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1 Future developments: *In vitro* growth (IVG) of human ovarian follicles

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19

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24 Key Message: In Vitro Growth (IVG) of immature oocytes from cryo-preserved ovarian

25 cortex has the potential to produce mature oocytes but is still at an experimental stage.

26 Further research is required before clinical application can be realised

27

28

29

30 **Abstract**

31 Removal and storage of ovarian cortical tissue is currently offered to young female cancer
32 patients undergoing potentially sterilizing chemotherapy and/or radiotherapy. For patients at
33 high risk of re-introduction of malignancy through auto-transplantation, the ultimate aim is to
34 achieve complete oocyte development from this tissue *in vitro*. The ability to develop human
35 oocytes from the earliest follicular stages through to maturation and fertilisation *in vitro*
36 would revolutionise fertility preservation practice. This has been achieved in mouse where *in*
37 *vitro* grown (IVG) oocytes from primordial follicles have resulted in the production of live
38 offspring. Systems that support growth and development of oocytes from human ovarian
39 cortex are being developed by several groups. This review focuses on the steps required to
40 recapitulate *in vitro* the process of human oocyte development from the primordial stage and
41 the systems currently available to support this.

42

43 **Introduction**

44 The ability to develop human immature oocytes *in vitro* would have many
45 potential applications but would be of particular relevance to Fertility Preservation. Ovarian
46 tissue cryopreservation is now an option for women with cancer prior to undergoing
47 gonadotoxic treatments (1). Re-implantation of cryo-preserved tissue is currently the only
48 option to use stored tissue but in many cases, particularly for women with Leukemia,
49 malignant cells are present in the ovary therefore, re-implantation is not an option, however,
50 the oocytes within this tissue could potentially be grown *in vitro* (1). Culture systems with the
51 aim of achieving *in vitro* growth (IVG) of immature oocytes to maturity and subsequent
52 fertilization *in vitro* (IVF) have been the subject of research for almost 40 years. Several
53 systems that support the growth of later stages of follicle development from rodents have
54 been developed (2-8) with some reporting the production of live young (3, 4, 6-8). Complete
55 development from the most immature oocytes (primordial stages) *in vitro* with subsequent
56 IVF of oocytes followed by embryo transfer and production of offspring has been achieved in
57 the mouse using a two step culture system (9, 10). The initial studies resulted in the birth of
58 one mouse which developed abnormalities as an adult (9). Following alterations to the culture
59 medium several mouse embryos and offspring have been obtained using IVG oocytes then
60 combined with *in vitro* maturation (IVM) and IVF (10). More recently, *in vitro* systems that
61 support complete development of murine oocytes starting from induced pluripotent stem cells
62 (11) and from primordial germ cells (12) have been reported and these systems have resulted
63 in competent oocytes that produce embryos and live young.

64 The work on rodents has provided proof of principle and has encouraged the challenge of
65 adapting these systems to support human oocyte development *in vitro*. IVG of primordial
66 follicles would be of particular benefit to pre-pubertal girls undergoing fertility preservation
67 before being exposed to damaging chemotherapy (13). Currently the only fertility
68 preservation option for pre-pubertal girls is storage of ovarian cortical tissue with the
69 potential for re-implantation at a later time (13). In cases where re-implantation is not
70 possible, IVG could provide another option to restore fertility. For IVG to be clinically
71 viable, the process would need to start with primordial follicles as cortical strips that are
72 stored contain predominantly this stage. Whilst there still remains much detailed research to
73 be carried out before IVG of primordial follicles could ever be clinically applied, a great deal
74 of progress has been made in developing culture techniques to support human oocyte

75 development *in vitro*. In this review the current status of human IVG from primordial
76 follicles will be considered.

77

78 **Stages of Follicle Development**

79 The majority of follicles within the ovary in all young mammalian females will be at the
80 primordial stage of development and these follicles are continually utilised throughout
81 reproductive life (14). It is not known whether the pool of primordial follicles represents a
82 homogeneous population but at this stage follicles have not yet been exposed to selection
83 processes that lead to follicle degeneration (15, 16). Primordial follicles represent a “resting”
84 population of germ cells (oocyte arrested at dictyate stage of Prophase 1 of meiosis
85 surrounded by a few flattened granulosa cells), that are formed pre-natally. Recruitment into
86 growth takes place throughout the woman’s reproductive life and a sequence of precisely
87 regulated processes is required for complete oocyte/follicle development to occur. The
88 sequence starts with (a) initiation of primordial follicle growth and development to the
89 preantral follicle stage; (b) growth of the preantral stage with formation of a fluid filled cavity
90 (antrum) and expansion to the pre ovulatory or Graafian follicle stage (c) rupture of the
91 Graafian follicle releasing a cumulus-oocyte complex at ovulation in response to the mid-
92 cycle LH surge (17, 18).

93 The oocyte is held in meiotic arrest as it grows within the follicle and it must acquire the
94 ability to resume meiosis (meiotic competence) as well as the ability to support fertilisation
95 and embryonic development (developmental competence). Oocyte development is dependent
96 upon the environment of the individual follicle for its function as a gamete and this is
97 regulated by inhibitory and stimulatory endocrine, paracrine and autocrine signalling by the
98 somatic cells of the follicle (granulosa and surrounding theca cells) enhanced by several
99 oocyte specific factors mediated through bi-directional communication (19, 20). The
100 physiological requirements of the oocyte, granulosa and theca cells are extremely complex
101 and dynamic therefore recapitulating the process of follicle activation and growth *in vitro* is
102 one of the greatest technical challenges in reproductive technology (18).

103

104 **Growing Human follicles *in vitro***

105 Several approaches have been taken to support early human follicle development *in vitro*
106 starting with primordial follicle activation (21-31). Whilst there are several culture systems
107 that support a specific stage of human oocyte development *in vitro*, there is so far only one

108 report that supports human primordial follicles to the stage of meiotic maturation (30). To
109 achieve complete development of human oocytes *in vitro* a multi-step culture system is
110 required (26, 30, 32). The first step in this process is to facilitate the initiation of primordial
111 follicle development and support early growth; the second step is to optimise the growth of
112 follicles from preantral to antral stages; step 3 supports the completion of oocyte growth
113 ready for *in vitro* maturation in step 4 (Figure 1). In optimising a culture system to obtain
114 developmentally competent oocytes the focus should be on oocyte development and this may
115 preclude the need to develop large follicular structures *in vitro*. The multi-step approach
116 needs to support the changing requirements of the developing oocyte and its surrounding
117 somatic (granulosa) cells whilst maintaining good oocyte-somatic cell interactions.
118 Therefore, providing conditions that support the maintenance of appropriately differentiated
119 somatic cells in contact with the developing oocyte similar to the oocyte granulosa cell
120 complexes in the rodent system is essential (4, 10).

121

122 **IVA: *In vitro* Activation of human primordial follicles**

123 The majority of follicles within ovarian cortical tissue will be at the quiescent primordial
124 stage. Activation of primordial follicles *in vitro* (IVA) and early follicle development are key
125 features of any IVG system. Human primordial follicles can be activated and grow well
126 within mechanically loosened cortical pieces, developing to multilaminar preantral
127 (secondary) stages within 6 days (26, 30). Central to this process is preparation of the
128 ovarian tissue. This involves removal of most of the underlying stromal tissue and any
129 growing follicles so that the cultured tissue consists of ovarian cortex containing primordial
130 and primary follicles. When these small fragments of human ovarian cortex are cultured there
131 is a significant shift of follicles from the quiescent to the growing pool over short periods of 6
132 – 10 days (26, 29-31).

133 It remains unclear how follicle activation is controlled but it is known to involve a
134 combination of inhibitory, stimulatory and maintenance factors (33). The importance of the
135 phosphatidylinositol-3'-kinase (PI3K-AKT) signalling pathway within the oocyte has been
136 implicated in regulating activation of primordial follicles using mouse knockout models (34)
137 and in human using culture of ovarian cortex (29, 35, 36). The phosphatase and tensin
138 homolog deleted on chromosome ten (PTEN) acts as a negative regulator of this pathway and
139 suppresses initiation of follicle development (34). The transcription factor Forkhead Protein
140 O3 (FOXO3) is a downstream effector of this pathway and acts to inhibit follicle recruitment

141 (37). Other components of this pathway are dependent on the mammalian target of rapamycin
142 complex 1 (mTORC1), a serine/threonine kinase that regulates cell growth and proliferation
143 in response to growth factors and nutrients and also regulates primordial follicle activation
144 (38). From knockout mouse data it appears that whilst PTEN within the oocyte suppresses
145 activation of primordial follicles mTORC promotes it. How these pathways regulate human
146 follicle development is unclear but culture models facilitate the study of these processes (29,
147 36, 39).

148 Significant primordial follicle activation occurs in step one of the multi-step culture system
149 (26, 30). This activation appears to be as a result of disrupting the Hippo signalling pathway
150 during the preparation of the tissue (36, 40, 41). Hippo disruption increases expression of
151 downstream growth factors but manipulation of the PI3K pathway results in further activation
152 (29, 35, 36, 40, 41). Inhibition of PTEN in cultured human ovarian cortex results in increased
153 activation of primordial follicles and more secondary follicles but subsequent growth and
154 survival of isolated secondary follicles is compromised (29, 36). The detrimental effect on
155 secondary follicles may be as a result of alterations in DNA damage and repair responses as
156 demonstrated recently in a bovine culture model (42).

157

158 Cortical strip culture removes follicles from the *in vivo* endocrine and paracrine processes
159 regulating growth rate; however, follicles will still be subject to the effect of follicle
160 interactions and the influence of stromal cell factors. It is clear that tissue shape and stromal
161 density are important factors that contribute to the regulation of follicle growth initiation in-
162 vitro, as solid cubes of cortical tissue show lesser growth initiation (21) than cortex cultured
163 as flattened “sheets,” where much of the underlying stroma is removed (26, 30). The physical
164 environment of the follicles within the cortical tissue affects their response to stimulatory and
165 inhibitory factors and therefore influences their ability to grow (43). Once follicle growth has
166 been initiated within cortical tissue they can develop to multi-laminar stages but do not
167 survive well within the cortical environment as growth is inhibited resulting in loss of follicle
168 integrity and oocyte survival (22, 26). Growing follicles need to be released from the cortical
169 stromal environment and cultured individually to limit the effect of follicle interactions (26,
170 30, 31, 44).

171

172 **IVG: *In vitro* Growth of human preantral follicles**

173 Preantral follicles can be isolated from cortical tissue post culture by mechanical dissection,
174 enzymatic isolation or a combination of both. Collagenase and DNase can be used to remove
175 preantral follicles from stromal tissue, however collagenase can cause damage which leads to
176 poor survival of follicles (45). The presence of theca layers is required for growing follicles
177 to retain their structure and survive the second stage of IVG and these may be compromised
178 by collagenase (45). More purified enzyme preparations such as Liberase may avoid the
179 damage that occurs with Collagenase (46, 47). Mechanical isolation of follicles has the
180 advantage of preserving follicular integrity by maintaining the basal lamina and thecal layers,
181 however the procedure is laborious and results in a low yield (26, 30).

182

183 Supporting the growth of human preantral follicles *in vitro* has led to the development of
184 matrices to maintain follicle structure. Alginate hydrogels has been used to encapsulate
185 human preantral follicles and support their growth *in vitro* (48). Alginate encapsulation is
186 thought to mimic the extra cellular matrix *in vivo* in terms of its ability to facilitate molecular
187 exchange between the follicle and the culture medium whilst its flexibility can accommodate
188 cell proliferation but its rigidity prevents dissociation of the follicular unit. The rigidity of the
189 alginate capsule affects follicle development as inhibition of growth and reduced
190 steroidogenesis have been reported in murine follicles embedded in 1% alginate gels (49)
191 whereas fully grown human oocytes have been produced using 0.5% gels (48).

192 The application of tissue engineering to support the growth of isolated follicles has been
193 making progress; decellularized ovarian tissue and 3D micro-porous scaffolds are being
194 explored as matrices to support preantral follicle growth (50, 51). Recent work has explored
195 the production of electrospun patterned porous scaffolds which may be more accessible and
196 reproducible than decellularised tissue (52). Engineered scaffolds clearly have great potential
197 and should be developed further to support human follicle growth *in vitro*.

198 The multi-step culture system that has been developed for human follicles (30) does not use
199 matrices or scaffolds to support the growth of isolated preantral follicles. Isolated growing
200 follicles are cultured in v-shaped micro-well plates and this has supported follicular
201 architecture *in vitro* whilst promoting growth, differentiation and antral formation (26, 30).

202

203 Once follicles are isolated from the ovarian cortex their progression *in vitro* is remarkable.
204 Secondary human follicles isolated enzymatically from fresh ovarian tissue and cultured in
205 the presence of Follicle Stimulating Hormone (FSH), become steroidogenically active and

206 complete oocyte growth within 30 days (48) and these oocytes have been shown to be
207 capable of meiotic maturation (53). Primordial follicles grown within fragments of ovarian
208 cortex to multi-laminar stages which are then isolated without the use of enzymes and
209 cultured in the presence of Activin and FSH become steroidogenic within 10 days of *in vitro*
210 growth (26, 30).

211 During step 2 of the multi-step system, isolated follicles cultured individually form antral
212 cavities within 6-8 days. At this stage oocyte-granulosa cell complexes can be retrieved by
213 applying gentle pressure to the follicle. Complexes with complete cumulus and adherent
214 mural granulosa cells are selected for step 3 of the multi-step system (Figure 1) (30). Step 3
215 involves culturing the complexes on membranes in the presence of Activin-A and rhFSH for
216 up to 4 days until oocytes reach a diameter of 100 microns (30).

217

218 That *in vitro* grown follicles can produce fully grown oocytes after a relatively short culture
219 period confirms that local ovarian factors inhibit follicle development *in vivo*. Oocyte size is
220 an indicator of its ability to resume meiosis therefore sustaining oocyte growth is the major
221 objective of any complete *in vitro* development system (17). There are clearly differences in
222 growth rate depending on whether the whole follicle is cultured (48, 53) or whether,
223 complexes are removed for further growth after an antral cavity has formed (26, 30).

224 Whether the growth rate observed *in vitro* should be characterised as accelerated is not clear.
225 The rate observed represents uninhibited growth without brakes that are required *in vivo* to
226 regulate follicle development within the context of the reproductive cycle. Comparisons of
227 culture systems is needed to determine optimal conditions but at this time there is only one
228 complete system that supports growth from human primordial to maturation (30). The next
229 step is to determine whether the growth pattern *in vitro* can support the development of
230 healthy oocytes or whether it is deleterious to oocyte function, epigenetic changes and health.

231

232 **Meiotic Maturation of Oocytes from IVG human follicles**

233 The final stage in the IVG process before IVF can take place is IVM to support resumption of
234 meiosis to the point of Metaphase II (Figure 1). IVM has been a successful strategy for
235 embryo production in domestic species and has been applied to human oocytes with varying
236 degrees of success (54, 55). The first live birth after IVM of immature oocytes was reported
237 in 1991 (56), although IVM was being utilised during the early development of IVF (57).

238 IVM is performed in a limited number of centres and success rates vary with the main factor

239 being oocyte source and stage (55). The rate of maturation of immature oocytes remains
240 below that of oocytes harvested from stimulated ovaries, indicating that the protocols are sub-
241 optimal or many of the harvested oocytes are intrinsically unable to undergo maturation (54,
242 55).

243

244 IVG oocytes derived from the multistep culture system undergo meiotic maturation following
245 an IVM protocol (30). These oocytes form Metaphase II spindles but emit abnormally large
246 polar bodies (30). Polar body size is influenced by the proximity of the spindle to the oocyte
247 cortex and the inter-chromosomal spacing within Metaphase II spindles (58). If there is a loss
248 of spindle contact with the oocyte cortex this can lead to extrusion of large polar bodies (59).
249 The cause of the large polar bodies in the IVG derived oocytes is not known but it indicates
250 that culture conditions need to be further optimised. The consequences of such abnormalities
251 on chromosome balance in mature oocytes needs to be investigated.

252 Whilst acknowledging that there are no fully optimised culture systems for human oocytes
253 there is now proof of concept that complete *in vitro* growth of human oocytes is possible
254 (30). The end point of any IVG system is to produce developmentally competent and
255 epigenetically normal oocytes therefore future research needs to focus on optimising each of
256 the stages and to gain further understanding of the epigenetic status of IVG oocytes and of
257 any embryos formed (60).

258

259 **Summary**

260 The most significant clinical application of *in vitro* growth (IVG) of human oocytes is in
261 Fertility Preservation given the widespread adoption of ovarian tissue cryopreservation for
262 cancer patients (1). It is clear that making a good egg is not an easy or straightforward
263 process (18). If reliable methodology could produce *in vitro* generated mature human
264 oocytes capable of fertilisation this would be a viable alternative to autologous
265 transplantation. Apart from the clinical implications and potential of the various *in vitro*
266 growth systems; each of them provide access to the process of human oogenesis in an
267 experimentally tractable paradigm. Through these systems we will gain greater understanding
268 of human oocyte development which will ultimately lead to improvements in Fertility
269 Preservation.

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424 List of Abbreviations:

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426 FSH Follicle Stimulating Hormone

427 FOXO3 Forkhead box protein O3

428 IVA In Vitro Activation

429 IVG In Vitro Growth

430 IVF In Vitro Fertilisation

431 IVM In Vitro Maturation

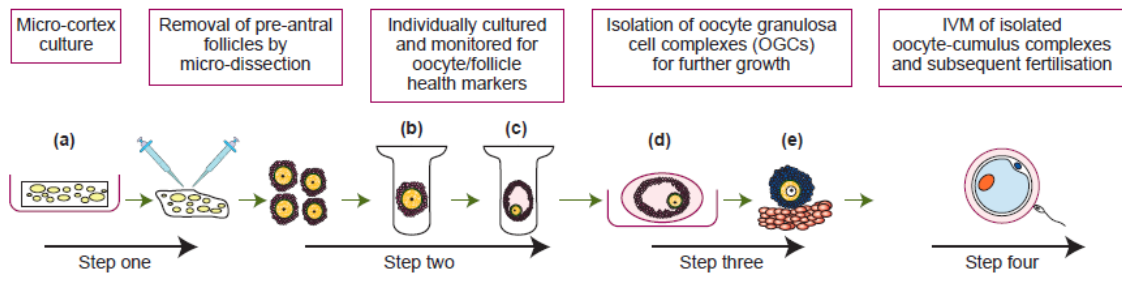
432 PI3K-AKT Phosphatidylinositol-3'-kinase and Protein Kinase B

433 PTEN The phosphatase and tensin homolog deleted on chromosome ten

434 mTORC1 Mammalian target of rapamycin complex 1

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441 **Figure 1:** Diagrammatic representation of a multi-step culture system to support in vitro
442 growth (IVG) of oocytes from human primordial follicles through to maturation as described
443 by McLaughlin et al., 2018. Step 1, in vitro activation within micro-cortex for 7 days (a) then
444 micro-dissection of multi-laminar growing follicles to be placed in step 2 (b) and cultured
445 individually until antral formation occurs (c) Step 3, Isolation of the oocyte granulosa
446 complex (d) from the intact follicle for further growth on membranes for up to 4 days (e).
447 Step 4, Oocyte-Cumulus complexes placed within medium for in vitro maturation (IVM).
448 Oocytes are then analysed for the presence of a Metaphase II spindle and a polar body.
449 Fertilisation of IVG human oocytes has not yet been tested.

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