



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Metaphase II oocytes from human unilaminar follicles grown in a multi-step culture system

Citation for published version:

McLaughlin, M, Albertini, DF, Wallace, WHB, Anderson, R & Telfer, E 2018, 'Metaphase II oocytes from human unilaminar follicles grown in a multi-step culture system' *Molecular Human Reproduction*, vol. 24, no. 3, pp. 135–142. DOI: 10.1093/molehr/gay002

Digital Object Identifier (DOI):

[10.1093/molehr/gay002](https://doi.org/10.1093/molehr/gay002)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

Molecular Human Reproduction

Publisher Rights Statement:

© The Author(s) 2018. Published by Oxford University Press on behalf of the European Society of Human Reproduction and Embryology.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



Metaphase II oocytes from human unilaminar follicles grown in a multi-step culture system

M. McLaughlin¹, D.F. Albertini², W.H.B. Wallace³, R.A. Anderson⁴,
and E.E. Telfer ^{1,*}

¹Institute of Cell Biology, University of Edinburgh, Edinburgh EH8 9XD, UK ²Center for Human Reproduction, New York, NY 10021, USA

³Department of Haematology/Oncology, Royal Hospital for Sick Children, Edinburgh EH9 1LF, UK ⁴MRC Centre for Reproductive Health, University of Edinburgh, Edinburgh EH16 4TJ, UK

*Correspondence address. Institute of Cell Biology, University of Edinburgh, Hugh Robson Building, Edinburgh EH8 9XD, UK.
E-mail: Evelyn.Telfer@ed.ac.uk  orcid.org/0000-0003-1468-4610

Submitted on June 30, 2017; resubmitted on January 10, 2018; editorial decision on January 17, 2018; accepted on January 23, 2018

STUDY QUESTION: Can complete oocyte development be achieved from human ovarian tissue containing primordial/unilaminar follicles and grown *in vitro* in a multi-step culture to meiotic maturation demonstrated by the formation of polar bodies and a Metaphase II spindle?

SUMMARY ANSWER: Development of human oocytes from primordial/unilaminar stages to resumption of meiosis (Metaphase II) and emission of a polar body was achieved within a serum free multi-step culture system.

WHAT IS KNOWN ALREADY: Complete development of oocytes *in vitro* has been achieved in mouse, where *in vitro* grown (IVG) oocytes from primordial follicles have resulted in the production of live offspring. Human oocytes have been grown *in vitro* from the secondary/multi-laminar stage to obtain fully grown oocytes capable of meiotic maturation. However, there are no reports of a culture system supporting complete growth from the earliest stages of human follicle development through to Metaphase II.

STUDY DESIGN, SIZE, DURATION: Ovarian cortical biopsies were obtained with informed consent from women undergoing elective caesarean section (mean age: 30.7 ± 1.7 ; range: 25–39 years, $n = 10$).

PARTICIPANTS/MATERIALS, SETTING, METHODS: Laboratory setting. Ovarian biopsies were dissected into thin strips, and after removal of growing follicles were cultured in serum free medium for 8 days (Step 1). At the end of this period secondary/multi-laminar follicles were dissected from the strips and intact follicles 100–150 μm in diameter were selected for further culture. Isolated follicles were cultured individually in serum free medium in the presence of 100 ng/ml of human recombinant Activin A (Step 2). Individual follicles were monitored and after 8 days, cumulus oocyte complexes (COCs) were retrieved by gentle pressure on the cultured follicles. Complexes with complete cumulus and adherent mural granulosa cells were selected and cultured in the presence of Activin A and FSH on membranes for a further 4 days (Step 3). At the end of Step 3, complexes containing oocytes $> 100 \mu\text{m}$ diameter were selected for IVM in SAGE medium (Step 4) then fixed for analysis.

MAIN RESULTS AND THE ROLE OF CHANCE: Pieces of human ovarian cortex cultured in serum free medium for 8 days (Step 1) supported early follicle growth and 87 secondary follicles of diameter $120 \pm 6 \mu\text{m}$ (mean \pm SEM) could be dissected for further culture. After a further 8 days, 54 of the 87 follicles had reached the antral stage of development. COCs were retrieved by gentle pressure from the cultured follicles and those with adherent mural granulosa cells ($n = 48$) were selected and cultured for a further 4 days (Step 3). At the end of Step 3, 32 complexes contained oocytes $> 100 \mu\text{m}$ diameter were selected for IVM (Step 4). Nine of these complexes contained polar bodies within 24 h and all polar bodies were abnormally large. Confocal immuno-histochemical analysis showed the presence of a Metaphase II spindle confirming that these IVG oocytes had resumed meiosis but their developmental potential is unknown.

LIMITATIONS, REASONS FOR CAUTION: This is a small number of samples but provides proof of concept that complete development of human oocytes can occur *in vitro*. Further optimization with morphological evaluation and fertilization potential of IVG oocytes is required to determine whether they are normal.

WIDER IMPLICATIONS OF THE FINDINGS: The ability to develop human oocytes from the earliest follicular stages *in vitro* through to maturation and fertilization would benefit fertility preservation practice.

STUDY FUNDING/COMPETING INTEREST(S): Funded by MRC Grants (G0901839 and MR/L00299X/1). No competing interests.

Key words: ovary / follicle / oocyte / *in vitro* growth / IVM / meiosis / human

Introduction

In vitro systems have been developed to support the growth and development of mouse oocytes from the most immature stage to produce developmentally competent oocytes (Eppig and O'Brien, 1996; O'Brien et al., 2003). The most immature stage of follicles (primordial) can be grown in a two-step culture system to produce oocytes that can be fertilized, and live offspring have been produced from these *in vitro* grown (IVG) oocytes (Eppig and O'Brien, 1996; O'Brien et al., 2003). Recent reports have shown that the process of mouse oocyte development can be recapitulated *in vitro* starting from primordial germ cells and induced pluripotent stem cells (Hikabe et al., 2016; Morohaku et al., 2016) but little is known about the potential for human IVG oocytes.

We previously developed a two-step culture system where human primordial follicle activation and growth to the secondary/multi-laminar stage was achieved within cortical pieces (Telfer et al., 2008). Development of follicles to the antral stage could be obtained when growing follicles were removed from the cortex and cultured individually in the presence of activin (Telfer et al., 2008). Other groups have demonstrated the developmental potential of growing isolated human multi-laminar follicles *in vitro* (Xu et al., 2009) and have confirmed that they can develop to the stage where they resume meiosis and reach Metaphase II (Xiao et al., 2015).

The aim of this study was to determine whether complete human oocyte growth from immature primordial/unilaminar follicles could be achieved *in vitro* by extending our two-step culture system (Telfer et al., 2008; Telfer and McLaughlin, 2012) to obtain antral follicles and mature oocytes. Herein we describe the addition of a third step to our culture system and demonstrate the acquisition and expression of meiotic competence of a subset of IVG human oocytes as evidenced by the progression to Metaphase II after a total of 20 days in culture starting from primordial/unilaminar follicles.

Materials and Methods

Ovarian cortical tissue

Fresh ovarian cortical biopsies ($n = 10$) of approximate size 5 mm \times 4 mm (with variable thickness) were obtained with informed consent from women undergoing elective caesarean section (mean age: 30.7 ± 1.7 ; range: 25–39 years). Approval of this study was given by the local ethics committee (ref LREC 10/S1101/2). Ovarian tissue was transported to the laboratory in pre-warmed dissection medium [Leibovitz medium (Invitrogen Ltd, Paisley, UK) supplemented with sodium pyruvate (2 mM), glutamine (2 mM) (both Invitrogen Ltd, Paisley, UK), HSA (3 mg/ml), penicillin G (75 μ g/ml) and streptomycin (50 μ g/ml) (Sigma Chemicals, Poole, Dorset UK)].

Tissue preparation and fragment culture (Step 1)

On arrival to the laboratory the tissue was transferred into fresh dissection medium, examined under light microscopy and any damaged, haemorrhagic

or excess stromal tissue was excised allowing the pieces to flatten and affording better microscopic visualization. The flattened tissue pieces were dissected into fragments of $\sim 1 \times 1 \times 0.5 \text{ mm}^3$, re-examined under light microscopy and any visible follicles measured using an eyepiece micrometer. Follicles with a mean diameter $> 40 \mu\text{m}$ were excised from the tissue fragments using fine needles, to ensure that the presumptive population of follicles contained within the fragments was unilaminar, i.e. primordial, transitional or primary. A total of 160 fragments were prepared and incubated individually in 24-well cell culture plates (Corning B.V. Life Sciences Europe, Amsterdam). Each well contained 300 μl of culture medium [McCoy's 5a medium with bicarbonate supplemented with HEPES (20 mM; Invitrogen Ltd, Paisley, UK), glutamine (3 mM; Invitrogen Ltd, Paisley, UK), HSA (0.1%), penicillin G (0.1 mg/ml), streptomycin (0.1 mg/ml), transferrin (2.5 $\mu\text{g/ml}$), selenium (4 ng/ml), human insulin (10 ng/ml) 1 ng/ml recombinant human FSH (rhFSH) and ascorbic acid (50 $\mu\text{g/ml}$) (all obtained from Sigma Chemicals, Poole, Dorset, UK, unless otherwise stated)]. Fragments were cultured for 8 days at 37°C in humidified air with 5% CO₂ with half the media being removed and replaced every second day according to our previously published system (Telfer et al., 2008; McLaughlin et al., 2011, 2014; Anderson et al., 2014).

At least two cortical fragments were taken from each biopsy at the start and end of the culture period ($n = 24$) to obtain an indication of the population of follicles present at each time. Fragments were fixed in Bouin's fluid and processed for histological evaluation as Days 0 and 8 controls following a previously published protocol (Telfer et al., 2008). Every histological section of each tissue fragment was examined under light microscopy and follicles were categorized according to their stage of development as previously described (Telfer et al., 2008; McLaughlin et al., 2014).

Follicle isolation and culture (Step 2)

During Step 1 growing follicles were observed on the surface of the cortical fragments and within 8 days of culture visible follicles of 100–150 μm in diameter were observed. These growing follicles were mechanically dissected with 25 gauge needles and those with an intact basement membrane were selected for further culture. Secondary follicles dissected from the cortical fragments were measured using an eyepiece micrometer and placed individually into 96-well V-bottomed culture plates (Corning Costar Europe, Badhoevedorp, The Netherlands) in 150 μl of culture medium supplemented with 100 ng/ml of human recombinant Activin A and 1 ng/ml rhFSH. Follicles were cultured individually for a further 8 days at 37°C in humidified air with 5% CO₂. Half the culture medium was replaced at 2-day intervals and at this time follicle diameter measurements were taken using a dissecting microscope with a crossed micrometer. Upon microscopic evaluation any damaged or degenerate follicles were excluded.

Isolation and culture of oocyte–granulosa cell complexes (Step 3)

Individual follicles were monitored and at the end of 8 days in Step 2, follicles with antral cavities were observed. COCs were retrieved from intact healthy cultured follicles by gentle pressure. The aim of this stage was to obtain oocytes that were at least a 100 μm in diameter for IVM. Complexes with complete cumulus and adherent mural granulosa cells were selected and cultured in the presence of 100 ng/ml of Activin-A and 1 ng/ml of rhFSH on

track-etched nucleopore membranes (Millipore) in 4-well culture plates with a maximum of six complexes per membrane. Complexes were cultured and the oocyte diameter monitored for a further 4 days (Step 3). If oocytes had not reached 100 μm by Day 4 they were cultured for an additional 2 days. At the end of Step 3 (total of 20 days *in vitro*), complexes containing oocytes >100 μm in diameter were selected for IVM.

IVM (Step 4)

Isolated complexes containing oocytes with a diameter >100 μm were placed in SAGE IVM medium (Cooper Surgical, Trumbull, Connecticut, USA) containing 75 mIU/ml of FSH and 75 mIU/ml of LH in humidified air and 5% CO_2 at 37°C. Complexes were examined after 24 h for the presence of a polar body and cumulus cell expansion. A selection of complexes with and without polar bodies were taken for confocal analysis.

Confocal analysis of oocytes

COCs were fixed in a microtubule stabilizing buffer supplemented with 3.7% (v/v) formaldehyde, 0.1% (v/v) Triton X-100, 1 mM taxol, 0.01% (w/v) aprotinin, 1 mM dithiothreitol and 50% (v/v) deuterium oxide as described previously (Wickramasinghe and Albertini, 1992).

Samples were stored at 4°C in a PBS blocking solution containing 1% (w/v) BSA, 0.2% (w/v) powdered milk, 2% (v/v) normal goat serum, 0.1 M glycine, 0.2% (w/v) sodium azide and 0.01% Triton X-100 until the time of specimen processing. The detection of microtubules, microfilaments and chromatin by was accomplished using a triple-stain strategy as follows. Oocytes were first incubated for 4 h at 37°C in a mixture of mouse monoclonal anti-tubulins (alpha and beta, Invitrogen, Sigma) at a final dilution of 1:500. Following several washes in blocking solution, samples were incubated in 1:400 dilution of Alexa 488 goat anti-mouse IgG (1:500; Invitrogen) and rhodamine-phalloidin (1:200, Invitrogen) for 3 h at 37°C. Samples were mounted in a glycerol-based medium containing Hoechst 33258 (1 ng/ml) on 1 mm posts of Vaseline to prevent compression of the oocytes and surrounding cumulus cells (Barrett and Albertini, 2010).

A Zeiss LSM Pascal system mounted on a Zeiss Axiovert II was used for confocal microscopy and all image collection was done with a 1.2 n.a. $\times 40$ water immersion objective with excitation lines at 405 nm (Diode, for Hoechst), at 488 nm (Argon laser for anti-tubulin) and 548 nm (HeNe for rhodamine phalloidin).

Statistical analysis

The proportions of the developmental stages observed by histological analysis pre and post culture were compared using chi-squared analysis. Mean follicle diameter measurements during stage 2 of culture and mean oocyte diameters during stage 3 were compared using a two sample t-test. *P* values of <0.05 were considered significant. All statistical analyses were carried out using EXCEL 2016 (Microsoft).

Results

Ovarian cortical tissue being prepared for culture contained no visible growing follicles (Fig. 1A) and this was confirmed by examining histological sections (Fig. 1B). Histological analysis showed the distribution of follicle stages to be 97.1% unilaminar (primordial or primary) at the start of culture. Counts of 385 follicles from histological sections at Day 0 showed 80.5% primordial, 16.6% primary and 2.9% secondary. Counts of 355 follicles after 8 days of culture showed 43.1% primordial, 46.1% primary and 10.8% secondary. Therefore, an increased ($P < 0.05$) percentage of growing follicles (primary and secondary) and a corresponding decrease ($P < 0.01$) in the percentage of non-growing follicles was observed in cultured tissue compared to freshly fixed tissue (Day 0).

Within 8 days of culture in serum free medium containing a low dose of FSH, growing follicles were observed on the surface of the cultured pieces (Fig. 1C), histological analysis of fragments cultured for 8 days showed the presence of growing multi-laminar follicles (Fig. 1D and g). Multi-laminar follicles can be observed under the dissecting microscope (Fig. 1E) and isolated by micro-dissection with attached stromal cells for individual culture in Step 2 (Fig. 1F).

A total of 87 follicles with a mean diameter of $121 \pm 2.5 \mu\text{m}$ (range: 100–150 μm) were dissected from the cultured fragments after 8 days and placed in Step 2 of the culture system. During the first 4 days of culture isolated follicles increased ($P < 0.005$) in size (Fig. 2). A further increase ($P < 0.005$) in size was observed between Days 4 and 6 during Step 2 of culture (Fig. 2) (total of 14 days *in vitro*). The mean follicle diameter increased to $248 \pm 4.5 \mu\text{m}$ (range: 198–314 μm ; Fig. 2) after 6 days in Step 2. Follicles were kept in Step 2 of culture for 8 days but no increase in diameter was observed between 6 and 8 days (Fig. 2). At the end of Step 2, 54 out of 87 follicles (62%) were identified as being intact and having reached the early-mid antral stage (Fig. 1H and I). From the 54 antral follicles, 48 oocyte granulosa cell complexes (Fig. 1J) were isolated for further culture on membranes (Step 3; Fig. 3). The mean oocyte diameter of complexes selected for further growth was $91.4 \pm 1.5 \mu\text{m}$ (range: 82.6–99 μm ; Fig. 4).

From the 48 oocyte-granulosa cell complexes grown on membranes (Step 3; Fig. 3) 32 remained intact and showed significant oocyte growth after 2 and 4 days on membranes ($P < 0.005$) (Fig. 4). Complexes that had not reached 100 μm in diameter after 4 days in Step 3 did not increase in size after 6 days in Step 3.

The results show that 36% of follicles isolated after Step 1 went on to form antral cavities and produce oocytes of diameter >100 μm which could be placed in maturation medium to determine their meiotic potential (Fig. 3).

Of 32 complexes isolated for IVM, 9 emitted polar bodies after 24 h in maturation media (Figs 3 and 5A). This result showed that complete oocyte growth could occur from primordial/unilaminar stages to antral stages, with 36.7% of isolated follicles producing fully grown oocytes after a total of 21 days in culture, and with 10% of the isolated follicles producing complexes that had polar bodies after IVM (Fig. 5A–D). In all cases the polar bodies were larger than that observed from *in vivo* grown oocytes, with the ratio between oocyte size to polar body size ranging from 4:1 to 3:1 (Fig. 5A–D). Whilst cumulus expansion did occur the degree of expansion was less than that reported from *in vivo* grown oocytes.

Confocal analysis showed the co-existence of a Metaphase II spindle and polar body confirming that these IVG oocytes had progressed through meiosis establishing the acquisition of meiotic competence for at least a fraction of the oocytes subjected to IVM (Fig. 5A–E). The spindles observed ranged from those with tapered poles (Fig. 5B and E) to that with more typical broad spindle poles (Fig. 5D).

Oocytes lacking a polar body after IVM were shown to be arrested at GV stage (Fig. 6A) or Metaphase-I, the latter showing chromosomes associated with aberrant spindles (Fig. 6B and C).

Discussion

The main objective of this study was to ascertain the ability of an *in vitro* system to support growth of immature human oocytes within the ovarian cortex to achieve a state of differentiation approaching that of a meiotically competent ovum. We describe here the successful development of a

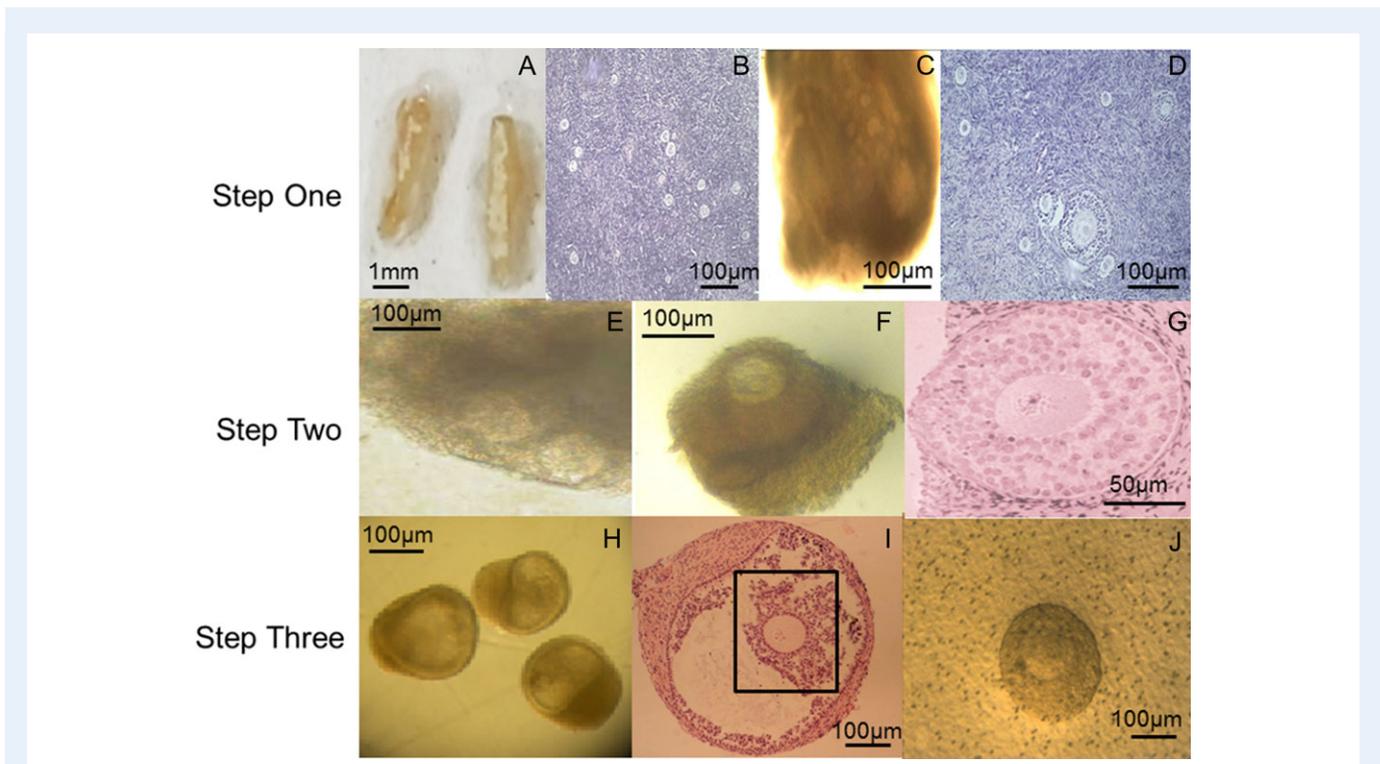


Figure 1 Photomicrographs illustrating each step in the multi-step culture system. (A–D) Illustrate the tissue during Step 1. Pieces of ovarian cortex are prepared by removing underlying cortex and creating strips that are then prepared into fragments. The cortex is prepared into strips of $5 \times 2 \times 1 \text{ mm}^3$ (A) and then cut into fragments of $\sim 1 \times 1 \times 0.5 \text{ mm}^3$. Tissue prepared for culture and examined histologically at Day 0 show the presence of predominantly primordial and primary follicles (B). After 8 days in culture growing follicles can be observed within the cultured ovarian cortex (C); histological evaluation of cultured pieces *in vitro* for 8 days shows the presence of growing follicles (D). (E–G) Illustrate Step 2. Pieces of ovarian cortex cultured for 8 days with growing follicles ready to be dissected out (E) multi-laminar follicles tend to be at the edge of the cultured fragments (E). Isolated follicle after dissection from a cultured fragment (F) the follicle is kept within stromal cells and cultured individually (Step 2). Histological section of a multi-laminar follicle dissected from cultured fragment after 8 days (G). Note the healthy oocyte and granulosa cells. Antral follicles at the end of Step 2 (H). Histological section of *in vitro* grown follicle/ oocyte showing mural and cumulus granulosa cells (I) and oocyte–granulosa/cumulus complex isolated from *in vitro* grown follicles and placed on membranes for further growth (J) during Step 3.

small number of human oocytes capable of acquiring and expressing a level of development approaching that of an ovulated ovum. The main criteria consistent with achieving our goals include: (i) the progressive growth of oocytes from primordial to early antral stage with maintenance of somatic cell support; (ii) cytological features of the nucleus (GV) and cytoplasm consistent with the differentiation of human oocytes within the intact ovary; and (iii) the acquisition of meiotic competence over the duration of the multi-step *in vitro* assay that resulted in $\sim 30\%$ of oocytes that survived the culture system emitting a polar body and reaching the Metaphase II stage of meiosis. Together, these results provide initial validation for a multi-step culture model capable of supporting the *ex vivo* propagation of human oocytes. This model system has the potential to advance our understanding of oogenesis in humans especially in relation to the treatment of infertility and regenerative medicine but clearly requires to be optimized.

The idea of obtaining viable oocytes by growing immature oocytes *in vitro* has been the subject of a great deal of research for almost 30 years (Eppig and Telfer, 1993). Complete growth *in vitro* from the most immature oocytes (primordial stages) with subsequent IVF and production of live young has only been achieved in mouse (Eppig and O'Brien, 1996; O'Brien et al., 2003). Early work on that two-step

culture system resulted in only one live offspring being obtained, and that mouse had many abnormalities as an adult (Eppig and O'Brien, 1996). Following improvements in the technique and after alterations to the culture medium several mouse embryos and offspring have been obtained using oocytes that have been IVG then combined with IVM and IVF (O'Brien et al., 2003). More recently, *in vitro* systems that support the complete development of mouse oocytes starting from induced pluripotent stem cells (Hikabe et al., 2016) and from primordial germ cells (Morohaku et al., 2016) have been reported and these systems have resulted in competent oocytes that produce embryos and live young.

Whilst there have been advances in culturing follicles and oocytes from humans, non-human primates and domestic species (McLaughlin and Telfer, 2010; Smitz et al., 2010; Hirao et al., 2013; Telfer and Zelinski, 2013; Xiao et al., 2015) there is no single system that has been fully optimized to support the complete *in vitro* growth and maturation of human follicles and oocytes from the earliest stages. There is a consensus that to achieve complete *in vitro* growth a dynamic culture system is required to support the three main transition steps (Smitz et al., 2010; Telfer and McLaughlin, 2012; Telfer and Zelinski, 2013). These are (i) activation of primordial follicles (Hovatta et al.,

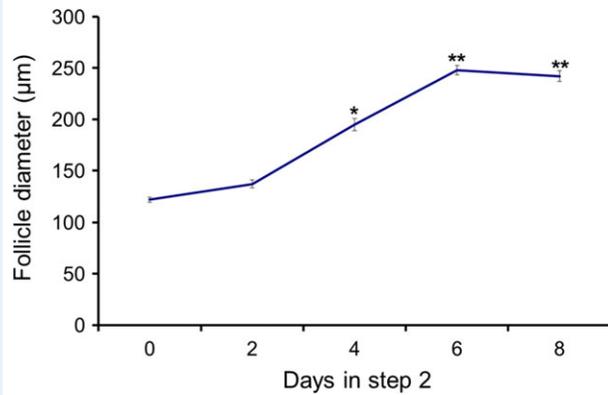


Figure 2 Growth rate of isolated follicles during Step 2 of culture. Multi-laminar follicles were isolated by micro-dissection from cultured ovarian cortex after 8 days *in vitro* and placed in individual v shaped culture wells. Follicle diameters were measured at 2-day intervals during an 8-day culture period. After 4 days in Step 2 follicles were larger (* $P < 0.005$) than at the start and increased in size up to 6 days (** $P < 0.005$) before plateauing after 8 days in Step 2.

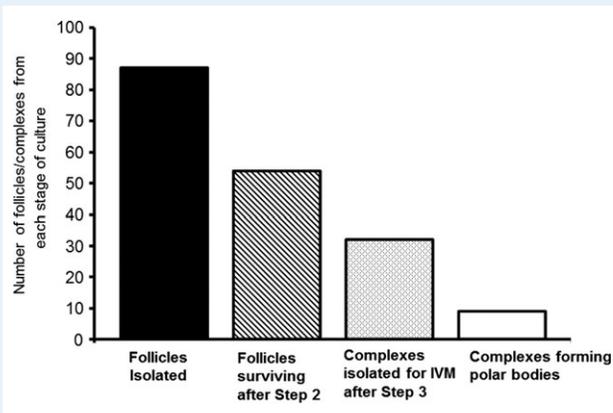


Figure 3 The number of follicles/complexes isolated at each stage of culture. At the end of Step 1 of culture 87 follicles were dissected from fragments to be placed into Step 2 (black bar). During Step 2 54 follicles reached the antral stage (62%) (diagonal striped bar). From 54 follicles 48 oocyte–cumulus cell complexes were retrieved and placed on membranes for Step 3 (55%). The 32 complexes contained oocytes $>100 \mu\text{m}$ (36%) at the end of Step 3 (hatched bar). The 32 complexes were placed in maturation medium and nine showed polar bodies after 24 h (white bar). This represents 10.3% of *in vitro* grown follicles isolated resulted in fully grown oocytes with some level of meiotic competence.

1999; Telfer *et al.*, 2008; Anderson *et al.*, 2014; McLaughlin *et al.*, 2014), (ii) isolation and culture of growing follicles to achieve oocyte growth and development (Hovatta *et al.*, 1999; Telfer *et al.*, 2008; Xu *et al.*, 2009; Anderson *et al.*, 2014; McLaughlin *et al.*, 2014; Xiao *et al.*, 2015), and (iii) removal from the follicle environment and maturation of oocyte cumulus complexes (Cavilla *et al.*, 2008; Chian *et al.*, 2013). The multi-step culture system described here integrates multiple

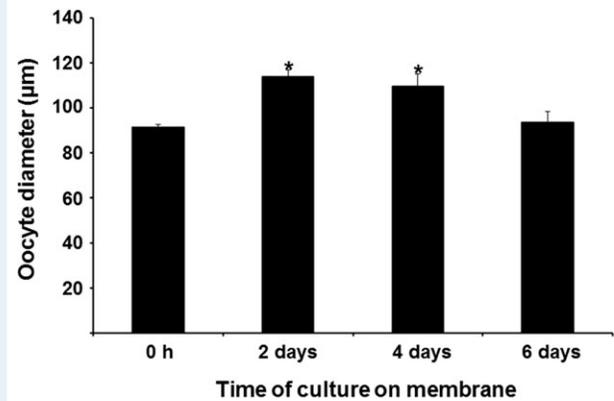


Figure 4 Mean oocyte diameter of oocytes placed on membranes (time 0) and after 2, 4 and 6 days of culture on membranes. Oocytes showed a significant (*) increase in diameter after 4 and 6 days ($P < 0.005$). Only oocytes $>100 \mu\text{m}$ after Day 4 of Step 3 were used for testing meiotic competence in the IVM step. Oocytes that did not reach $100 \mu\text{m}$ within 4 days did not show significant growth if left for 6 days.

approaches resulting in the propagation of functional human oocytes based on assessment of meiotic competence acquisition.

Previous studies have cultured human multi-laminar follicles encapsulated in alginate hydrogels to support follicle growth *in vitro* (Xu *et al.*, 2009; Xiao *et al.*, 2015). Alginate encapsulation mimics the extracellular matrix in terms of its ability to facilitate molecular exchange between the follicle and the culture medium. Isolated secondary human follicles grown in this way become steroidogenically active and complete oocyte growth in 30 days (Xu *et al.*, 2009) and have been shown to be capable of meiotic maturation (Xiao *et al.*, 2015).

In the present multi-step culture system, quiescent follicles activated to grow within cultured fragments of cortex and mechanically isolated as secondary follicles became steroidogenic and exhibited signs of follicle differentiation after a total of 16 days *in vitro* (Telfer *et al.*, 2008). Once follicles reach the antral stage a further period of growth of the isolated oocyte–granulosa cell complex is required before maturation can take place (McLaughlin and Telfer, 2010). To achieve this final growth phase the oocyte somatic cell unit is removed from the antral follicle: this is technically demanding but 48 from 54 IVG follicles were successfully isolated. In developing these complexes further, maintaining contact between the oocyte and somatic cells is key to supporting oocyte development (Li and Albertini, 2013). Maintenance of oocyte somatic cell interactions and cytoskeleton stability *in vitro* is a challenge in *ex vivo* systems. The use of membranes for the final stages of oocyte development has been shown to be successful in the rodent model (Eppig and O'Brien, 1996) and appears to support human oocyte development in this system. We have previously demonstrated that the correct balance of activin and FSH *in vitro* imparts a stabilizing influence on oocyte–somatic cell connections in bovine IVG follicles (McLaughlin *et al.*, 2010) and this knowledge has informed the culture system described here. While the pattern of cell contacts was not followed throughout the various steps employed in this study, it was noted by confocal microscopy that few trans-zonal projections were present in cumulus oocyte complexes following IVM. A lingering

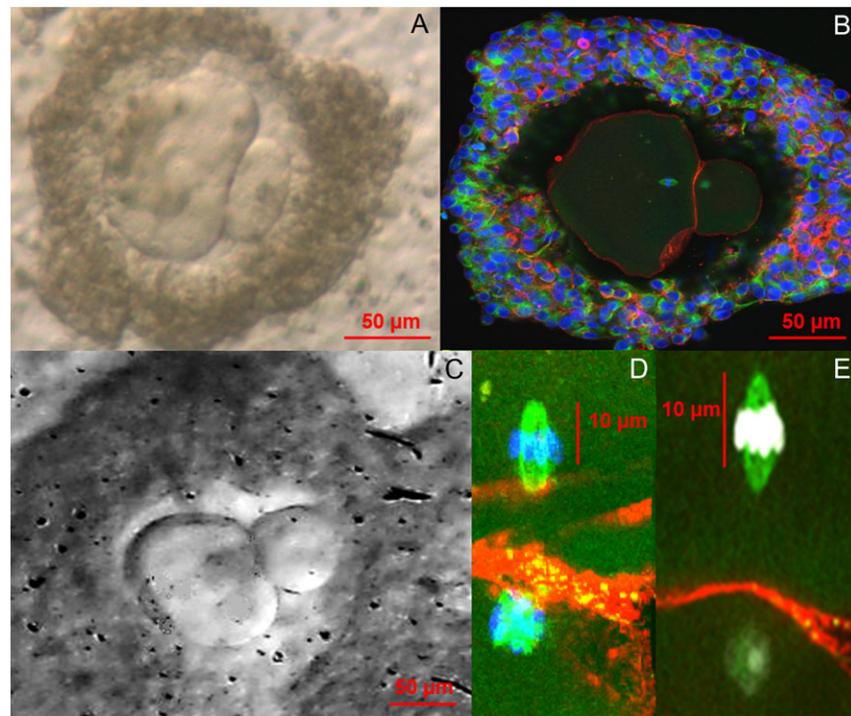


Figure 5 Bright field (**A, C**) and confocal images (**B, D, E**) of *in vitro* grown and *in vitro* matured metaphase-II human oocytes. The compact cumulus is separated from the oocytes (**A–C**) that display an enlarged first polar body (**A–C**). The spindle (green) with tapered poles anchored to the oocyte cortex (red) in juxtaposition to the polar body (**B**). Equatorially aligned chromosomes (blue in **B, D**; white in **E**) on the spindle. An enrichment of f-actin is seen at the interface between the polar body and oolemma where the spindle is attached (**D, E**). (**D**) An additional MII with more typical broad spindle poles (green) and chromosome alignment (blue) on the metaphase spindle relating to the polar body seen by light microscopy in (**C**).

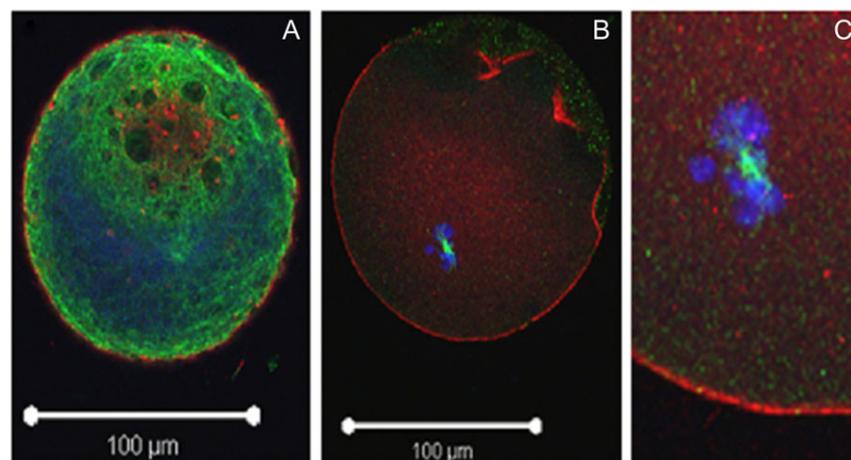


Figure 6 Representation of oocytes that failed to emit a polar body after IVM. (**A**) Illustrates a germinal vesicle (GV) arrested oocyte containing a dense cytoplasmic microtubule network (green) indicative of retention in an interphase G2 state of the cell cycle. In (**B**), an oocyte arrested at MI has undergone germinal vesicle breakdown and chromosomes (blue) are located around a small monopolar meiotic spindle; note the displacement of the spindle remnant from the oocyte cortex and appearance of f-actin foci in the vicinity of the spindle (**C**, 3.3 \times zoom of **B**).

question has been whether the growth rate observed in this system represents acceleration of growth or is the consequence of removing *in vivo* inhibitory forces. While it appears that development of a

significant proportion of oocytes occurs at a faster rate relative to *in vivo* estimates our system gives some insight into the process of oogenesis in humans that will require further experimentation.

The final goal of an *in vitro* system is to produce oocytes capable of fertilization and developmental competence. The IVG human oocytes need to be matured *in vitro* and in this study a total of 32 complexes were selected for IVM with 9 showing emission of a polar body after 24 h in IVM medium. For those oocytes examined by confocal microscopy, it was noted that abnormally large polar bodies were present in oocytes demonstrating Metaphase II spindles. Previous studies on mouse oocytes have shown that both the proximity of the spindle to the oocyte cortex and the inter-chromosomal spacing within Metaphase II spindles will influence polar body size (Barrett and Albertini, 2010). Wider spacing of chromosomes and loss of spindle contact with the oocyte cortex lead to extrusion of polar bodies of unusually large sizes compared to controls (Coticchio *et al.*, 2013). The present observations clearly warrant closer examination of these relationships within IVG human oocytes and the likely consequences such abnormalities would have on chromosome balance in mature oocytes.

In this study a relatively low number of oocytes reached Metaphase II. All tissue came from women within a similar age range and at the end of pregnancy thus we are unable to correlate any particular characteristics with culture success. Each stage of the culture system requires optimization and even when relatively mature GV oocytes are used clinically it is widely accepted that the rate of maturation *in vitro* is still well below that of oocytes harvested from stimulated ovaries, indicating that the protocols for IVM are sub-optimal. It may also be that many of the harvested oocytes are intrinsically deficient in their ability to reinitiate and progress through meiosis (Nogueira *et al.*, 2012). The results presented here provide proof of concept in developing a complete *in vitro* growth system to support human oocyte development but highlight the need to optimize each of the stages of development and to gain further understanding of how the culture system affects development, particularly the epigenetic status of any embryos formed from IVG oocytes (Anckaert *et al.*, 2013).

The most significant clinical application of this work is in Fertility Preservation. Given the widespread adoption of ovarian tissue cryopreservation for cancer patients (Anderson *et al.*, 2017), our results make plausible the utilization of *in vitro* generated mature human oocytes for embryo production and thus an alternative to autologous transplantation particularly where there is a threat of tumour reintroduction as a result of grafting. Whether the multi-step model will be useful for other applications such as screening for potentially gonadotoxic therapies or environmental toxins is not yet known but these are becoming realistic prospects for future applications. Apart from the clinical implications of this work, the system described here provides direct access to the process of human oogenesis in an experimentally tractable paradigm for future research.

Acknowledgements

We are very grateful to Anne Saunderson and Norma Forson for patient recruitment, and to our obstetric colleagues for taking the ovarian biopsies.

Authors' roles

All authors were involved in designing and planning experiments, preparing and reviewing the article. M.Mc. carried out all the cultures, E.E.T. and

M.Mc. analysed data, D.F.A. carried out confocal analysis. All others approved the final article.

Funding

MRC Grants (G0901839 to E.E.T. and R.A.A.) and (MR/L00299X/1 to R.A.A.).

Conflict of interest

None of the authors have any conflicts of interest to declare.

References

- Anckaert E, De Rycke M, Smits J. Culture of oocytes and risk of imprinting defects. *Hum Reprod Update* 2013;**19**:52–66.
- Anderson RA, McLaughlin M, Wallace WH, Albertini DF, Telfer EE. The immature human ovary shows loss of abnormal follicles and increasing follicle developmental competence through childhood and adolescence. *Hum Reprod* 2014;**29**:97–106.
- Anderson RA, Wallace WH, Telfer EE. Ovarian tissue cryopreservation for fertility preservation: clinical and research perspectives. *Hum Reprod Open* 2017;**1**. <https://doi.org/10.1093/hropen/hox001>.
- Barrett SB, Albertini DF. Cumulus cell contact during oocyte maturation in mice regulates meiotic spindle positioning and enhances developmental potential. *J Assist Reprod Genet* 2010;**27**:29–39.
- Cavilla JL, Kennedy CR, Byskov AG, Hartshorne GM. Human immature oocytes grow during culture for IVM. *Hum Reprod* 2008;**23**:37–45.
- Chian RC, Uzelac PS, Nargund G. *In vitro* maturation of human immature oocytes for fertility preservation. *Fertil Steril* 2013;**99**:1173–1181.
- Coticchio G, Guglielmo MC, Dal Canto M, Fadini R, Mignini Renzini M, De Ponti E, Brambillasca F, Albertini DF. Mechanistic foundations of the metaphase II spindle of human oocytes matured in vivo and in vitro. *Hum Reprod* 2013;**28**:3271–3282.
- Eppig JJ, O'Brien MJ. Development *in vitro* of mouse oocytes from primordial follicles. *Biol Reprod* 1996;**54**:197–207.
- Eppig JJ, Telfer EE. Isolation and culture of oocytes. *Methods Enzymol* 1993;**225**:77–84.
- Hikabe O, Hamazaki N, Nagamatsu G, Obata Y, Hirao Y, Hamada N, Shimamoto S, Imamura T, Nakashima K, Saitou M *et al.* Reconstitution in vitro of the entire cycle of the mouse female germ line. *Nature* 2016;**539**:299–303.
- Hirao Y, Naruse K, Kaneda M, Somfai T, Iga K, Shimizu M, Akagi S, Cao F, Kono T, Nagai T *et al.* Production of fertile offspring from oocytes grown *in vitro* by nuclear transfer in cattle. *Biol Reprod* 2013;**89**:57–61.
- Hovatta O, Wright C, Krausz T, Hardy K, Winston RM. Human primordial, primary and secondary ovarian follicles in long-term culture: effect of partial isolation. *Hum Reprod* 1999;**14**:2519–2524.
- Li R, Albertini DF. The road to maturation: somatic cell interaction and self-organization of the mammalian oocyte. *Nat Rev Mol Cell Biol* 2013;**14**:141–152.
- McLaughlin M, Bromfield JJ, Albertini DF, Telfer EE. Activin promotes follicular integrity and oogenesis in cultured pre-antral bovine follicles. *Mol Hum Reprod* 2010;**16**:644–653.
- McLaughlin M, Kinnell HL, Anderson RA, Telfer EE. Inhibition of phosphatase and tensin homologue (PTEN) in human ovary in vitro results in increased activation of primordial follicles but compromises development of growing follicles. *Mol Hum Reprod* 2014;**20**:736–744.
- McLaughlin M, Patrizio P, Kayisli U, Luk J, Thomson TC, Anderson RA, Telfer EE, Johnson J. mTOR kinase inhibition results in oocyte loss characterized by empty follicles in human ovarian cortical strips cultured *in vitro*. *Fertil Steril* 2011;**96**:1154–1159.

- McLaughlin M, Telfer EE. Oocyte development in bovine primordial follicles is promoted by activin and FSH within a two-step serum-free culture system. *Reproduction* 2010;**139**:971–978.
- Morohaku K, Tanimoto R, Sasaki K, Kawahara-Miki R, Kono T, Hayashi K, Hirao Y, Obata Y. Complete *in vitro* generation of fertile oocytes from mouse primordial germ cells. *Proc Natl Acad Sci USA* 2016;**113**:9021–9026.
- Nogueira D, Sadeu JC, Montagut J. *In vitro* oocyte maturation: current status. *Semin Reprod Med* 2012;**30**:199–213.
- O'Brien MJ, Pendola JK, Eppig JJ. A revised protocol for *in vitro* development of mouse oocytes from primordial follicles dramatically improves their developmental competence. *Biol Reprod* 2003;**68**:1682–1686.
- Smitz J, Dolmans MM, Donnez J, Fortune JE, Hovatta O, Jewgenow K, Picton HM, Plancha C, Shea LD, Stouffer RL et al. Current achievements and future research directions in ovarian tissue culture, *in vitro* follicle development and transplantation: implications for fertility preservation. *Hum Reprod Update* 2010;**16**:395–414.
- Telfer EE, McLaughlin M. Strategies to support human oocyte development *in vitro*. *Int J Dev Biol* 2012;**56**:901–907.
- Telfer EE, McLaughlin M, Ding C, Thong KJ. A two-step serum-free culture system supports development of human oocytes from primordial follicles in the presence of activin. *Hum Reprod* 2008;**23**:1151–1158.
- Telfer EE, Zelinski MB. Ovarian follicle culture: advances and challenges for human and nonhuman primates. *Fertil Steril* 2013;**99**:1523–1533.
- Wickramasinghe D, Albertini DF. Centrosome phosphorylation and the developmental expression of meiotic competence in mouse oocytes. *Dev Biol* 1992;**152**:62–74.
- Xiao S, Zhang J, Romero MM, Smith KN, Shea LD, Woodruff TK. *In vitro* follicle growth supports human oocyte meiotic maturation. *Sci Rep* 2015;**5**:17323.
- Xu M, Barrett SL, West-Farrell E, Kondapalli LA, Kiesewetter SE, Shea LD, Woodruff TK. *In vitro* grown human ovarian follicles from cancer patients support oocyte growth. *Hum Reprod* 2009;**24**:2531–2540.