Review Article

In Vitro Growth of Human Ovarian Follicles for Fertility Preservation

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Abstract

Young female cancer survival rates significantly increased due to the great progress of cancer therapy. In fact, cryostorage and transplantation of ovarian tissue have already resulted in the birth of healthy babies. Follicle *in vitro* growth (IVG) has the great potential of restoring fertility by achieving functional oocytes from the most immature stages to maturation. This is suitable for a wide range of patients, from pubertal to perimenopause women. Notable achievements have been achieved in human follicle IVG in the past decade. Mature oocytes have been successfully collected from long-term sequential follicle IVG. However, it is still a major challenge to establish a stable and efficient follicle IVG system able to generate mature and competent oocytes. Hereby, we review the approaches being taken so far using ovarian tissue to support follicle growth at different stages *in vitro*.

Key words: Follicle Isolation; Human Follicle; *In vitro* Growth; Primordial Follicle Activation

Introduction

With the remarkable progress achieved in the diagnosis and treatment of cancer, many cancer patients have obtained a surpassing survival rate and a better life quality. However, radiotherapy and chemotherapy, which are the principal strategies for cancer treatment, have high toxicity to gonads and lead to diminished ovarian reserve and infertility in women.[1] Therefore, fertility preservation methods become critical to preserve the ability of having children in cancer survivors.

There are different fertility preservation strategies to satisfy the different types of cancer patients, including embryo and oocyte cryopreservation, ovarian tissue cryopreservation and transfer, and follicle *in vitro* culture, some of which are still at the research level. Embryo and oocyte cryopreservation are the assisted reproductive techniques used routinely in clinical practice, but often require ovarian stimulation which may increase hormone levels, placing hormone-sensitive cancer patients in higher risk situations and increasing the waiting time to undergo cancer treatment. In addition, these techniques are not suitable for prepubertal patients.[2] Ovarian tissue cryopreservation and transfer has been successfully reported to result in a live birth 2004,[3] and more than 130 babies were subsequently born by this technique all over the world since then.[4] Nevertheless, transferring of the cryopreserved ovarian tissues poses the risk of reintroducing the malignant cells back to the body, leading to the recurrence of cancer.[4]

Follicle *in vitro* growth (IVG) aims to isolate all phases of follicular development before ovulatory stage from cryopreserved ovarian tissues and production of the competent oocytes to follow *in vitro* maturation (IVM) or *in vitro* fertilization (IVF). This is an ideal alternative to avoid reintroduction of malignant cells into the patients and to potentially increase the number of oocytes, as there are thousands of various classes of follicles in ovarian tissues. Moreover, it does not require hormone stimulation, which is feasible for both reproductive-age women and prepubertal children. However, no live births have been yet

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achieved with IVG techniques in humans. There are still several challenges to overcome before follicle growth can be applied as an approach for accessing competent oocytes. In recent years, much progress has been made in establishing IVG approaches in humans. This review aims to summarize follicle activation and IVG methods and discusses the prospects as well as the limitations of applying them in clinical practice.

Human Folliculogenesis

Formation and development of primordial follicles begin in the fetal phase. This pool of primordial follicles represents a population of germ cells from which recruitment for growth occurs throughout the reproductive life of females. Follicular development involves a series of complex and precisely regulated biological events consisting of three main distinct phases: first, formation and beginning of primordial follicles activation; second, growth of primary follicles with cuboidal granulosa cells, in which the oocyte experiences a rapid growth in size; third, rupture of the ovulatory follicle, releasing a cumulus–oocyte complex at ovulation in response to the luteinizing hormone surge.[5]

Oocytes arrest at the meiotic stage of prophase I within the early follicles and must acquire the ability to resume meiosis and acquire the developmental competence necessary to support fertilization and embryonic growth. Cell-to-cell bidirectional communication between the oocyte and granulosa cells is crucial for oocyte competence and correct embryo fertilization and development.[6]

Primordial Follicle Activation and Growth *In vitro*

Primordial follicle activation

The majority of follicles in the human ovary are primordial follicles during the whole individual lifespan.[7] This large proportion of primordial follicles makes them the most abundant resource to obtain the fertilizable oocyte. Most of the primordial follicles are at dormant state before they are recruited to the growing follicle pool. A primordial follicle encloses a meiotically arrested oocyte at the dictyate stage of prophase I surrounded by flattened granulosa cells. Only a small number of these follicles are recruited for further growth into Graff follicles in each cycle. The main feature of primordial follicle activation is the change in morphology of granulosa cells, which change from a flat shape to cuboidal, initiating oocyte growth.[8] It is very critical to activate primordial follicles effectively and synchronically to establish a reliable IVG system. To achieve this goal, extensive efforts have been made on revealing the mechanism underlying follicular activation during the past decades. For instance, two of the major pathways involved in primordial follicle activation, PI3K-PTEN-AKT-FOXO3-mTOR pathway and the Hippo signaling pathway have been investigated in the past decades.

PI3K-PTEN-AKT-FOXO3-mTOR pathway

PI3K-PTEN-AKT-FOXO3-mTOR pathway is the main pathway involved in primordial activation. It is based on PI3K-AKT-mTOR pathway which has many molecules, including kinases, phosphatases, and transcription factors that regulate cell proliferation, apoptosis, growth and metabolic activity.[9] In the previous studies, phosphatase and tensin homolog (PTEN) and forkhead box O-3 (FOXO3) have been revealed to regulate primordial activation with the coordination of PI3K-AKT-mTOR pathway. The mechanism of this pathway involved in mammalian primordial follicle activation has been reviewed comprehensively in the past[10,11] and is summarized briefly in Figure 1a.

The Hippo signaling pathway

The Hippo pathway has been identified to have a pivotal role in growth regulation and suppress the overgrowth of organs.[12] It consists of a serine/threonine protein kinase cascade that includes several negative growth regulators which dispute the key transcriptional coactivators, Yes-associated protein (YAP), and transcriptional coactivators PDZ-binding motif (TAZ) to achieve the nuclear access.[13] When the Hippo pathway is disrupted, YAP phosphorylation is inhibited and results in the accumulation of dephosphorylated form of YAP in the nucleus, which enhances the CCN growth factors and expression of other transcription factors.[14] Details of Hippo pathway with follicle activation are summarized in Figure 1b.

Primordial follicle growth *in vitro*

Multiple studies have investigated human primordial follicle activation and growth by manipulating the involved pathways. One of the star molecules is PTEN inhibitor. Liu *et al*. incubated human ovarian cortex strips with PTEN inhibitor *in vitro* for 24 h and found that primordial follicles were activated and led to mature oocytes after xenotransplantation into immunodeficient mice.[15] Likewise, other studies showed that the PTEN inhibitor increased activation of primordial follicles, while compromising development of growing follicles in the ovary.[16] Sun *et al*. proposed an alternative approach with mammalian target of rapamycin (mTOR) and phosphatidylinositol-3-kinase (PI3K) stimulators which increased the human growing follicles in ovarian cortex with histological analysis.[17] Notably, Kawamura *et al*. treated primary ovarian insufficiency patients by fragmenting ovaries followed by Akt stimulator treatment and autografting. Follicular growth was successfully promoted, and the first live births were announced after IVF-ET with the mature egg collected after the treatment.[14] Following this study, several groups had succeeded in achieving live births either with cryopreserved[18] or fresh tissues.[19] In the most recent study, PI3K/Akt activators, mTORC1 inhibitor, and Hippo signaling pathway disruption were compared in human ovarian cortex, confirming that PI3K/Akt pathway and Hippo pathway accelerate primordial follicles recruitment while mTORC1 inhibitor partially prevents massive spontaneous activation.[20]

Developmental Follicle Growth *In vitro*

Isolation of follicles

Follicle isolation is one of the crucial steps toward follicle IVG. The most challenging part is how to effectively isolate follicles

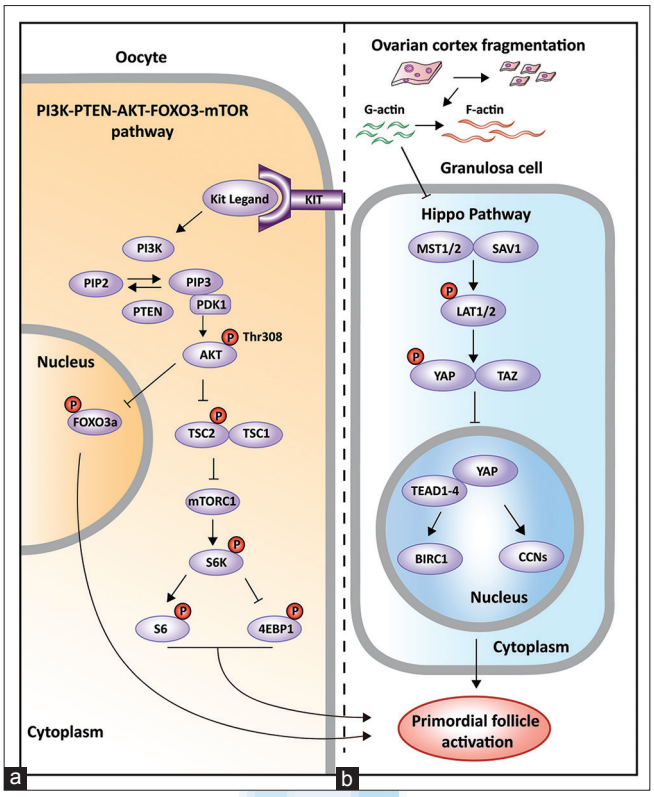

Figure 1: Pathways in primordial follicle activation (adapted from[5,20]). (a) PI3K-PTEN-AKT-FOXO3-mTOR pathway in primordial follicle activation. The initiating factors of this process are largely unknown. Upgraded Kit legand from GCs of primordial follicle binds to its KIT/tyrosine kinase receptor, KIT on the surface of oocyte and activate the PI3K complex.[65] PI3K complex convert PIP2 to PIP3 which is the key element of PI3K pathway and activate the nuclear translocation and phosphorylate AKT (p-AKT).[66,67] p-AKT enhances primordial follicle activation by inhibition of the phosphorylation of FOXO3a[68] as the deletion of FOXO3a initiated the activation of primordial follicle in mouse model.[69,70] p-AKT activates mTOR complex 1 (mTORC1) by inhibiting tuberous sclerosis complex 1/2 (TSC1/2, or tuberin). mTORC1 then phosphorylates S6K which activates S6 to phosphorylates S6 as a sequence. Also, mTORC1 inhibits the phosphorylation of 4EBP1, which functions together with p-S6 are highly involved in cell growth and proliferation.[71,72] PTEN is the regulator to convert PIP3 to PIP2 and which keep the primordial follicle as dormant.[73,74] PDK1 is a master kinase, which is another regulator for the activation of AKT/PKB and many other AGC kinases.[75] PI3K-mediated signaling partially converges at PDK1. PDK1 activates AKT via phosphorylating AKT at T308.[68] (b) The Hippo signaling pathway in primordial follicle activation. Ovarian tissue fragmentation transforms G-Actin to F-Actin by inducing the actin polymerization which disrupts the Hippo signaling pathway by decreasing the expression of MST1/2 and SAV1 complex.[5] It then phosphorylates LATS1/2 which inhibit the phosphorylation YAP and TAZ, two major downstream effectors of the Hippo pathway. Dephosphorylated YAP/TAZ translocate into the nucleus and interact with the transcription factors such as TEAD1-4 then stimulate the expression of downstream CCN growth factors and BIRC apoptosis inhibitors, leading to initiates primordial follicle activation and development.[20] PI3K: Phosphatidylinositol-3-kinase; AKT: Protein kinase B; mTOR: Mammalian target of rapamycin; GC: Granuloca cells; KIT: KIT Proto-Oncogene Receptor Tyrosine Kinase; mTORC1: Target of Rapamycin complex 1; 4EBP1: 4E-Binding Protein 1; PIP3: Phosphatidylinositol 3,4,5 trisphosphate; PIP2: Phosphatidylinositol (4,5)-bisphosphate; PDK1: 3-Phosphoinositide-dependent protein kinase-1; PKB: Protein kinase B; F-actin: Filamentous actin; G-actin: Globular actin; MST1/2: Mammalian sterile 20 -like kinase-1/2; homologs of Drosophila Hippo; SAV1: Homolog of Drosophila Salvador; LATS1/2: Mammalian homologs of Drosophila Warts; YAP: Yes-associated protein; TAZ: Transcriptional coactivator with PDZbinding motif; TEAD transcriptional factors: Transcription factors containing the TEA/ATTS DNA binding domain; CCN growth factors – Acronym derived from three of its members: CCN1 (cysteine-rich angiogenic protein 61), CCN2 (connective tissue growth factor), and CCN3 (nephroblastoma overexpressed); BIRC apoptosis inhibitors: Baculoviral inhibitors of apoptosis repeat-containing proteins.

with a great balance of both quality and quantity. The ideal isolation approach should allow for harvesting the maximum number of follicles while causing the minimum damage. There are two main methods currently in use, mechanical isolation and enzyme digestion. Mechanical isolation is more often used to isolate larger follicles[21] as they are visible under the microscopy. The main advantage of mechanical isolation is avoidance of detrimental effects caused by enzyme digestion. However, this strategy comes with some disadvantages. First, it has relatively low efficiency. Second, it is only capable to isolate large-sized growing follicles. Finally, it is hard to remove potential malignant cells during the follicle isolation when artificial ovary is established and transferred.[22]

Enzyme digestion is widely used for follicle isolation yielding high numbers of isolated preantral follicles.[23,24] In the earlier studies, the enzymes used were often collagenases, which were originally employed for digestion of tissues. However, collagenases may contain high levels of endotoxins that could significantly affect culture and grafting outcomes, as shown for pancreatic

islets.[25] Oocyte exclusion from IVG follicles were observed often, which may be caused by endotoxicity and overexposure to the enzymes. Furthermore, collagenases also show a considerable batch effect when assessing effectiveness.[26] Because of this, second-generation enzymes, including Liberase™[23] and Liberase DH,[24] were tested and proved to have a better efficiency and result in less damage to follicles.[22,27]

Culture systems

Once activation of primordial follicles is accomplished, the next major challenge for follicular IVG is to ensure growth of the primary follicle, development of granulosa and theca cells, and subsequently the development of an atrium.[28] Several culture strategies have been proposed in different species and with variable success, each of these with their own advantages and drawbacks. These techniques can be readily divided into two major categories: two-dimensional (2D) and 3D culture systems.

Two-dimensional culture systems

The first attempts for follicular culture in mammals were conducted using 2D culture systems, given its apparent less complex nature and its similarity with already well-established cell culture protocols.[29,30] Moreover, the easiness to monitor cell development and morphology also makes 2D systems more appealing than the 3D counterparts, which offer an environment where visualization of the sample in question is very much limited.[31] On the other hand, 2D systems come with several disadvantages, which, unsurprisingly, have rendered development of most mammalian follicles *in vitro* unsuccessful and have only provided promising results in rodents.[28,29]

Perhaps the most critical of these disadvantages is failure to maintain the follicular spherical structure. 2D systems behave like canonical monolayer cell cultures.[32] In fact, it has been reported that granulosa cells undergo cell growth, leading to horizontal spread across the culture flask rather than organizing in a sphere around the germ cell in a multilayer manner.[30] As a result, pivotal cellular interactions between the oocyte and granulosa cells are disrupted and further *in vitro* development is compromised.[33,34]

Three-dimensional culture systems

3D culture systems have become the preferred and most utilized platforms to sustain *in vitro* follicle culture due to the challenges mentioned above.[28] As previously discussed, a system in which the follicular architecture is preserved allows for undisturbed cellular interactions and for more optimal follicle development. For instance, a recent study investigated the potential of a 3D-printed microporous scaffold to sustain follicle development and eventually restore ovarian function in rodents.[35] Briefly, a porous structure made out of gelatin was designed in which the size of the micropores was carefully controlled. In addition, when isolated follicles of several stages were added to the scaffold for *in vitro* culture, it was noted that follicle survival and development was significantly enhanced when the spherical structure of the follicle was maintained. Although this technology was mainly thought as a bioprosthetic alternative to restore folliculogenesis *in vivo*, it indeed offers a novel approach to be exploited in the *in vitro* follicle culture field.

Nonetheless, 3D strategies still present some disadvantages that cannot be ignored. For instance, some commonly use matrices for 3D culture are too rigid and as a result become resistant to follicle growth, as seen in rodents.[36,37] In humans, a grown antral follicle *in vivo* could reach 10 mm,[38] which means that the ideal chosen 3D matrix should have either enough elasticity or be prone to digestion by enzymatic activity from the growing follicle.[39] In addition, once the follicle has reached an acceptable stage of development, the 3D structure should be easily dismantled to retrieve the mature follicle, and many of the currently used materials for 3D culture cannot effortlessly removed.[37]

Furthermore, another important issue when establishing 3D systems for *in vitro* follicle culture is selecting the most appropriate material. Collagen was one of the first materials used to build matrices for 3D culture due to its abundant presence in the *in vivo* extracellular matrix. Although some attempts have been successful in humans,[40] collagen comes with the drawback that visibility is highly hampered for monitoring follicular development, not to mention the accelerated shrinkage of the gel during prolonged culture.[31] Moreover, another popular choice for 3D matrix is alginate, a material of natural origin that in the form of a hydrogel has provided promising results in culture of human[41] and other primate follicles.[42,43] Matrigel, on the other hand, is a protein mixture that when coupled with fibrin renders the system highly elastic and therefore allows the follicle to expand and increase in size.[44,45] Finally, other materials available for this kind of culture systems are polyethylene glycol hydrogels and mixtures of the aforementioned materials in different ratios.[46-48]

Although an enormous effort has been made to investigate and discover the ideal scaffold for follicle culture, it is important to mention that culture of follicles in V-bottom-shaped wells has also provided very promising results. In fact, a very recent study reported for the first time the development of metaphase II oocytes with polar bodies and normal spindles from human primordial/unilaminar follicles *in vitro* without the use of any 3-D matrix, indicating that other alternatives can be followed to circumvent the challenge of maintaining the *in vivo* architecture of the follicle *in vitro*.[21,28] Nevertheless, it is worth mentioning that this study should provide additional evidence to make the claim that all starting follicles were at the primordial stage. This because the pool of immature follicles cultured in the study included different stages of development and not only primordial.

Improving *In vitro* Growth of Human Preantral Follicles

The selection of the optimal culture system is paramount for successful culture of human follicles *in vitro*. Nonetheless, another very important aspect of *in vitro* culture beyond selecting the right scaffold is the culture components, either

cellular or chemical, that are added to the culture to mimic the *in vivo* niche of these follicles.

For instance, addition of follicle-stimulating hormone (FSH) in the medium for follicular growth has been widely studied and optimized in both animal models and humans. Due to its pivotal role in follicular recruitment and development *in vivo*,[49] its presence in *in vitro* culture systems is perhaps one of the most obvious strategies to improve preantral follicular growth. Indeed, the first report of meiotically mature oocytes after *in vitro* human follicle culture was obtained with FSH as part of the culture cocktail.[50] Likewise, another elegant study on rhesus monkey follicles revealed that although FSH does improve growth and survival of *in vitro* growth follicles, the amounts required in the culture system vary depending on the developmental stage of the follicle. High levels of FSH showed to improve follicular growth at early stages, whereas lower doses resulted optimal to sustained follicular survival *in vitro* in later stages of development only.[43]

Moreover, the protein fetuin has also been widely used in follicle culture systems with the scope of generating serum-free media, thus avoiding the presence of undesired contaminates.[28] For these systems, fetuin has proved to be indispensable for follicular growth and survival, not to mention for the generation of meiotically competent oocytes.[43] Furthermore, Telfer *et al*. in 2008 reported that addition of Activin results in higher percentages of antral follicles, average increase of size, and achievement of morphologically normal oocytes compared to control medium without this supplement.[51]

In addition, some attempts have been made to enhance 3D culture systems by combining the use of a scaffold with coculture with other cell types, following the hypothesis that a paracrine effect could improve follicle survival and mimic the niche in the gonads. For instance, a significant benefit in follicle survival and perfused vasculature was observed after adding mesenchymal stem cells (MSCs) to cryopreserved human ovarian cortex transplanted in mice.[52] Due to the improved follicle survival, the same group also reported that coculture of human preantral follicles with MSCs increased not only *in vitro* survival and growth but also expression of genes vital for proper folliculogenesis, such as *GDF9, BMP15,* and Activin A.[53]

Finally, other compounds such as basic fibroblast growth factor (bFGF) and endothelial growth factor have been tested in cattle, goats, and felines with significant improvements in *in vitro* follicular development.[54-56] With respect to human follicle culture, bFGF was added to the culture medium of a study assessing follicular growth in alginate beads of follicles isolated from frozen and thawed ovarian tissue compared to fresh. Follicular survival rates were significantly improved with the presence of bFGF.[57,58]

Follicle Growth Quality Assessment

There are no standard evaluation criteria for *in vitro* follicle development. The ultimate success of the *in vitro* culture of follicles is determined by whether the grown oocytes derived can reach the MII stage after IVM and eventually achieve fertilization, embryo production, and live births. However, it is also necessary to assess follicular growth efficiency and quality during the cross sections of IVG procedure. Commonly used measurements include morphology, viability assays, follicle and oocyte dimensions, survival rate, growth rates, hormone secretion, cell signaling, and molecular markers. These measurements help predict the outcome of *in vitro* follicle development.

The most basic way to assess follicles is their morphology. First, follicle growth could be evaluated by increase in diameter according to the duration of culture. Second, growth into advanced stages could be assessed according to the criteria of follicle classification: primordial follicle (an oocyte surrounded by a single layer of flattened granuloca cells [GCs]), primary follicle (an oocyte surrounded by a single layer of cuboidal GCs), secondary follicle (a growing oocyte surrounded by two to five layers of GCs), and preantral follicle (six or more layers of GCs or diameter >120 mm).[59,60] Third, the isolated follicles could also be classified into "healthy" follicles (with a distinct oocyte and intact basement membrane), "damaged" follicles (with a broken basement membrane), and "atretic" follicles (with a dark and intact basement membrane).[61] Follicle atresia or degeneration occurs when the oocyte within the "healthy" follicle becomes dark or disappears. Otherwise, the follicles are considered alive. Moreover, histological evaluation is also another approach to assess *in vitro*-cultured follicles within ovarian tissues. Common parameters of histological measurements include measuring the diameter of the follicle, assessing the morphology of the oocyte, measuring the thickness of the zona pellucida, and checking the presence of theca cells and the layers of granulosa cells.

Hormone production of the isolated follicle during *in vitro* culture can serve as another indicator of follicle growth quality. Studies showed that estradiol and progesterone levels were both positively correlated to follicle development.[42] Follicles become steroidogenically active and entered the antral stage increasing secretion of the anti-Mullerian hormone.[62]

Follicle viability is another important parameter to assess follicle development. The follicle viability test is often achieved with a live dead stain of calceinAM and Methidium homodimer-I. Based on the percentage of dead granulosa cells, the follicles can be classified into four categories: V1 follicle (with no dead granulosa cells), V2 follicle (with <10% of dead granulosa cells), V3 follicle (with 10%–50% of dead granulosa cells), and V4 follicle (with >50% of dead granulosa cells).[23] In addition, follicle development can also be evaluated at the molecular level with gene expression analyses, such as single cell quantitative real-time polymerase chain reaction[57] or even on genome level with RNA-seq analysis.[63,64]

Conclusion

Human ovarian follicle IVG is a fascinating technique for fertility preservation as it does not need hormone stimulation, has a low risk of reintroducting malignant cells in cancer

patients, and could increase the pool of fertilizable oocytes in premenopausal and pubertal females. Nonetheless, in human beings, retrieval of mature oocytes from *in vitro*-cultured follicles is highly inefficient and evidence of their reproductive competence is lacking. In future studies, the mechanism of follicle activation and growth should be further investigated and the efficiency of follicle isolation should be enhanced. Long-term culture systems of follicle growth should be optimized, and health and safety of offspring derived from these approaches must be monitored to reach clinical translation.

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Conflicts of interest

There are no conflicts of interest.

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