REPRODUCTION
ANNIVERSARY REVIEW

FERTILITY PRESERVATION

Progress and prospects for developing human immature oocytes *in vitro*

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Abstract

Ovarian cryopreservation rapidly developed from basic science to clinical application and can now be used to preserve the fertility of girls and young women at high risk of sterility. Primordial follicles can be cryopreserved in ovarian cortex for long-term storage and subsequently autografted back at an orthotopic or heterotopic site to restore fertility. However, autografting carries a risk of re-introducing cancer cells in patients with blood-born leukaemias or cancers with a high risk of ovarian metastasis. For these women fertility restoration could only be safely achieved in the laboratory by the complete *in vitro* growth (IVG) and maturation (IVM) of cryopreserved primordial follicles to fertile metaphase II (MII) oocytes. Culture systems to support the development of human oocytes have provided greater insight into the process of human oocyte development as well as having potential applications within the field of fertility preservation. The technology required to culture human follicles is extremely challenging, but significant advances have been made using animal models and translation to human. This review will detail the progress that has been made in developing human *in vitro* growth systems and consider the steps required to progress this technology towards clinical application.

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Introduction

It is now over 40 years since the birth of the first IVF baby (Niederberger *et al.* 2018) and since then several advances have been made in the area of assisted reproductive technologies particularly the emergence of the new field of fertility preservation (Anderson & Baird 2019). Women now have the opportunity to have pieces of their ovarian cortex removed and stored prior to damaging chemotherapy treatments with the goal of future re-implantation, however, for some patients re-implantation carries a risk of re-introducing malignant cells (Anderson *et al.* 2017). The development of an 'artificial ovary' system is ongoing in an attempt to avoid the risk of re-introducing malignant cells. The artificial ovary involves isolating follicles and reconstituting them with somatic cells within a matrix in the hope that any remaining malignant cells would be removed before transplantation (Dolmans & Amorim 2019). The ability to support oocyte development entirely *in vitro* would offer an alternative strategy by avoiding the need for transplantation to restore fertility. This review outlines the potential and difficulties of developing culture systems to support the development of human oocytes.

The ovarian tissue that is harvested for cryopreservation contains non-growing follicles at the primordial stage of development. These follicles contain the earliest stage of oocyte and research on how to develop them entirely *in vitro* has been ongoing for many years. Culture systems for *in vitro* gametogenesis/growth (IVG) need to support all stages of oocyte development from activation of dormant primordial follicles to a stage where oocytes can undergo meiotic maturation and be fertilised. If this methodology could be demonstrated to be safe it would maximise the potential of cryopreserved ovarian tissue and have many clinical applications relevant to fertility preservation and assisted reproduction. Culturing immature oocytes would negate the need for hormonal stimulation of the patient, avoid multiple surgical procedures to harvest oocytes but most importantly there would be options for fertility preservation/restoration to women who currently have none (Anderson *et al.* 2017, Telfer 2019).

The clinical application of growing primordial follicles *in vitro* would be especially relevant to prepubertal girls having ovarian tissue removed and cryopreserved prior to being exposed to potentially gonadotoxic treatments (Anderson *et al.* 2015, 2017). Freezing mature oocytes or embryos is not possible for prepubertal girls therefore their only option for fertility preservation is cryopreservation of ovarian cortical tissue with the

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potential for subsequent re-implantation (Anderson *et al.* 2015, 2017). If re-implantation was contra-indicated, IVG would provide an additional option for restoration of fertility in such cases. Given that cryopreserved cortical strips contain predominantly primordial follicles, it is the obvious starting material for a culture system; however, this also presents many challenges. The realisation of clinical applications of IVG will require a great deal of research to optimise culture systems and to demonstrate the safety of these techniques. Nonetheless progress in developing culture techniques to support *in vitro* development of human oocytes has been made.

Producing competent oocytes for assisted reproduction technologies (ARTs) is the main objective of IVG systems, but the development of these methods provides models of human gametogenesis that allow for detailed analyses of the sequential stages of human oocyte development of which we still know very little about. In addition, culture systems facilitate analysis and assessment of cryopreserved ovarian tissue prior to transplantation, making IVG central to fertility preservation programmes (Picton *et al.* 2008, Telfer & McLaughlin 2011).

Follicle/oocyte development

The most immature stage of oocytes are contained within structures known as primordial follicles (Fig. 1). In most mammals follicle formation occurs before birth and these form the store of female germ cells that will be utilised throughout reproductive life (Telfer & Zelinski 2013). Recruitment of follicles to growth is controlled by a defined sequence of events regulated by paracrine and endocrine factors (Hsueh *et al.* 2015). Initiation of follicle growth is also influenced by the intra ovarian environment and in particular biomechanical factors (Shah *et al.* 2018). The key stages that need to be supported can be summarised as (1) activation of primordial follicle growth, (2) development to multi-laminar pre-antral follicle stage, (3) antral cavity formation and differentiation of granulosa cells to mural and cumulus cells, (4) Proliferation of granulosa cells and expansion of the fluid filled antral cavity leading to the formation of the pre-ovulatory or Graafian follicle, and (5) release of the cumulus-oocyte complex after rupture of the Graafian follicle in response to the surge of luteinising hormone (LH) triggering ovulation (Fig. 1) (Telfer & Zelinski 2013).

The majority of follicles in all mammals will be at the primordial stage and during follicle formation the oocyte is arrested at Prophase I of meiosis and remains in meiotic arrest until ovulation. During follicle growth and development the oocyte needs to acquire the competence to resume meiosis as well as the ability to support events during fertilisation and early embryonic development. This process is referred to as the acquisition of developmental competence and the development of culture conditions to support these processes represents an enormous technical challenge (Anderson & Telfer 2018) that requires a good understanding of the complex requirements of the various compartments within the ovary that is, oocyte, granulosa, theca and stromal cells. These requirements are dynamic and complex (Anderson & Telfer 2018). To ensure the development of healthy oocytes, it is important to consider how the culture conditions might alter DNA integrity and stability and to be able to alter conditions appropriately (Martin *et al.* 2019).

Growing follicles *in vitro*

Complete IVG of oocytes from primordial stages with subsequent *in vitro* maturation of oocytes (IVM) and

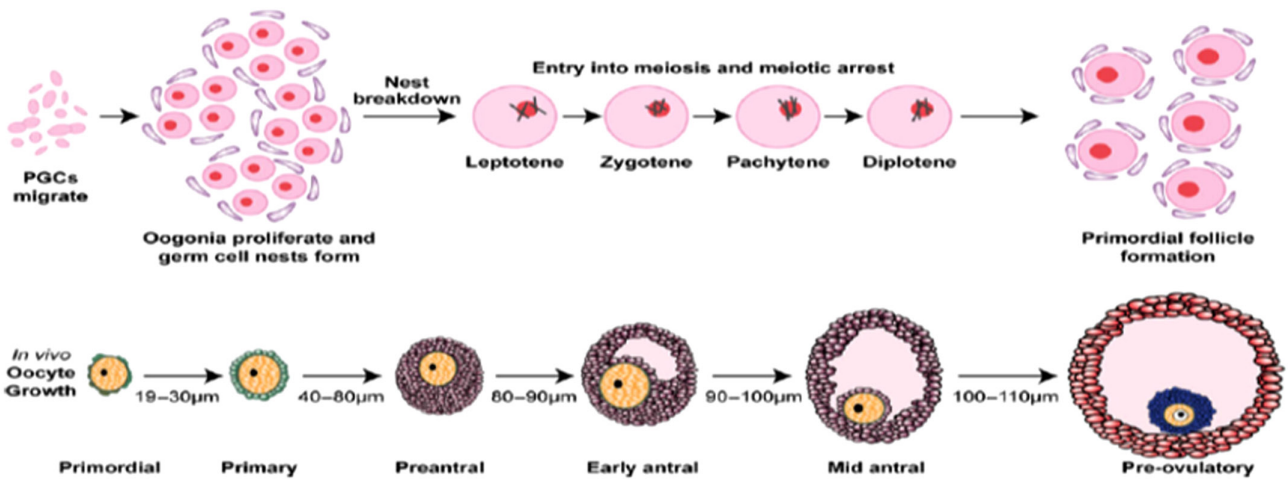

Figure 1 Representation of stages of oocyte formation and development. Upper panel: proliferation of primordial germ cells (PGCs) which form nests and then enter meiosis to the dictyate stage of Prophase 1 forming oocytes associated with somatic cells to form primordial follicles. In humans this occurs during fetal life. Lower panel: Primordial follicles are continually activated to grow throughout reproductive life and progress through follicular development with co-ordinated oocyte development. Sizes shown illustrate oocyte development. Reproduced from Anderson & Telfer (2018) with permission.

fertilisation (IVF) has been achieved in the mouse resulting in the production of embryos and live young after embryo transfer (ET) (Eppig & O'Brien 1996, O'Brien *et al.* 2003). This was achieved using a two-step culture system in which primordial follicles were activated in step one and then growing oocytes developed within oocyte-granulosa cell complexes (OGCs) in step two (Eppig & O'Brien 1996, O'Brien *et al.* 2003). The first study reported the birth of one mouse derived from IVG oocytes but this animal subsequently developed many abnormalities as an adult although it is not clear if these can be attributed to IVG (Eppig & O'Brien 1996). Further work on the culture system led to improvements in the medium and physical environment resulting in several mouse embryos and offspring from *in vitro*-grown oocytes (O'Brien *et al.* 2003). Indeed these reports have been the only ones to date that have demonstrated complete oocyte development using a rodent system from the primordial stage *in vitro*, indicative of the complexity and technical challenges involved (Eppig & O'Brien 1996, O'Brien *et al.* 2003). Further studies have recently demonstrated that complete *in vitro* development of mouse oocytes starting from stages before primordial follicle formation is possible and developmentally competent oocytes capable of being fertilised and forming embryos have been produced from induced pluripotent stem cells (iPSCs) (Hikabe *et al.* 2016) and primordial germ cells (PGCs) (Morohaku *et al.* 2016). The studies using rodent models provide proof of principle that it is possible to achieve complete oocyte development *in vitro* and have highlighted the challenge of translating these systems to support human oocyte development (Herta *et al.* 2018).

Several culture systems supporting defined stages of human oocyte development have been reported but only a single report describes the growth of human oocytes from primordial stages to maturity within a multi-step culture system (Fig. 2) (McLaughlin *et al.* 2018). Whilst this system is not yet optimised it highlights the sequence of environments that may mimic the changing niche required to facilitate development of human oocytes *in vitro* (Anderson & Telfer 2018, McLaughlin *et al.* 2018).

Supporting initiation/activation of primordial follicles *in vitro*

Isolated primordial follicles do not activate to grow *in vitro*. Oktay and colleagues (1997) demonstrated that primordial follicles that were isolated from thawed human cortical tissue were as viable as those isolated from fresh tissue (Oktay *et al.* 1997) but culturing isolated primordial follicles resulted in poor survival rates (Hovatta *et al.* 1997, Abir *et al.* 1999). Therefore culture systems where primordial follicles are the starting stage need to start with pieces of human ovarian cortex rather than isolated primordial follicles (Hovatta *et al.* 1997, 1999, Picton & Gosden 2000, Telfer *et al.* 2008, Garor *et al.* 2009, McLaughlin *et al.* 2018). For over 20 years there has been the development of systems supporting the initiation of human primordial follicle growth *in vitro* (Hovatta *et al.* 1997, 1999, Wright *et al.* 1999, Picton & Gosden 2000, Hreinsson *et al.* 2002, Telfer *et al.* 2008, Garor *et al.* 2009, Ding *et al.* 2010, Anderson *et al.* 2014, McLaughlin *et al.* 2014, 2018). Different rates of follicle activation have been observed in each of these systems and this appears to be associated with the preparation and architecture of the starting tissue, indicative of the importance of mechanical signalling to cell pathways regulating primordial follicle activation (Shah *et al.* 2018).

Whilst the activation of primordial follicles *in vitro* and early follicle development are critical features of an IVG system, the mechanisms regulating follicle activation are still not well understood (Picton & Gosden 2000,

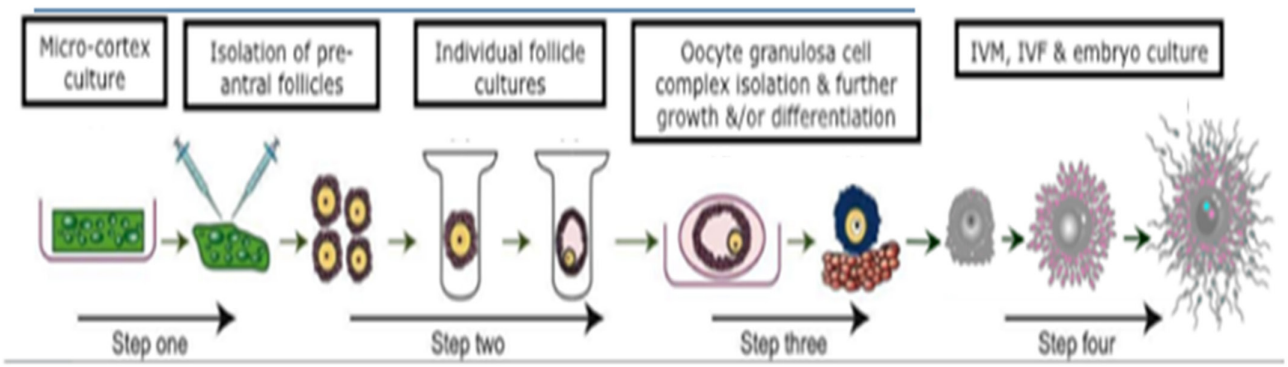

Figure 2 Steps needed in a multi-step culture system (McLaughlin *et al.* 2018) to support *in vitro* growth (IVG) of oocytes starting from human primordial follicles. Step 1: culture of ovarian cortex for 7 days to promote activation of primordial follicles. At the end of the culture period any multi-laminar follicles present are isolated by micro-dissection. Step 2: Isolated follicles are cultured individually in v-shaped wells until antral cavities are formed. Step 3: Oocyte-granulosa cell complexes are removed from intact antral follicles and placed on membranes for up to 4 days. Step 4: Maturation media (IVM) is added to Oocyte-granulosa cell complexes overnight and analysed for the presence of a Metaphase II spindle and a polar body.

Herta *et al.* 2018). Regulation of activation requires a complex interaction of inhibitory, stimulatory and maintenance factors (Zhang & Liu 2015).

Anti-Mullerian hormone (AMH) produced in the granulosa cells of growing follicles has been shown to have an inhibitory effect on primordial follicle activation in mice (Durlinger *et al.* 2002) and to attenuate the action of FSH on growing follicles (Durlinger *et al.* 2001). Human ovarian tissue xenografted to immunodeficient mice with engineered endothelial cells (ECs) to deliver AMH leads to a reduction in primordial follicle activation and growth during a 2-week grafting period (Man *et al.* 2017). In contrast, human ovarian cortical tissue cultured in the presence of AMH resulted in increased activation and improved development of growing follicles (Schmidt *et al.* 2005). These results illustrate the differences of action *in vitro* and *in vivo* and also of timings and mode of delivery.

Components of the phosphatidylinositol-3′-kinase (PI3K-AKT) signalling pathway within oocytes have been shown to be key regulators of primordial follicle activation in mouse (Reddy *et al.* 2008, Zhang & Liu 2015), human (Li *et al.* 2010, McLaughlin *et al.* 2014, Grosbois & Demeestere 2018) and cow (Maidarti *et al.* 2019). The PI3K-AKT signalling pathway is the main non-gonadotrophic growth factor signalling pathway regulating growth and differentiation of ovarian follicles (Dupont & Scaramuzzi 2016).

It has been demonstrated that a component of the PI3K pathway, the phosphatase and tensin homolog deleted on chromosome ten (PTEN), negatively regulates this pathway resulting in the suppression of follicle initiation/activation (Reddy *et al.* 2008). A member of the forkhead transcription factors family (FOXO3a) acts as downstream effector of the PI3K pathway resulting in inhibition of follicle activation (Castrillon *et al.* 2003). Another component of the PI3K pathway is the mammalian target of rapamycin complex 1 (mTORC1), a regulator of cell growth and proliferation and involved in regulating primordial follicle activation (Adhikari & Liu 2010). Data obtained from knockout mouse models have confirmed that PTEN within the oocyte inhibits follicle activation whilst mTORC1 promotes activation.

Significant activation of primordial follicles occurs when pieces of ovarian cortex are prepared by fragmentation and this appears to be a consequence of disrupting the Hippo signalling pathway (Badouel & McNeill 2011) during tissue preparation (Kawamura *et al.* 2013, Hsueh *et al.* 2015, Grosbois & Demeestere 2018). This emphasises the importance of biomechanical forces on these processes (Shah *et al.* 2018).

Observations on the role of the Hippo signalling pathway may explain differences in results observed between culture systems. Human ovarian cortex prepared as dense cubes *in vitro* results in low levels of follicle activation (Hovatta *et al.* 1997, 1999, Wright *et al.* 1999). In contrast, significant primordial follicle activation occurs in human ovarian cortex prepared as micro-cortex, where underlying stroma is removed and tissue interactions are disrupted (Telfer *et al.* 2008, McLaughlin *et al.* 2018). The combination of disruption of the Hippo pathway by fragmentation and manipulation of components of the PI3K pathway (PTEN and mTORC1) results in enhanced activation of follicles (Li *et al.* 2010, Kawamura *et al.* 2013, McLaughlin *et al.* 2014, Hsueh *et al.* 2015, Grosbois & Demeestere 2018, Maidarti *et al.* 2019). These observations confirm the importance of tissue preparation in these systems.

The action of PTEN can be inhibited pharmacologically using vanadium compounds such as bisperoxovanadium (Spinelli *et al.* 2015). Inhibition of PTEN in human tissue prior to transplantation has been used for the treatment of premature ovarian insufficiency (POI) and has led to increased follicle activation (Kawamura *et al.* 2013). PTEN inhibition in cultured human ovarian cortex also results in more primordial follicles being activated and an increase in the number of secondary follicles that can be isolated (McLaughlin *et al.* 2014) but further development of these follicles is compromised as demonstrated by poor growth and survival after isolation (McLaughlin *et al.* 2014, Grosbois & Demeestere 2018). The observed effects on secondary follicles following inhibition of PTEN at the activation stage could be attributed to direct effects on DNA repair response mechanisms within the oocyte/follicle and increased DNA damage (Martin *et al.* 2019). Primordial follicle activation has been shown to be increased in cultured bovine ovarian cortex where PTEN has been inhibited pharmacologically (Maidarti *et al.* 2019). This increased activation rate is accompanied by a resultant increase in DNA damage and alterations in DNA repair mechanisms (Maidarti *et al.* 2019). These deleterious effects are not observed when bovine follicles are activated in media containing activin and FSH without inhibiting PTEN (McLaughlin & Telfer 2010).

Any culture system removes follicles from *in vivo* control mechanisms (paracrine and endocrine) that will determine their rate of growth, but tissue architecture and stromal density have been shown to have an influence on follicle activation and subsequent growth rate. Follicles within cortical tissue prepared as solid cubes with dense stroma show little growth initiation (Hovatta *et al.* 1997) when compared with cortex prepared as flattened ‘sheets’, where much of the underlying stroma is removed (Telfer *et al.* 2008, McLaughlin *et al.* 2018). How follicles respond to stimulatory and inhibitory factors and therefore their growth rate is influenced by the tissue environment (McLaughlin & McIver 2009). Follicles initiated to grow within cortical tissue can develop to multi-laminar stages, but their growth is inhibited if they are not removed from the cortical tissue (Hovatta *et al.* 1999, Telfer *et al.* 2008). Follicles that reach multi-laminar stages start to degenerate if they are not removed from the cortical stromal environment and

cultured individually and show a loss of oocyte-somatic cell connections (Telfer *et al.* 2008, McLaughlin & Telfer 2010, Anderson *et al.* 2014, McLaughlin *et al.* 2018).

Growth of multi-laminar follicles *in vitro*

Isolation of multi-laminar stages from the ovarian cortex can be achieved by using enzymes, mechanical isolation or by combining both methods. Multi-laminar follicles can be isolated from ovarian stroma using a mixture of collagenase and DNase; however, this combination can cause damage resulting in decreased survival of growing follicles (Telfer *et al.* 2000). The use of liberase for follicle isolation is gentler than collagenase and leads to less damage (Dolmans *et al.* 2006, Rice *et al.* 2008). Isolated follicles need to maintain their structure during the second stage of IVG and the presence of theca cells is essential for this. Given that theca cells can be compromised by enzymatic preparations (Wandji *et al.* 1996, Telfer *et al.* 2000) it is better to isolate follicles by using needles to remove them from the stroma whilst preserving follicular integrity by maintaining the basal lamina and thecal layers (Telfer *et al.* 2008, McLaughlin *et al.* 2018). There are now several culture systems that have been developed to support the growth of isolated multi-laminar (pre-antral) follicles (Telfer & Zelinski 2013).

Tissue engineering has been applied to the challenge of growing multi-laminar structures that could grow up to several millimetres in diameter (Shea *et al.* 2014, Rajabi *et al.* 2018a). Human pre-antral follicles can be encapsulated in bio-matrices such as alginate which supports follicle structure and growth *in vitro* (Xu *et al.* 2009). Alginate is a polysaccharide that is found in the cell wall of algae and forms a gel which follicles can be embedded in. Alginate forms controlled set gels with ridgidity that can be manipulated. It is important to balance flexibility of the gel to accommodate cell proliferation with its rigidity to avoid collapse of the follicle structure (Skory *et al.* 2015). The degree of rigidity affects follicle development as demonstrated by reports that show poor growth of mouse follicles embedded in 1% alginate gels (Heise *et al.* 2005) whilst 0.5% gels support the development of fully grown human oocytes (Xu *et al.* 2009). These studies demonstrate that the physical environment of the ovary is a regulating force and that biomechanical signals can be mimicked by an appropriate support matrix (West-Farrell *et al.* 2009).

Other scaffolds such as de-cellularised ovarian tissue (Laronda *et al.* 2015) and 3D microporous scaffolds are being developed as matrices and are being applied to support human pre-antral follicle growth (Laronda *et al.* 2017, Pors *et al.* 2019). Electrospun patterned porous scaffolds are being explored to support follicle development and these may be more accessible and reproducible than de-cellularised tissue (Liverani *et al.* 2019).

No matrices or scaffolds are utilised in the multi-step culture system developed for human follicles (McLaughlin *et al.* 2018). During step 2 of the multi-step system isolated follicles are placed free floating in v-shaped micro-well plates. This system supports follicular structure *in vitro* as well as growth, differentiation and antral formation during a relatively short culture period of up to 10 days (Telfer *et al.* 2008, McLaughlin *et al.* 2018).

Following initiation of follicle growth within the strip, follicles develop to multi-laminar stages but do not survive within the cortical environment (Hovatta *et al.* 1997, Telfer *et al.* 2008). Therefore follicles need to be released from the cortical stromal environment and cultured individually (Telfer *et al.* 2008, McLaughlin *et al.* 2018). Pre-antral follicles isolated from ovarian cortex and grown *in vitro* undergo significant growth and development and whilst this can be rapid it represents growth without brakes since the follicle has been removed from all regulatory mechanisms. Two approaches have been taken by researchers developing culture systems to support the growth of pre-antral follicle, one is a 'slow' grow system where isolated follicles are grown for protracted periods in an attempt to mimic growth rates *in vivo* (Chambers *et al.* 2010). Multi-laminar follicles isolated enzymatically from fresh human ovarian tissue can undergo complete oocyte growth within 30 days (Xu *et al.* 2009) and some are capable of meiotic maturation (Xiao *et al.* 2015). Whilst these systems may be presented as slow growth systems they still support a more rapid development than that observed *in vivo*.

Developing pre-antral human follicles to pre-ovulatory diameters present technical challenges but oocyte development may not require the expansion of follicle size associated with pre-ovulatory stages and so a 'fast' growth system that focuses on oocyte development has been developed. Follicles activated to grow within fragments of ovarian cortex reach multi-laminar stages within 7 days and when they are removed from the cortex and cultured individually achieve antral formation and significant oocyte growth within a further 7–10 days of *in vitro* growth in the presence of activin A and FSH (Telfer *et al.* 2008, McLaughlin *et al.* 2018).

Developmentally competent mouse oocytes can be obtained from primordial stages using a two-step culture system where follicles are activated within ovarian fragments and then oocyte-granulosa cell complexes (OGCs) are isolated and cultured during step 2 (O'Brien *et al.* 2003). This system does not require antral follicles to be formed but by maintaining oocyte-somatic cell connections within oocyte–granulosa cell complexes (OGCs) complete oocyte development can be supported (O'Brien *et al.* 2003). The mouse system has informed the development of step three of the human multi-step culture system (McLaughlin *et al.* 2018). Whilst isolated human follicles cultured individually form antral cavities

within 6–8 days, rather than attempt to maintain the development of large antral follicles, oocyte–granulosa cell complexes are removed from the follicle for further growth and development. The removal of complexes at this stage is technically demanding and is achieved by applying gentle pressure to the antral follicle (Telfer *et al.* 2008, McLaughlin *et al.* 2018). Intact complexes with complete cumulus and adherent mural granulosa cells are selected for further growth in step 3 of the multi-step system (Fig. 2). These complexes can produce oocytes of at least 100 microns in the presence of activin-A and rhFSH, and some of these can reach metaphase II (McLaughlin *et al.* 2018).

Optimising conditions to support activation and early growth

A multitude of factors affect follicle/oocyte development *in vivo*, either by promoting growth or degeneration. There is still a lack of knowledge on the exact requirements to support *in vitro* growth therefore optimising conditions has been extremely difficult (Telfer & Zelinski 2013, Rajabi *et al.* 2018b). Early culture systems to support activation and growth used medium with serum but defined media containing human serum albumin (HSA) and a combination of insulin, selenium and transferrin (ITS) is now being used (Telfer *et al.* 2008, McLaughlin *et al.* 2018). Several types of basal culture media including MEM alpha, Waymouths medium and McCoys 5A have been used with our preference being for McCoys 5A (Telfer *et al.* 2008, McLaughlin *et al.* 2018). An essential component of the culture media is ascorbic acid which is added fresh at each media change to reduce cell death (Thomas *et al.* 2001). The activation and growth of primordial follicles occurs in the presence of basic medium, and this process can be inhibited by the addition of growth factors such as activin (Ding *et al.* 2010). A greater understanding of how specific factors influence the process of activation is essential and will lead to improvements in number and quality of IVG follicles. Another approach that is being developed to increase the number of follicles activated *in vitro* is the manipulation of key signalling pathways controlling this process. As discussed above the PI3K/PTEN/Akt pathway has a major role in activation of primordial follicles but balancing increasing activation by manipulating this pathway with possible damage and effects on DNA repair mechanisms needs to be determined (Maidarti *et al.* 2019).

The addition of several growth factors and hormones has been tested in a range of species but there are conflicting reports and still no consensus on the timing and concentration of key additives (Rajabi *et al.* 2018b). It is well recognised that oocyte–somatic cell interactions need to be maintained to support oocyte development. Cell–cell communication is affected by members of the TGF beta superfamily, in particular oocyte-specific factors, growth differentiation factor-9 (GDF-9) and bone morphogenetic protein 15 (BMP-15) (Sanfins *et al.* 2018). Human GDF-9 and BMP 15 act in a synergistic fashion to promote cell proliferation and cell–cell interaction (Reader *et al.* 2016). In humans GDF-9 and BMP-15 is expressed in the oocyte and cumulus granulosa cells and increased mRNA levels in cumulus cells has been correlated with an increase in oocyte maturation and subsequent fertilisation rates in human (Li *et al.* 2014). The addition of either GDF-9 or BMP-15 to cultured human ovarian cortex can increase the activation of primordial follicles with BMP-15 having more beneficial effects on early growth (Kedem *et al.* 2011).

Successful growth of multi-laminar follicles in step 2 of the multi-step system is dependent on maintaining oocyte–somatic cell interactions during this stage (Li & Albertini 2013). Experiments using human tissue demonstrated that the addition of activin resulted in antral cavity formation *in vitro* (Telfer *et al.* 2008) and the combination of activin and a low-dose FSH during stage 2 of growth maintains oocyte–somatic cell interactions leading to improved health of multi-laminar stages and antral formation in a bovine model (McLaughlin *et al.* 2010) and has been successfully applied to a human system (McLaughlin *et al.* 2018). Given the range and complexity of factors involved in oocyte/follicle development it is clear that the concentration, mode of delivery and timings of exposure to specific factors need to be determined. Co-ordinating such a complex system will require the development of systems that can facilitate a dynamic culture system. Researchers are working on bioreactor systems to regulate timings, concentration and oxygen availability in a dynamic process (Catapano *et al.* 2019).

Since oocyte size indicates its ability to resume meiosis (Fair *et al.* 1995, Sirard 2011) complete *in vitro* development systems must support oocyte growth rather than follicle growth (McLaughlin *et al.* 2018). Differences in growth rate of oocytes have been observed between those cultured within intact follicles for the entire culture period (Xu *et al.* 2009, Xiao *et al.* 2015) and those that are removed as complexes after an antral cavity has formed and cultured on membranes (Telfer *et al.* 2008, McLaughlin *et al.* 2018). Comparisons of culture systems with different growth rates is required to determine optimal conditions. It will be essential to determine whether *in vitro* growth is capable of supporting the development of healthy human oocytes. Any deleterious effects on oocyte function and epigenetic changes are as yet unknown and will require a large animal model such as cow or sheep to determine the safety of these techniques. In addition, metabolic studies such as those carried out on sheep follicles over a prolonged culture period should be conducted for human follicles to determine how growth period affects metabolism as an indicator

of oocyte health and competence (Harris *et al.* 2010, Hemmings *et al.* 2012).

Meiotic maturation of oocytes from IVG human follicles

Once IVG oocytes are fully grown they need to be placed in maturation media (IVM) to determine their capacity to resume meiosis and reach metaphase II (Fig. 2). IVM has been successfully utilised in domestic species such as cows (Van den Hurk *et al.* 2000, Hirao *et al.* 2013, Dieci *et al.* 2016) and pigs (Hirao *et al.* 1994) for embryo production. IVM of human oocytes has been applied but success rates have been variable (Nogueira *et al.* 2012, Chian *et al.* 2013). Whilst IVM of immature human oocytes resulted in a live birth in 1991 (Cha *et al.* 1991), this technique was being used whilst IVF was being developed (Edwards *et al.* 1969). IVM is now used clinically in a small number of centres with variable success rates (Chian *et al.* 2013). Success rates are related to the source of oocytes selected for IVM with it being well recognised that successful maturation of immature oocytes is lower than that of oocytes obtained from stimulated ovaries (Nogueira *et al.* 2012, Chian *et al.* 2013). Many of the oocytes harvested for IVM will be intrinsically unable to undergo maturation therefore optimisation of IVM conditions is also required if IVG is ever to be applied clinically (Shirasawa & Terada 2017).

Some oocytes that survive the multi-step culture system are capable of meiotic maturation following an IVM protocol (McLaughlin *et al.* 2018). Whilst some IVG oocytes form metaphase II spindles their polar bodies are larger than normal (McLaughlin *et al.* 2018). It is not known what causes the formation of large bodies in IVG-derived oocytes, but polar body size can be affected by the position of the spindle in relation to the oocyte cortex (Barrett & Albertini 2010). It has been shown that large polar bodies are extruded from human oocytes undergoing IVM when spindle contact with the oocyte cortex is lost (Coticchio *et al.* 2013). The goal of any IVG system is to support the development of oocytes that are competent to be fertilised and are epigenetically normal. Research focusing on optimising each of the stages of IVG is required, and it is essential to determine the epigenetic status of IVG oocytes and of any embryos that might be formed (Anckaert *et al.* 2013).

Steps towards clinical translation

The main clinical application of IVG is within fertility preservation therefore it is important to determine whether cryopreserved/thawed ovarian tissue can be cultured. Human follicles isolated from cryopreserved tissue have been shown to be viable and the birth rate from tissue re-implantation is now more than 130 babies (Donnez & Dolmans 2017). Analysis of frozen thawed tissue indicates high viability of follicles (Kristensen *et al.* 2018) and activation *in vitro* of primordial follicles in frozen thawed ovarian cortex can be comparable to fresh tissue (Grosbois & Demeestere 2018). Whilst follicles isolated from frozen thawed tissue after activation and culture appear to grow in a similar way detailed testing is still required.

Future developments: making new oocytes from stem cells

The starting material for the *in vitro* growth systems described above is the primordial follicle that has been formed *in vivo* before birth. There is now a focus on trying to produce *in vitro*-derived oocytes, from sources such as embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) or putative oogonial stem cells (OSCs). The ability to produce oocytes in this way would provide women that have few or no oocytes with the possibility of genetically related offspring and would alleviate the need for donor eggs (Ilic *et al.* 2019). Several studies have shown that putative OSCs can be isolated from ovarian cortex of adult women (White *et al.* 2012, Anderson & Telfer 2018, Clarkson *et al.* 2018, Silvestris *et al.* 2018). These cells have the capacity to form what appear to be oocytes when they are grown *in vitro* and provided with a somatic cell support (White *et al.* 2012, Clarkson *et al.* 2018, Silvestris *et al.* 2018). Controversy has been generated over the biological significance of these cells and this remains unknown but the identification and isolation of populations of cells from the adult human ovary that have germ line potential is a significant finding. These cells could have the potential to improve infertility treatments if new oocytes can be formed and grown *in vitro* (Anderson & Telfer 2018). Whilst the production of oocytes from stem cells is promising, it is clear that some cells under certain conditions can form morphological oocytes, but these do not enter meiosis. The process of oocyte differentiation can be dissociated from meiosis, so if functional artificial oocytes are to be obtained, then it will be essential to understand the connection between oocyte differentiation and entry into meiosis (Handel *et al.* 2014).

The development of functional oocytes from stem cells is dependent on the methodologies discussed in this review to grow human immature oocytes from primordial stages to mature oocytes entirely *in vitro*. As highlighted here there are many practical and conceptual obstacles remaining before clinical application of any of these technologies can ever be fully realised.

Summary

The practice of fertility preservation is a new and fast developing field. Cryopreservation and re-implantation of ovarian tissue developed by Roger Gosden laid the

foundations and IVG systems would be an important addition. The capacity to successfully develop human oocytes from immature stages through to maturation and fertilisation *in vitro* would offer an alternative to autologous transplantation. In addition it has the potential to be the next generation IVF. Proof of concept that complete *in vitro* growth of human oocytes is possible has been achieved but optimisation is now required (McLaughlin *et al.* 2018). There is an overarching need to avail models of human gametogenesis under conditions that will allow for detailed analyses of the sequential stages of oogenesis in the human. By culturing human oocytes/follicles we have gained greater insight to the process of human oogenesis and better understanding of human oocyte development.

There are many women for whom current methods of fertility preservation are unsuitable and the process of IVG would be beneficial to them but cautious optimism needs to be tempered with realism. There is a long road ahead before optimisation and thorough testing can be carried out to demonstrate the safety of these techniques (Anderson & Telfer 2018).

Declaration of interest

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

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