods of identifying cells at different stages of MPI, and distinguishing them from somatic and degenerating cells, are fraught with difficulty, raising queries about the comparability and interpretation of earlier studies which raise many conflicting data. More recently, the application of molecular techniques has improved the confidence with which oocytes can be identified. Our work in mice confirmed that of McClellan et al. (2003), that apoptosis occurs in all stages of oocytes (Ghafari et al., 2007); however, similar analyses of human oocytes are not yet available.

We have also shown (Ghafari et al., 2009) that the lack of p53 gene in knockout mice is associated with a changed profile of progression through MPI at 15.5 and 16 dpc, possibly associated with reduced surveillance and an increased proportion of visually abnormal axial elements. Therefore, a gene that is profoundly involved in the control of the genetic integrity of cells and a regulator of apoptosis (Pietsch et al., 2008) is implicated in both the rate of meiotic progression and the quality of the resulting oocytes, possibly adding weight to the idea that failure of MPI owing to the timing of entry or the rate of progress might predispose to elimination. p63 gene is likely to be a dominant player here (Suh et al., 2006); however, its role in response to exogenous stimuli, such as radiation, does not exclude a role of p53 in the absence of such stimuli. p53 gene...
product was detected in half of oogonia in human ovaries at 8 weeks of gestation and all at 10–12 weeks of gestation (Quenby et al., 1999), and p53 has been reported to increase with in vitro duration of culture (Wu et al., 2002).

A further issue with human oocyte analyses comprises the genetic diversity of donors. The impact of genetic background on aspects of oogenesis has been noted by several authors (Koehler et al., 2002; Canning et al., 2003; Ibáñez et al., 2005) and we have also documented differences between B6CB mice of F1 and F2 generations in terms of meiotic progression (Ghafari et al., 2009). For humans, where the supply of tissue is relatively limited, inter-individual variability can be accommodated by controlled experiments utilizing tissue culture replicates. Therefore, much of our future work is aimed at characterizing tissue responses in vitro and modelling prenatal oogenesis in a manner that facilitates functional analyses.

**Discussion**

Even after many decades of study, oogenesis retains many intriguing mysteries. The concepts presented here highlight some of the knowledge that we need in order to understand the processes involved. In this article, we have presented new data on the timing of prenatal oogenesis and apoptosis, through TUNEL staining and identification of meiotic progress at different gestational ages in human, with comparative data in mice. We have also presented preliminary data on the variable capacity for human oocytes and ovarian tissue to move and expand in vitro. The large capacity for oocyte movement at early stages is a striking finding that we shall follow up.

We emphasize the need to study human material, because the results obtained using model animals differ from humans in our experience. An improved understanding of oocyte formation and selection holds the key to many human applications, with potential to improve our ability to control unwanted fertility or promote fertility treatments, suspend or accelerate the menopause, protect the germ line from toxic agents and pharmaceutical or radiological destruction and better understand the origins of maternally inherited genetic diseases, such as Trisomy 21 Down syndrome.

One reason why progress in this area has been slow is the difficulty of accessing prenatal ovarian tissue for research. Ethical and procedural issues make completing the governance requirements a protracted task, even where the legal and regulatory framework is permissive. Additional hurdles have included a limited array of sufficiently informative research techniques. However, recent progress in molecular markers for MPI, and for oocytes and stem cells, has helped immensely in the identification and classification of oocytes. Similar progress in apoptotic markers and many other pathways relevant to ovarian cellular functions now leaves us poised for substantial and rapid progress towards understanding oogenesis. The final piece of the jigsaw is the establishment of culture methods that support human oogenesis and folliculogenesis in vitro, thereby permitting experimentation under controlled conditions, on live cells. The basic model has been developed, and optimization is under way. Oocytes are known to be sensitive to perturbations in culture, and local ovarian signals in vivo are crucially important for survival and determination of cell fates. However, a live-cell model is the only way to study the decisions of individual oocytes responding to selection criteria applied for entry to the ovarian reserve.

In this way, factors affecting both the quality and the quantity of oocytes will become accessible for study. The concept of a trade-off between quantity and quality of oocytes, where resources are limited, is attractive but has not been substantiated. However, it is important to understand fully how individual local cell death decisions accumulate to result in reproductive function at the level of the whole organism. This systems biology approach cannot readily be tackled until more basic molecular information is available, but it remains on the horizon as a potentially fruitful research area.

Clearly, the internal environment of the pregnant woman during key stages of fetal ovary development has the potential to directly affect both the fertility of her future daughter (by controlling the size of the ovarian reserve) and the quality of the oocyte that will eventually become her granddaughter (by influencing the extent of selection and apoptosis). Prenatal oogenesis thus extends the well-known concept of fetal origins of adult disease (Barker, 1998) to include the next generation, whose building blocks are dependent upon events up to 80 years earlier. This unique concept helps to underline the importance of gaining a full understanding of prenatal oogenesis for the benefit of future generations.

**Materials and Methods**

**Tissues**

Mice were housed in licensed premises at the University of Warwick and killed at different gestational ages or within 2 days of birth. Ovaries were dissected from fetuses or neonates under microscopic observation and placed in culture medium. Full details are available in Ghafari et al. (2007).

Human fetal ovaries were obtained from second-trimester terminations of pregnancy in patients who consented to their use in research. The pregnancies were normal (i.e. no abnormalities had been identified) and were dated by foot length measurement, with additional information from ultrasound scan and/or dates of last menstrual period, if available. The researchers had no contact with the patients. Tissues were maintained at 4°C until ovaries were removed aseptically, and placed into culture medium for transport to the laboratory. For details, see Hartshorne et al. (1999).

**Detection of SC proteins**

The SC is present uniquely during MPI and can be detected using specific antibodies to its component parts on cells spread on microscope slides (Hultén et al., 2001; Tease et al., 2002). The orientation of the SC proteins often provides sufficient detail to be confident of the stage of MPI. Cell spreads were prepared by chopping ovarian tissue to a fine suspension. A drop of suspension on a microscope slide was mixed with a drop of hypotonic solution (0.3% Liposol) and allowed to stand for 30 min. Cells were fixed by adding two drops of 1% formaldehyde in 0.4% sodium dodecyl sulphate (SDS) for 20 min. Cells were blocked for 30 min in PBT buffer before primary antibodies to either synaptonemal complex protein 3 (SCP3) or core (COR-I) protein were added at dilutions of 1:1000. Anti-SCP3 antibody, raised in rabbit, was kindly provided by Crista Heyting, Wageningen University, The Netherlands, and polyclonal mouse anti-hamster COR-I antibody was kindly provided by Barbara Syropoulos and Peter Moens, University of Toronto. Fluorescently labelled secondary antibodies were applied at a dilution of 1:200, and the mounted slides were viewed under fluorescence microscopy. Full details are available in Hartshorne et al. (1999) and Ghafari et al. (2007).
Detection of apoptotic nuclear markers

Cleaved PARP-1
The primary antibody against cleaved PARP-1 (rabbit anti-mouse, polyclonal antibody) was applied at a concentration of 1:50 in PBT, simultaneously with the anti-COR-1 antibody described earlier. Slides were placed in a moist chamber at 4°C overnight. Fluorescently labelled secondary antibodies were then applied and visualized as described.

TUNEL
DNA cleavage was detected by TUNEL using the Apop Tag Fluorescein Direct In Situ Apoptosis Detection Kit (Intergen, USA). We applied it to study DNA breaks on the same micro-spread oocytes that had already been assessed using antibodies to COR-1 and cleaved PARP-1. All washing and incubation processes were therefore performed in the dark. Slides were washed in PBT, and then processed according to the manufacturer’s protocol for TUNEL. Positive control slides were treated with 0.9 μg/ml DNase I in DN buffer for 10 min at room temperature. Slides were mounted with Vectashield mounting medium without DAPI.

Enzymatic detection of AP
AP is a cytoplasmic marker routinely used for detecting germline cells. We applied the method of Buehr and McLaren (1993) to suspensions of ovarian tissue prepared by fine chopping, followed by spreading on a microscope slide and air drying. The same staining method was also applied to membranes containing cultured cells.

Slides or membranes were fixed for 20–30 s in buffered acetone, washed in tap water and allowed to dry. Slides were stained with AP substrate for 15 min, rinsed in dH2O and mounted in aqueous mountant. The stain comprised 25 mg Fast Texas Red and 5 mg alpha-naphthyl phosphate in 44.6 ml dH2O; 0.3 ml 10% MgCl₂ and 5 ml 4.5% borax. These ingredients were mixed in order and used immediately. The presence of AP resulted in red staining.

Culture of fetal ovarian tissue
Fragments of ovarian tissue, ~0.3 x 0.3 x 0.3 mm³, were placed individually on permeable polyester membranes (Costar) in pre-prepared tissue culture wells containing 1 ml culture medium in 12-well plates. The tissue was supported near to the surface of the medium, and any empty wells were filled with medium to maintain humidity. The culture medium was minimal essential medium (MEM) α supplemented with insulin, transferrin and selenium, and 2.5–5% serum, with or without SCF (10 ng/ml) and IGF-I (5 ng/ml). Growth factors were prepared in insulin, transferrin and selenium, and 2.5–5% serum, with or without medium was minimal essential medium (MEM).

Wells were filled with medium to maintain humidity. The culture wells containing 1 ml culture medium in 12-well plates. The washings and incubation processes were therefore performed in the same wells.

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