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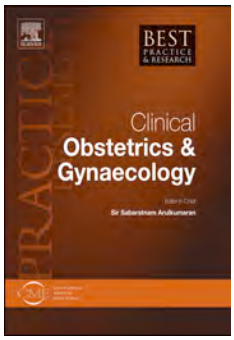

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Oocytes from stem cells

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Abstract

Folliculogenesis describes the process of activating an oocyte-containing primordial follicle from the ovarian reserve, and its development to the mature ovulatory stage. This process is highly complex and is controlled by extra- and intra-ovarian signalling events. Oocyte competence and capacity for fertilisation to support a viable pregnancy is acquired during folliculogenesis. Cancer, and cancer-based therapies can negatively affect this process, compromising fertility. Currently, preservation of fertility in these patients remains limited to surrogacy, oocyte freezing, oocyte donation or in vitro maturation (IVM). Recent reports of stem cells being used to produce fully competent oocytes, and subsequently healthy offspring in mice, has opened up a novel avenue for fertility preservation. However, translating these findings into human health first relies on enhancing our understanding of follicle growth, and mimicking its intricacies in vitro. Indeed, the future of oocytes from stem cells in humans comes with many possibilities, but currently faces several technical and ethical obstacles.

Key Words:

Oocytes, folliculogenesis, fertility, stem cells, cancer, chemotherapy

A. Overview of folliculogenesis and building a competent oocyte

The ovarian follicle containing an oocyte surrounded by somatic cells is the niche for the female germline which must be nourished and protected from sustained damage. The germ cells that eventually form follicles are first identified at 4 weeks gestation among stem cells in the embryonic epiblast. After migration to the gonadal ridge and proliferation, they enter meiosis and form nests surrounded by pre-granulosa cells. The nests breakdown between 25 and 40 weeks gestation to form primordial follicles, each of which contains an oocyte arrested in prophase of the meiotic cell cycle, surrounded by a single layer of squamous or flattened pre-granulosa cells. This process called oogenesis occurs over 150 and 250 days in the fetal ovary (Figure 1) (1, 2). At birth, the human female has approximately 300,000 primordial follicles (range 35,000-2.5 million), defined as the ovarian reserve (1, 3).This reserve is not replenished after birth under normal physiological circumstances. The size of the ovarian reserve declines with age until <1000 primordial follicles are present at the time of menopause (3, 4). The length of the fertile period from puberty to menopause is determined by (a) the rate of death of primordial follicles before being selected to activate, (b) their rate of activation, and (c) the size of the ovarian reserve at birth (1, 5). It follows that genetic or environmental factors such as chemotherapeutic agents or radiation that reduce the size of the ovarian reserve will shorten or even eliminate the fertile period.

Folliculogenesis is the process by which primordial follicles in the ovarian reserve undergo morphological and functional growth and development until such time as a follicle achieves dominance [Graafian follicle], contains a competent oocyte, and is ovulated (Figure 1). It is estimated that <1% of selected primordial follicles will ever reach the dominant stage, the remainder undergoing atresia at each of the stages of folliculogenesis (6, 7). Once a primordial follicle is selected for folliculogenesis, its morphological characteristics change; the pre-granulosa cells transform from squamous to cuboidal granulosa cells, which proliferate and the oocyte diameter increases. Thereafter, folliculogenesis is divided into two morphological stages, preantral and antral, based on the appearance of an antrum filled with follicular fluid. The selection and activation of primordial follicles and the growth of primary and secondary preantral follicles can occur independently of the pituitary gonadotrophins, FSH and LH. Formation of the theca layer outside the basement membrane, antrum formation

and subsequent development of antral follicles to ovulatory Graafian follicles is FSH and LH dependent.

A major discovery has been the essential role of the oocyte in all stages of folliculogenesis. It plays a critical role in the differentiation of the granulosa cells into cumulus cells surrounding the oocyte and mural granulosa cells lining the basement membrane. Bidirectional communication between the oocyte and cumulus cells is subsequently involved in maintaining meiotic arrest until ovulation, and is required for oocyte development and quality, ultimately establishing the capacity for ovulation (8).

The morphological characteristics of folliculogenesis mask two important facts; (a) the time taken for a selected primordial follicle to reach the ovulatory stage is >300 days (Figure 1), the majority of which is spent in the preantral stage (>150 days); transformation of antral follicles to the tertiary stage takes >75 days, and the development of those selected by the rise in FSH at menstruation, and which eventually become dominant and ovulate, takes only 15 days, and (b) there are a complex series of cellular pathways essential for folliculogenesis that are regulated by local growth factors originating from the oocyte and the somatic cells of the follicle, and by the intra- (e.g. estrogens, androgens) and extra - ovarian (e.g. FSH, LH) hormones (9, 10). Taken together, this implies that under normal physiological conditions, transforming a stem cell in the embryonic epiblast into an oocyte competent for fertilization and subsequent embryonic development is a complex and time consuming process requiring input from the oocyte, the follicular somatic cells and the steroid and pituitary hormones. Achieving this *in vitro* is a major challenge.

A. *Cancer treatments compromise folliculogenesis and the ability to build a competent oocyte*

It is well established that cancer treatments, including both radiation and chemotherapy, can damage the ovary and compromise endocrine function and fertility (11). Radiation, when delivered in close proximity to the ovary, depletes the ovarian reserve of primordial follicles and thus poses a substantial risk to fertility (12, 13). In the case of chemotherapy, the extent of ovarian damage and risk of infertility is highly dependent on drug class. Alkylators, such as cyclophosphamide, and platinum agents, such as cisplatin are known to be especially harmful to the ovary and may result in permanent loss of fertility and early menopause (14).

Both of these agents are capable of inducing atresia of growing follicles and can also damage the DNA of meiotically-arrested oocytes in primordial follicles, thereby directly depleting the ovarian reserve (13). In contrast, the antimetabolites, such as 5FU, likely only induce atresia of growing follicles, which contain proliferating granulosa cells (15). The death of these hormone producing follicles can result in transient amenorrhea. However, the ovarian reserve of primordial follicles is not depleted and so ovarian function is expected to return shortly after the cessation of treatment. In addition to causing oocyte and follicle death, cancer treatments have the potential to reduce oocyte quality, either by damaging the oocyte directly, or by compromising the ability of the follicle and ovarian microenvironment to support oocyte development and maturation.

A. Current fertility preservation options

In recent years there has been a dramatic increase in survival rates for many cancer patients and with this has come a heightened focus on quality of life post-treatment. Young women are particularly concerned about losing their future reproductive potential and so a number of options have become routinely available to female cancer patients wishing to preserve their fertility. These include the cryopreservation of embryos and unfertilized oocytes, both of which can now be performed on a cycle-day independent schedule, meaning that there is little time delay before cancer treatment is commenced for women to decide to use these options (16, 17). Of note, the latter option may be especially desirable to women who ethically object to embryo freezing, and those who do not have a male partner or do not wish to use donor sperm. Another option is the cryopreservation of ovarian tissue, followed by transplantation back into the patient after successful treatment (17, 18). Whilst still designated experimental in many countries, this approach offers a number of potential benefits because it does not require ovarian stimulation and is the only option available to pre-pubertal girls. Furthermore, this option may be particularly appealing because it has the potential to preserve both fertility and ovarian endocrine function. However, for patients with leukemia, the possibility of reintroducing cancers cells with the transplanted tissue remains a concern (19, 20). In an effort to overcome this potential problem, a number of laboratories are developing novel methods to support the *in vitro* growth of follicles obtained from the cryopreserved ovarian tissue (21, 22). The objective is to then use optimised IVM protocols followed by IVF or ICSI to generate embryos for transfer. However, the efficacy of the IVM procedure still needs further improvement (23). Finally, in situations where the aforementioned methods of fertility

preservation are not possible, ovarian suppression using gonadotropin-releasing hormone agonists (GnRHa) may be offered. However, there is still considerable controversy regarding the ability of GnRHa to reduce the likelihood of chemotherapy-induced ovarian insufficiency (24).

A. The potential application of stem cells to fertility preservation

There is considerable interest in the application of stem cells for fertility preservation. The hope is that in the future, autologous stem cells could be differentiated in oocytes, which could then be used to restore the fertility of women whose follicular reserves were depleted.

B. Oogonial stem cells

With this goal in mind, it has been proposed that oogonial stem cells (OSCs) could be used to generate new oocytes to repopulate follicle-depleted ovaries or developed to maturity *in vitro*. There have been a number of studies reporting the isolation of putative OSCs from adult ovaries from a variety of species, including mice (25) and humans (26). Furthermore, the *in vitro* development of OSCs has been studied in mice (25) and the generation of offspring derived from OSCs has been described in rats (27). However, the method of OSC isolation employed by many of these studies, and the criteria used for the identification and characterisation of these presumptive OSCs, have been met with considerable criticism (28). Thus, the existence of functional OSCs is still hotly debated and the dogma persists within the reproductive biology community that the ovarian reserve in women is determined by birth. Nevertheless, some researchers continue to actively work towards refining protocols for the isolation and differentiation of OSC (29, 30). If this on-going research bears out, then the source of the OSCs for fertility preservation could theoretically be ovarian biopsies specifically obtained for this purpose, or ovarian tissue previously cryopreserved with the intention of transplantation at a later time point.

B. Embryonic and induced pluripotent stem cells

Alternatively, embryonic stem (ES) cells (31) or induced pluripotent stem (iPS) cells could be used to generate granulosa cells and oocytes in the laboratory (32). These oocytes and granulosa cells could then be used to build new follicles that could subsequently be

transplanted back into the ovary, or combined with IVM and IVF/ICSI protocols to create embryos. This possibility is exciting, but the field faces huge obstacles that must be overcome in order for (pluripotent) stem cells to become a viable fertility preservation option. Not only must protocols for the differentiation of oocytes and granulosa cells from stem cells be developed, but the *in vitro* development of follicles through to maturity must be supported. This necessitates the development of validated multi-phase protocols, where each step needs to be fully optimised to recapitulate follicle development as it occurs *in vivo*. As described above, the process of follicle development and oocyte maturation in women is extremely lengthy and complex and relies on physical and molecular interactions between the oocyte and granulosa cells, and requires a suite of nutrients, growths factors and hormonal support, which varies dynamically across follicular development. As such, the extensive manipulation required for the development of stem cells into fully functional oocytes *in vitro* raises concerns over the viability, genetic integrity and epigenetic programming of the gametes produced.

Despite these challenges, there has been remarkable progress towards generating gametes from ES and iPS cells in mice. In 2011, Hayashi and Saitou reported the production of live pups from sperm derived from pluripotent stem cells (31), and the following year, a similar strategy was used to produce offspring from oocytes derived from ES or iPS cells (32, 33). In these studies, ES or iPSCs were used to produce primordial germ cell like cells (PGCLCs) *in vitro*, and these were subsequently transplanted into mice to produce oocytes (or sperm). In a further advance, a landmark study by Hikabe et al in 2016 extended these protocols in order to derive mature, fertilisable, developmentally competent oocytes from stem cells entirely *in vitro (34).* Hikabe reported a three step process, referred to as *in vitro* differentiation, *in vitro* growth and *in vitro* maturation, each of which was supported by a specific and defined cocktail of growth factors, hormones and conditions (Figure 2). In the first phase of the process, PGCLCs were generated from ESCs or iPSCs using media containing a variety of growth factors (bFGF, ActA, BMP4, KL, LIF and EGF) and then mixed with embryonic ovarian cells to form a re-aggregated ovary. The re-aggregated ovaries facilitated the differentiation of PGCLCs into oocytes, and supported their progression through the initial stages of meiotic prophase I and the assembly of diplotene arrested oocytes into follicles, the process to this point taking 3 weeks. One notable addition to the media used for the *in vitro* differentiation phase was the estrogen inhibitor ICI182780, which prevented the formation of multi-oocyte follicles. Follicles were then grown to the secondary stage *in vitro*. The *in vitro*

growth phase was 11 days long and involved the isolation of secondary follicles and the further stimulation of their growth by supplementing the media with GDF-9, BMP15 and FSH, leading to the formation of cumulus oocyte complexes containing fully grown germinal vesical stage oocytes. The complexes were then transferred to standard IVM conditions for 16 hours, which facilitated their development to MII. Overall, the complete process from stem cell to fertilisable mouse oocyte took almost 33 days.

Significantly, the pups produced following IVF using MII oocytes grown entirely *in vitro* appeared to be phenotypically normal, were fertile and remained healthy to at least 11 months of age. Furthermore, an analysis of select imprinted loci suggested that the epigenetic status of the offspring was similar to that of normal wild-type mice (34). However, it should be noted that for *in vitro* generated oocytes, embryonic development was often arrested at the cleavage stage, and early and late gestation resorptions were common. These latter observations clearly illustrate that while a relatively small number of healthy offspring could be achieved, unhealthy oocytes were often generated and the current *in vitro* development methodology is far from optimal. Indeed, the success rate of full-term development using 2-cell embryos from *in vitro*-generated oocytes was 3.5%, which was significantly lower than the 61.7% success rate that was achieved when *in vivo*-generated oocytes were used. It is expected that ongoing work will now focus on refining the culture conditions in order to improve oocyte quality.

A. Practical issues in research and ethics

Creating oocytes from stem cells may have seemed near impossible a decade ago, but advances in the last five years have reopened the conversation surrounding the possibilities, and challenges that lie ahead. As it stands, the recent strides in creating oocytes from stem cells in mice give us important insight into new reproductive technologies, with a better understanding of the processes of oocyte maturation and fertilisation. However, there are still major issues with proving the functionality, feasibility, and understanding the long-term effects of this currently highly experimental biology. There are no reports of being able to generate human PGCLCs and differentiating these into viable oocytes for humans.

Current available methods for fertility preservation have only been achieved in humans for the third step of the process, the IVM phase, and require further optimisation

(Figure 2) (23, 35). The technology required for robust delivery of step 2, enabling human follicle growth and development in vitro, is still very much in its infancy (36). In order to recapitulate human oogenesis and follicle growth, to produce viable oocytes in vitro requires a plethora of different cell types and tightly regulated somatic cell-oocyte interactions during follicle development. Furthermore, compared to mice, human oocyte and follicular growth is a lengthy process and requires developing a tightly regulated culture system with adequate nutrients, cytokines, growth factors and developmental-stage dependent hormones that can be sustained potentially over several months. Additionally, epigenetic programming in oocytes occurs in a concerted manner throughout the process of folliculogenesis. These epigenetic changes affect the long-term viability of the pregnancy and the health of the offspring. Currently, the extent of these epigenetic changes in human oocytes is not fully understood, making it difficult to recapitulate in vitro (37-39). These factors pose a significant number of technical hurdles before this process could be applied to preservation of human fertility.

Further to recapitulating follicle growth in vitro, culture systems also need to support the ability of the oocyte to gain competence in order to resume its meiotic potential through fertilisation and preimplantation development. Meiotic competence is essential for IVM, IVF, and yielding healthy viable offspring (40, 41). Currently, IVF success is dependent on using oocytes from large follicles (12-20 mm in diameter) that have fully developed to the metaphase II (MII) phase in vivo. Producing meiotically competent oocytes, let alone viable embryos from follicles prior to this stage, in humans, has not been described. Whilst the ability to grow follicles from PGCLCs and PGCs to fully competent oocytes in rodents is a notable feat, the efficiency of gaining competent oocytes was quite low (28.9% for MII oocytes, but only 3.5% for number of pups) (34, 42). Being able to replicate this in humans requires a more profound understanding of the maturation process, and the role played by the supporting cells of the follicle on oocyte competence.

Another issue with this process in humans is the source of the stem cells and process of reaggregation. The technique employed by Hikabe et al. to create competent, fully grown follicles relied on using reconstituted ovaries, such that the somatic cells were provided by embryonic day 12.5 (E12.5) ovaries (34). It is unlikely that human embryonic gonadal cells would be available for the re-aggregation step of this process, therefore, another source is required. One potential route is the production of ovarian somatic cells from pluripotent stem cells, but such a protocol has not yet been developed. Additionally, whilst controversial, it

remains a possibility that oogonial stem cells could be isolated from cryopreserved ovarian tissue (Figure 2).

The use of stem cells to produce oocytes is not limited to the technical barriers, but also the ethical concerns that arise from it. The ethical parameters surrounding the field of reproductive medicine have always been under the scrutiny of both the scientific community and the general public. Some of the major concerns surrounding the use of stem cells to form oocytes include the source of the stem cells and the wellbeing of the offspring (43). The most rigorous validation of oocytes derived from stem cells is the ability to produce healthy, fertile offspring. The ability to translate the mouse studies into human studies has several obstacles in the process. Despite the lack of gross abnormalities seen in the mouse offspring, the leap from mouse to human is large and requires several levels of understanding before it could be translated into human use. Our ability to validate the long-term health and fertility of human offspring from stem cell derived oocytes depends on our current understanding of in vivo human oocytes. However, given our access to research on human oocytes is limited, our ability to translate knowledge to stem cell derivatives remains an insurmountable hurdle.

For women requiring methods of fertility preservation in the instance of cancers, and associated treatments, the future of oocytes from stem cells holds both new possibilities but also practical challenges. The ability to produce oocytes from stem cells would open the doors to more choice, and less time-bound decisions. Current fertility preservation techniques are time-consuming and invasive, which can be dangerous for the woman undergoing treatment. Furthermore, decisions regarding fertility preservation are under time constraints. The prospect of generating oocytes from stem cells increases the independence and freedom of choice when making reproductive choices. However, this hypothetical situation can also raise issues concerning criteria for eligibility, and who funds this process.

The future use of oocytes derived from stem cells still has a long way to go before, if ever, it can be implemented to human health. The field of ART evolves at a tremendous rate; and insights into deriving oocytes from stem cells will contribute substantially to the betterment of these technologies. For oocytes derived from pluripotent stem cells, the priority lies in developing culture and technologies to support the development in vitro of earlier stage follicles leading to competent oocytes. With this achievement, we may be one step closer to fostering the production of oocytes from stem cells and changing the future of

human reproduction.

Summary

The ability to convert mouse ESC or iPSC into competent oocytes and subsequently to embryos and offspring has been achieved, but success rates remain low. Further research is needed to improve these success rates and to confirm that the offspring are genetically normal. There is no evidence that human ESC or iPSC can be converted into competent oocytes. Should this become possible and practicable, then the ensuing ethical and safety issues will need careful investigation and discussion. In addition, further research is needed to improve the safety, efficacy and success rates of IVM of human oocytes, one of the key steps in transforming stem cells into competent oocytes. Until these barriers are overcome, it will remain necessary to use the current methods of fertility preservation.

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Conflict of interest

The authors have no conflicts of interest.

Practice Points

- Using human stem cells as a source of oocytes for ART is not an option at this stage
- Preservation of fertility remains limited to surrogacy, embryo freezing, oocyte freezing, and oocyte donation or IVM.

Research Agenda

- Improving the success rates of IVM of human oocytes
- Improving the success rates of building mouse oocytes from stem cells
- Investigating the factors and culture conditions necessary to convert human stem cells into oocytes
- Generating scientific and community discussion about the application of stem cells in ART

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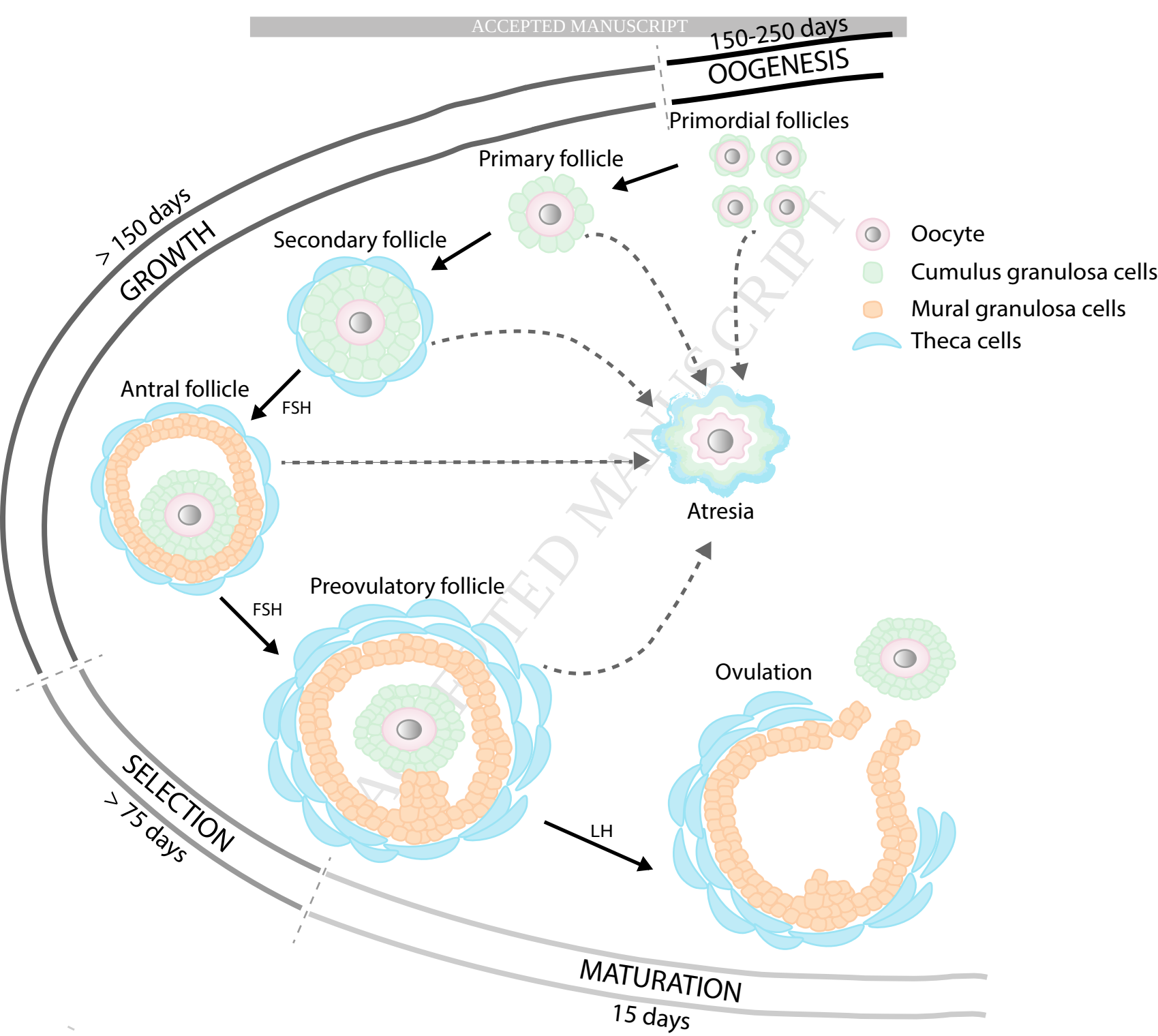
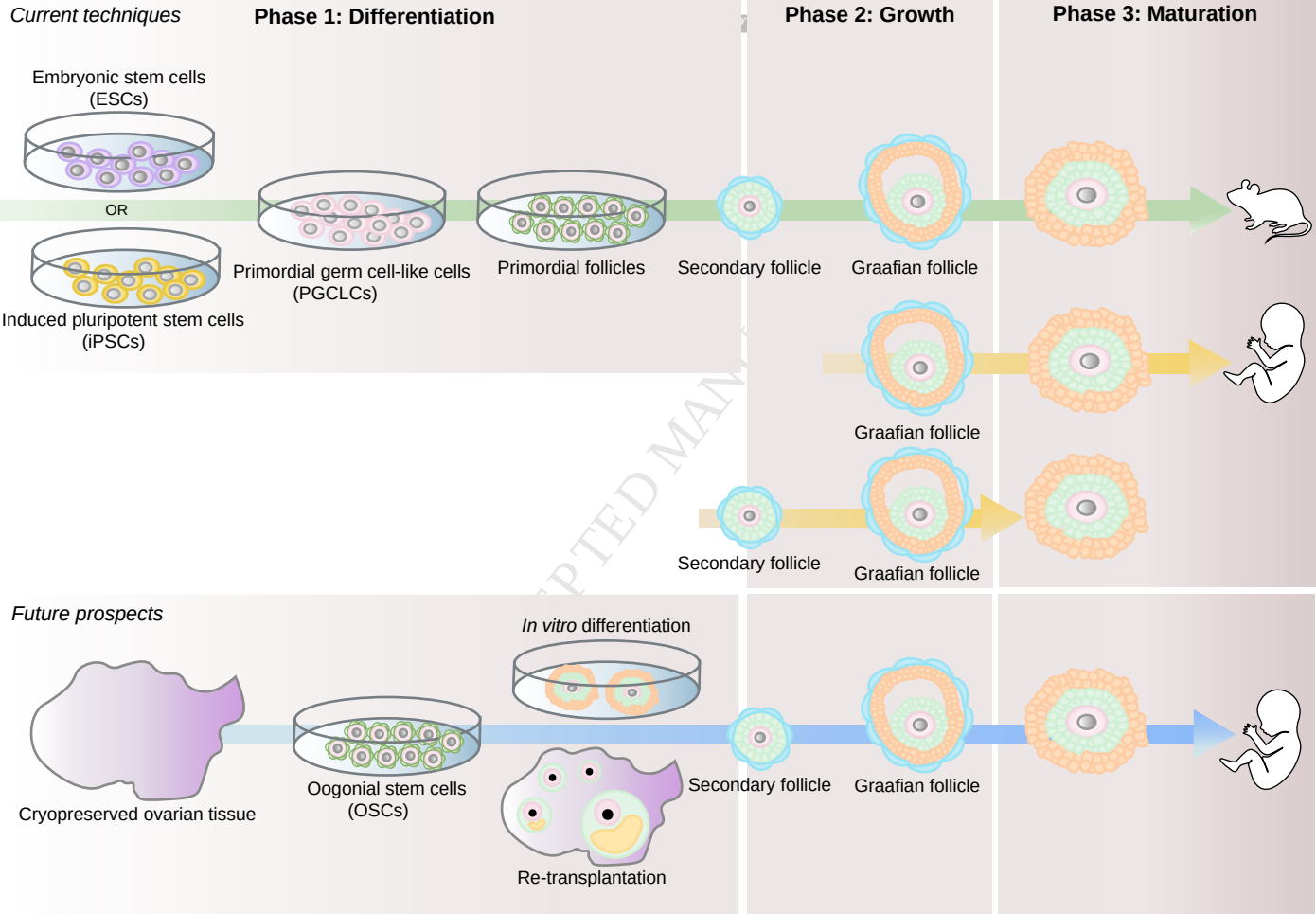
Figure 1: Stages of follicle growth during folliculogenesis.

Formation of the primordial follicle pool starts during fetal development in a process known as oogenesis, and takes between 150-250 days. Upon initiation of follicle growth, the squamous granulosa cells surrounding the oocyte in primordial follicles transition to cuboidal granulosa cells, forming primary follicles. Granulosa cells proliferate and form several layers around the oocyte as the follicle grows through to the secondary follicle stage. Theca cells aggregate to form an outer layer with endocrine function. Gonadotropins, FSH and LH stimulate the formation of antral follicles, distinguished by a fluid filled antral cavity. There are two types of granulosa cells, cumulus granulosa cells (green) found closest to the oocyte and mural granulosa cells (peach) which surround the antral cavity. These granulosa cells respond differently to gonadotropins. This process of follicle growth takes > 150 days. A preovulatory follicle is selected over a > 75 day period, after which it matures for 15 days. The remaining granulosa and theca cells luteinise to form the corpus luteum. Throughout the process of folliculogenesis, growing follicles are lost at all stages through atresia, shown with dotted lines. There are a plethora of other intra- and extra-ovarian factors that are involved in process of follicle growth and maturation.

Figure 2: Current technologies and future prospects in deriving oocytes from stem cells.

Currently, embryonic stem cells and induced pluripotent stem cells have been differentiated into oocytes, and follicles have grown to maturity culminating in the successful birth of offspring in mice. In humans, follicle maturation through IVM to produce healthy offspring has been achieved. Follicle growth from secondary to Graafian follicle has been achieved, but unsuccessful offspring production. Future prospects in the field requires the optimisation of the differentiation, growth and maturation processes before iPSCs, ESCs or follicles prior to Graafian follicle stage can be used to produce healthy human offspring. Another future prospect lies in the contested field of cryopreserved ovarian tissue. This includes validating the presence of OSCs, which can be grown *in vitro* or retransplanted following treatment to produce mature oocytes and healthy offspring. These technologies are limited by technical and ethical hurdles. Modified from Smitz and Gilchrist (43).




Current techniques
Phase 1: Differentiation
Phase 2: Growth
Phase 3: Maturation
Embryonic stem cells (ESCs)
OR
Induced pluripotent stem cells (iPSCs)
Primordial germ cell-like cells (PGCLCs)
Primordial follicles
Secondary follicle
Graafian follicle
Graafian follicle
Secondary follicle
Graafian follicle
Future prospects
In vitro differentiation
Cryopreserved ovarian tissue
Oogonial stem cells (OSCs)
Re-transplantation
Secondary follicle
Graafian follicle

Issue on CMRC (GE Quinn)

Oocytes from stem cells

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Highlights

- Oogenesis and folliculogenesis are lengthy and complex processes.
- Fully functional mature oocytes have been generated from stem cells in mice.
- It is currently not possible to generate oocytes from stem cells in humans.