



# Human Follicle *in vitro* Culture Including Activation, Growth, and Maturation: A Review of Research Progress

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Fertility preservation has received unprecedented attention nowadays. In addition to cryopreservation and re-implantation of embryos, oocytes, and ovarian tissue pieces, *in vitro* culture system for follicles/oocytes has been considered as an alternative strategy for fertility preservation. Since the metabolic dynamics and required nutrients are not entirely the same in different stages of follicular development, optimization of each culture step is needed. In this paper, literature regarding culture conditions in three steps were analyzed. Known additives in activation stage included 740Y-P, bpV(HOPic), follicle stimulating hormone (FSH), human serum albumin (HSA), ITS, growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15), and cyclic adenosine monophosphate (cAMP), with different degrees of activation promotion and potential detrimental effect on DNA integrity. For isolated follicles growth stage, actin A, FSH, basic fibroblast growth factor (bFGF), estradiol were proved to improve development or proliferation. As for maturation, addition of growth hormone, melatonin, C-type natriuretic peptide (CNP), GDF9, cilostamide, or forskolin helped to regulate maturation rate or improve oocyte quality. Based on previous sequential culture system for human follicles, optimization is needed to achieve higher maturation rate and better oocyte quality, pursuant to current review, which demonstrated the effects of various additives on different stages.

**Keywords:** *in vitro* culture, human follicle, human oocyte, *in vitro* maturation, fertility preservation

## INTRODUCTION

In recent decades, considerable progress has been made in the field of assisted reproductive technology (ART). Objectively, with improvement of the prognosis of malignant diseases and progress in ART, an increasing number of female cancer patients have the opportunity to preserve fertility through advanced technologies. Subjectively, young cancer patients are no longer satisfied simply with the continuation of life span, but become aware of their fertility needs. Therefore, fertility preservation has received unprecedented attention (1, 2). Thanks to current technology, part of the ovarian tissue of the women who need chemotherapy due to malignancies could be removed for cryopreservation before receiving treatment, so as to avoid the reproductive toxicity of chemotherapy drugs, and then be auto-transplanted at the end of treatment. However, the risk of re-implantation of tumor cells is faced by some patients during transplantation (3, 4). To solve this problem, an *in vitro* culture (IVC) system for follicles and oocytes has been considered as an

alternative fertility preservation strategy without the risk of re-implanting maternal somatic tumor cells (5).

For most mammals, the formation of follicles takes place pre-natally and constitutes the basic reserve unit of female reproductive cells, which are recruited for growth throughout the female's reproductive age. Specifically, development of follicles includes (1) initiation of primordial follicle growth and development to the preantral follicle stage; (2) the formation of antral follicles; and (3) rupture of the Graafian follicle releasing a cumulus-oocyte complex (COC) at ovulation in response to the mid-cycle luteinizing hormone (LH) surge (6). The IVC system should support the development of oocytes in all stages, from activation of primordial follicle to oocyte maturation. Once the safety and effectiveness of the system are confirmed, the maximum use of the ovarian follicle reserve would be achieved, and the risk of tumor cells carried by transplanted ovarian tissue would be eliminated. Moreover, for younger patients like prepubertal girls, neither mature oocytes nor embryo cryopreservation are available. Therefore, fertility preservation may be achieved by the IVC system (3, 7, 8).

Most follicles are in the primitive stage, and their recruitment is regulated by both endocrine factors and the ovarian internal environment (9, 10). During *in vivo* follicle growth, oocytes acquire the competence to resume meiosis, complete fertilization, and support early embryonic development. Thorough comprehension to this complicated growth procedure is essential for researchers to construct an IVC system meeting all requirements, with consideration for maintaining DNA integrity and stability (11, 12). In previous studies, a two-step culture system was designed for follicular development in mouse models, where the IVC oocytes were fertilized and healthy offspring were successfully obtained (13, 14). Similarly, mature oocytes from IVC cattle follicles were fertilized and a healthy calf was born with higher birth weight (15). These results in animal models proved the feasibility of follicular IVC. However, the inconsistency of follicular growth environment between humans and animals and challenges of applying the system to human follicles still need to be considered (16). A single research reported the multi-step IVC system for the whole course of human oocyte development, and some second meiotic metaphase (MII) oocytes were obtained (17). Since the metabolic dynamics and required nutrients are not entirely the same in different stages of follicular development, optimization of each step is needed to achieve a higher maturation rate and better oocyte quality, based on the sequential culture system. In this paper, the effects of various additives on different stages are demonstrated, and the current research progress of different stages of human follicular IVC are reviewed, respectively.

## INITIAL STAGE: *IN VITRO* ACTIVATION (IVA) OF PRIMORDIAL FOLLICLES

Primordial follicle activation is the key initial step of oocyte development, involving the interaction of inhibitory factors, stimulating factors, and maintenance factors (18). Early studies showed that the viability of primordial follicles isolated from

thawed human ovarian cortical tissues was comparable with that from fresh tissue. However, a lower survival rate was observed in IVC of isolated primordial follicles (19, 20). Consequently, a consensus has been reached that the culture system based on primordial follicles needs to start from human ovarian cortex tissue, not isolated primordial follicles (17, 20–23).

Several pathways, including the phosphatidylinositol 3 kinase (PI3K) pathway, the mechanistic target of rapamycin complex 1 (mTORC1) pathway, and the p27Kip1 (p27)-cyclin dependent kinase (CDK) system, have been shown to regulate the activation of dormant oocytes in the mammalian ovary (18). Some components in these pathways were well-studied and used as regulatory targets for IVC optimization.

The PI3K-PTEN-AKT-FOXO3 signaling pathway is the main non-gonadotrophic growth factor signaling pathway regulating the growth and differentiation of ovarian follicles (24–27). The phosphatase and tensin homolog deleted on chromosome 10 (PTEN), as a negative regulator of PI3K, inhibits the levels of phosphatidylinositol 3,4,5-triphosphate (PIP3), leading to suppressed activation of PI3K signaling (28, 29). The Forkhead box O3 (FOXO3), a downstream effector, also regulates this pathway negatively and suppresses follicle activation, based on mice experiments (24, 30). In women with premature ovarian insufficiency (POI) or premature ovarian failure (POF), mutation in FOXO3 genes was identified and lower FOXO3a expression in ovarian tissue was detected (31, 32). Another component is the mTORC1, a regulator of cell growth and proliferation, involved in promoting primordial follicle activation (33). In previous studies, IVA of primordial follicles was promoted through regulating the pathway by mTOR and PI3K activator, thus improving ART outcomes of patients with POI (26, 34). PTEN inhibition of human ovarian tissue before auto-transplantation has been proved to significantly enhance activation of residual follicles in POI patients, and healthy baby was born (35). Noteworthy, more than 50% POI patients in this study contained no residual follicle and did not respond to the IVA agents, limiting the application of IVA procedure in infertility treatment.

During IVC of the human ovarian cortex, more primordial follicles were activated, and more secondary follicles were isolated with PTEN inhibition. Unfortunately, the survival and development of isolated follicles were poor (26, 36), which may be related to the effect of PTEN inhibition on the DNA repair mechanism in follicles (12). The study involving IVC of bovine ovarian cortex demonstrated that PTEN inhibition activated bovine non-growing follicles, but simultaneously increased DNA damage and reduced DNA repair response (27). Recently, a drug-free IVA method was used in infertility treatment for POI patient, leading to successful pregnancies (37). This method may eliminate the negative effect of activation agents on follicles, but it may simultaneously reduce the yield of follicles, which makes it insufficient for subsequent culture use.

The activation rate of primordial follicles varies with different culture conditions, which seems to be related to the preparation of the ovarian cortical tissue, indicating that mechanical signal was also an important influencing factor (10). The Hippo signaling pathway, which is known for its role in conserving optimal organ size *via* growth

inhibition, consists of several negative growth regulators acting in a kinase cascade that ultimately phosphorylates and inactivates key Hippo signaling effectors, Yes-associated protein (YAP)/transcriptional coactivator with PDZ-binding motif (TAZ) (26, 38). Fragmentation of human ovarian cortical tissue can dramatically promote the activation of primordial follicles, which was proved to be the result of interruption of Hippo signal pathway. Ovarian fragmentation increased actin polymerization and disrupted Hippo signaling by decreasing pYAP levels together with increased nuclear localization of YAP, leading to increased expression of CCN growth factors and BIRC apoptosis inhibitors (26, 35). Intuitively, follicles at the most advanced developmental stage were observed at the periphery of the tissue, close to the section site (26). Taken together, the activation of follicles can be significantly promoted by intervening the important components of PI3K pathway, while the Hippo pathway is blocked through the fragmentation of the ovarian cortex (9, 25–27).

At present, common basic media for IVA include MEM- $\alpha$ , Waymouth's medium, D-MEM, and McCoys 5a (17, 34, 39, 40). Different laboratories seem to choose the medium used according to their preferences and practices, because no study comparing all media exists. In an early research, two complex media ( $\alpha$ -MEM and Waymouth's) were compared with a simple salt solution (EBSS), all of which contained 10% human serum. Results showed that in ovarian tissue cultured in  $\alpha$ -MEM, follicle growth was significantly greater and development was improved compared to that seen in follicles cultured with Waymouth's medium. A proportion of follicles cultured with both  $\alpha$ -MEM and Waymouth's media reached secondary stages on day 10 of culture. However, only follicles cultured with  $\alpha$ -MEM reached preantral stages until days 15 (41). Both media contained amino acids, vitamins, and inorganic salts, but  $\alpha$ -MEM has some unique components, including alanine, serine, lipoic acid, ribonucleosides, and deoxyribonucleosides. Meanwhile, the vitamin concentration in  $\alpha$ -MEM are higher than that in Waymouth's medium. Unfortunately, the study did not discuss the specific reason of different effects of the two media. It would be interesting to investigate how various nutrients in different media act on primordial follicle IVA.

Further understanding of effect of specific factors on the activation process was another hot topic to improve the quantity and quality of IVC follicles. For example, on the basis of  $\alpha$ -MEM, the culture system with human albumin and ITS (insulin, transferrin, selenium) is more favorable to the activation and development of human follicles, leaving less atretic follicles, than that with serum alone. And the addition of follicle stimulating hormone (FSH) to the media greatly reduces atresia and increases follicle diameter simultaneously (41). However, the findings in different reports could be contradictory. One study in 2005 showed that human follicular recruitment and activation were promoted in culture media with 300 ng/ml anti-Mullerian hormone (AMH) (40), while another research in 2006 disclosed the inhibitory effect of 100 ng/ml AMH on human follicles activation (42). The reason for the difference between findings was unclear. It is possible that AMH influences primordial follicle activation in a dose-dependent manner as shown in a study using

an ovine pre-pubertal ovarian cortex culture system (43). In fact, no unified standard for the timing and dosage of important additives has been developed. Currently, a consensus has been reached that the interaction between oocytes and somatic cells is essential for the activation and development of follicles (18). The intercellular communication is influenced by the members of TGF beta superfamily, especially the oocyte specific factors: growth differentiation factor (GDF)-9 and bone morphogenetic protein (BMP)-15 (44). In human follicles, GDF-9 and BMP-15 are expressed both in oocytes and cumulus granulosa cells. The increase of mRNA levels in cumulus cells is related to the oocyte maturity and fertilization rate (45), and the addition of GDF-9 or BMP-15 in IVC system can promote the activation of human primordial follicles, with seemingly more beneficial effects of GDF9 (46). Other factors, such as basic fibroblast growth factor (bFGF), kit ligand (KITL), keratinocyte growth factor (KGF), leukemia inhibitory factor (LIF), stem cell factor (SCF), vascular endothelial growth factor (VEGF), and cyclic adenosine monophosphate (cAMP), have also been proved to improve the activation and survival of cultured follicles *in vitro* (47–49). Shown in **Table 1**.

## DEVELOPMENT STAGE: *IN VITRO* GROWTH (IVG) OF SEPARATED SECONDARY FOLLICLES

As mentioned above, the culture system based on primordial follicles needs to start from human ovarian cortex tissue, in which the activated follicles are capable of developing to secondary stage, with multi-layer granular cells. However, further development will be inhibited if secondary follicles are not separated from the cortex (17, 23, 39). Secondary follicles can be separated by an enzymatic method, mechanical method or combination of the two methods. Mechanical isolation, with a fine syringe needle, has been commonly used for follicle isolation (17, 50, 51). This method preserves the natural follicular morphology, maintaining an intact theca cell layer and a normal basement membrane. However, it is inevitably laborious and inefficient (52), especially for denser human ovarian tissue. Enzymatic follicle isolation typically involves collagenase and DNase enzymolysis, by which the COCs were obtained from mice ovaries, and offsprings were produced following appropriate culture, indicating that intact follicle structure is not an absolute requirement during follicle IVC (53). A later study on molecular level showed that theca cells of mechanically-isolated secondary follicles induced the expression of IGF1 and promoted follicular growth in mice, while many follicles with theca cells still showed no growth during culture (54). It seems that theca cells affect follicular development to some extent, but may not be the only and necessary factor, as long as migration of granulosa cells from oocyte could be minimized (53). Interestingly, one study even demonstrated that although incubated with enzymes for 1 h, most enzymatically isolated follicles contained more theca cells compared with mechanically isolated follicles, which is contrary to our general thought (55). However, the influence of the operating technology of the experimenters cannot be excluded.

**TABLE 1** | Reported additives in human follicle *in vitro* activation stage.

| Additives                  | Species | Dose                  | Mechanism  | Effect  | Detection method  |
|----------------------------|---------|-----------------------|--|---|---|
| 740Y-P<br>(26, 34, 35)     | Human   | 150 µg/ml             | PI3K/AKT activator   | More follicles activated, no survival difference;<br>Increase in p-PTEN levels and Akt and rpS6 phosphorylation;<br>Higher serum AMH;<br>Healthy baby achieved. | Histological analysis;<br>TUNEL apoptosis assay;<br>Immunohistochemistry for Ki67, GDF9, rtPCR for TSC1, LATS1, Kit Ligand; E2 and AMH assay. |
| bpV(HOPic)<br>(26, 34, 35) | Human   | 30 µM                 | PTEN inhibitor   |   |   |
| bpV (HOPic) (36)           | Human   | 1 µM                  | PTEN inhibitor   | More follicles activated, with limited growth and reduced survival of isolated follicles.   | Histological analysis;<br>Immunohistochemistry for FOXO3.   |
| rhAMH (40)                 | Human   | 300 ng/ml             | May rescue some follicles from entering atresia  | Enhance follicles recruitment, survival and growth.   | Histological analysis   |
| rrAMH (42)                 | Human   | 100 ng/ml             | Act as a negative paracrine feedback signal  | Suppress the initiation of the growth of primordial follicles, without detrimental effect on viability or follicle density.                                     | Histological analysis   |
| FSH (41)                   | Human   | 300 mIU/ml            | Prevent apoptotic atresia;<br>Mitogenic function                                       | Reduce the proportion of atretic follicles; Increase follicle size and healthy follicles.   | Histological analysis   |
| HSA+ITS (41)               | Human   | 2.5% HSA;<br>1%ITS*   | Promote cell proliferation; Act as free-radical scavengers.                            | Reduce the proportion of atretic follicles; Increase follicle size and healthy follicles.   | Histological analysis   |
| GDF9 (46)                  | Human   | 10 ng/ml<br>100 ng/ml | BMP15 activates the intracellular signal-mediated pathways<br>Smad1, Smad5, and Smad8; | More follicles activated;<br>More follicles activated; increase PCNA expression;  | Histological analysis;<br>Immunohistochemistry  |
| BMP15 (46)                 | Human   | 10 ng/ml<br>100ng/ml  | GDF9<br>activates Smad2 and Smad3  | More follicles activated;<br>Increase PCNA expression;  |   |
| cAMP (48)                  | Human   | 0.5 mM                | Induce formation of the FSH receptor, as an intracellular second messenger             | More follicles activated;   | Histological analysis   |

\*1% ITS includes 10 mg/ml insulin, 5.5 mg/ml transferrin, and 6.7 ng/ml sodium selenite.

rhAMH, recombinant human anti-Mullerian hormone; rrAMH, recombinant rat anti-Mullerian hormone; FSH, follicle stimulating hormone; HSA, human serum albumin; GDF9, growth differentiation factor 9; BMP15, bone morphogenetic protein 15; cAMP, cyclic adenosine monophosphate.

Most recently, a research compared four isolation methods (two mechanical and two enzymatic) with mice, concluding that mechanical methods were preferable, with better follicular growth, survival, and MII rate. In addition, mincing isolation with a cell dissociation kit (SigmaAldrich) was more effective than conventional mechanical isolation (56). Overall, there is no one method that is absolutely superior to the others. And considering the difference between ovaries of mice and humans, both methods need to be improved and well-designed comparative researches on human samples are required.

IVG of isolated secondary follicles also depends on oocyte somatic interaction (57). At this stage, addition of activin and low-dose FSH, based on IVA medium, can help to maintain the intercellular connection, improve the quality of oocytes and promote the formation of antrum, which has been verified in dog, bovine, and human experiments (17, 39, 58, 59). Addition of 200 ng/ml bFGF in IVG medium also promotes the development of human ovarian early follicles (60), as shown in **Table 2**.

In addition to optimizing the composition of culture medium, the physical environment of isolated follicle culture is also widely

concerned. Conventional planar 2D culture plates may destroy the connection between human oocytes and granulosa cells, and inhibit the development of follicles (61). Therefore, some 3D culture systems were established to support the IVG of pre-antral follicles, and tissue engineering technology was also applied to this stage (62). Human preantral follicles can be encapsulated in bio-matrices such as alginate to complete growth and development *in vitro* (63). Alginate is a polysaccharide found in algal cell walls, which can form rigid and controllable gel. Studies have shown poor growth of mouse follicles embedded in 1% alginate gels (64), while 0.5% alginate gel supports the growth of human oocytes well (63). The research using microfluidic technology to encapsulate follicles also proved the function of mechanical heterogeneity in follicle development and ovulation (65). This indicated the effect of rigidity of the gel and the significance of balancing flexibility of the gel to accommodate cell proliferation with its rigidity to avoid collapse of the follicle structure (66). Other scaffolds such as de-cellularised ovarian tissue and 3D microporous scaffolds were also applied to support human pre-antral follicle growth (67, 68).

**TABLE 2** | Reported additives in human follicle *in vitro* growth stage.

| Additives    | Species | Dose      | Mechanism   | Effect  | Detection method  |
|--------------|---------|-----------|---|---|---|
| Actin A (39) | Human   | 100 ng/ml | Regulate granulosa cell growth and differentiation  | Increase follicles diameter; Higher estradiol secretion; More healthy follicles | Histological analysis; Diameter measurements; Detection of estradiol in culture media by enzyme immunoassay |
| FSH (59)     | Human   | 1.5 U/ml  | Increase E2 production  | Promote follicle antrum formation and growth                                    | Histological analysis; E2 assay.  |
| bFGF (60)    | Human   | 200 ng/ml | Promote GC proliferation, suppress apoptosis in preantral follicles, and enhance early follicle cell differentiation. | Higher diameter and survival rate, with good viability.                         | Histological analysis; Confocal analysis.   |

FSH, follicle stimulating hormone; bFGF, basic fibroblast growth factor.

In previous multi-step IVC system of human follicles, isolated secondary follicles were directly placed in a 96 well V-bottomed plate, without any extracellular matrix or scaffold. Sixty-two percent of the follicles completed the process of growth, differentiation and follicle antrum formation, but only 8% of the follicles finally reached MII stage (17). Another team's research showed that the follicles encapsulated in alginate can reach 110  $\mu\text{m}$  in diameter after 30 days of culture, while the oocytes in these follicles cannot reach MII stage (63). Considering that human immature follicles gradually move from the rigid collagen-dense cortex zone to the less dense perimedullary region as they grow (69, 70), this team released a portion of follicles from the alginate hydrogels at antral stage, and then continued the culture in low-attachment plates for up to 40 days in further study. Results showed that follicles cultured with only alginate encapsulation produced oocytes that either remained in the germinal vesicle (GV) stage or degenerated, while 20% (four out of 20) of follicles cultured using the two-step strategy produced meiotic competent MII oocytes (61). In the future, more research is needed to compare different culture environments to find out which one best supports the development of follicles *in vitro*.

## MATURATION STAGE: *IN VITRO* MATURATION (IVM) OF OOCYTES

Since human immature oocytes were cultured by IVM technology, resulting in a live birth after fertilization in 1991 (71), many studies regarding the establishment and optimization of the IVM system have been reported. The source of oocytes selected for IVM seems to be a crucial factor as the successful maturation of immature oocytes is lower than that of oocytes obtained from stimulated ovaries (72, 73). The developmental competence of IVG derived oocytes is different from that of natural or controlled ovulation cycle derived oocytes. Therefore, if IVG is to be used in clinical practice, IVM conditions need to be optimized (74).

In 2008, Telfer et al. developed a two-step culture system, which initially demonstrated that individual human pre-antral follicles grown *in vitro* from the primordial stage, within human ovarian cortical strips, can be isolated and have the potential to grow to the antral stage (39). From then, scientists have been

working on bridging the gap between IVG of follicles and IVM of oocytes. The oocytes produced by IVG need to be transferred to the maturation medium when they reach a certain stage, so as to complete the final meiotic resumption and maturation. In 2015, a study comparing alginate encapsulation culture and two-step culture of isolated follicles revealed that the diameters of oocytes that reached the MII stage was not significantly different from that of oocytes that remained in GV stage, indicating that the oocyte meiotic competence could be achieved although the terminal follicle size cannot reach the size of preovulatory follicles *in vivo* (61). In the later modified multi-step IVC system, follicular antrum formation was observed within 6–8 days of isolated follicle culture, and at the time COCs were removed for subsequent culture, without waiting for the follicle diameter to increase to the preovulatory follicle level. After IVM, oocytes with a diameter over 100  $\mu\text{m}$  were obtained and few of them developed to MII stage (17). These results indicated that a more direct and efficient IVC process could be achieved based on the development of oocytes rather than the diameter of follicles.

However, the maturation rates of IVG derived oocytes were still not ideal. In the most recent multi-step culture system, 32 COC from IVG were selected for IVM, and nine oocytes emitted polar bodies after 24 h of maturation (17). Optimization is required not only for the IVM stage, but also for the whole culturing course. Nevertheless, most of the studies focused merely on the maturation stage of the oocytes, so the oocytes used in the experiments were obtained mainly from immature oocytes in the COH cycle, while IVG derived oocyte maturation was less reported. While almost all the literature on IVM optimization was based on oocytes harvested from stimulated ovaries, a review of the results of these literature still helps to optimize IVM following IVG.

Common basic media for IVM include  $\alpha$ -MEM, SAGE, TCM-199 (17, 61, 75), although which medium is better for oocyte maturation is still uncertain. To explore the best conditions of IVM, a good understanding of the mechanism of oocyte maturation is needed. cAMP plays a crucial role in regulating oocyte maturation. The granulosa cells in the outer layer contain C-type natriuretic peptide (CNP), while the natriuretic peptide receptor (NPR2) is expressed in the cumulus cells around the oocytes. The paracrine factors of oocytes promote the activation of NPR2 in the cumulus cells, and the CNP binds to the

**TABLE 3** | Reported additives in human follicle *in vitro* maturation stage.

| Additives        | Species | Dose           | Mechanism  | Effect   | Detection method   |
|------------------|---------|----------------|--|--|--|
| Melatonin (75)   | Human   | 10 $\mu$ mol/L | Maintain the mitochondrial oxidative phosphorylation function in the oocytes by effectively inhibiting environmental stress, providing sufficient ATP for subsequent embryo development. | Increased high-quality blastocyst formation rate, with low aneuploidy rate.  | Blastocyst grading and examination of aneuploidy; Single-cell RNA-sequence analysis; Detection of ROS and calcium levels in human oocytes; Mitochondrial function assay in human oocytes |
| GH (81)          | Human   | 200 ng/ml      | Accelerate meiotic progression, balance redox homeostasis of cellular environment, and promote oocyte developmental competence   | Promote maturation of human oocytes  | Oocyte RNA Sequencing; Real-Time PCR for Validation  |
| CNP (80)         | Human   | 25 nM          | Binds the Natriuretic peptide receptor 2 (NPR2), induces the production of cGMP  | Increase the meiotic maturation rate   | Evaluation of blastocyst yield; Documentation of aneuploidy rate in blastocysts; Assessment of cumulus-oocyte TZPs; Evaluation of chromatin configuration in GV oocytes                  |
| GDF9 (82)        | Human   | 200 ng/mL      | Promote cumulus expansion  | Increase the embryo formation rate and blastocyst viability  | Assessment of maturation, fertilization, embryo formation, blastocyst formation rate, and blastocyst viability (by Propidium iodide/Hoechst staining).                                   |
| Cilostamide (79) | Human   | 20 $\mu$ M     | PDE3 specific inhibitor  | Positively influence oocyte developmental competence by exhibiting a synergistic effect on the prevention of gap junction communication loss and resumption of meiosis | Intercellular gap junction communication and maturational status were examined by fluorophotometry;  |
| Forskolin (79)   | Human   | 50 $\mu$ M     | Adenylate cyclase activator  |  |  |

GH, growth hormone; CNP, C-type natriuretic peptide.

NPR2 receptor in the cumulus cells to produce cyclic guanosine monophosphate (cGMP), which enters the oocytes through gap junctions, inhibits the activity of phosphodiesterase (PDE3A), maintains a high level of cAMP, and thus keeps the meiosis arrest of oocytes. When PDE3A is activated by LH, the cAMP level in oocytes would be down-regulated, resulting in resumption from meiotic arrest of the immature oocytes in the GV stage or the first meiotic metaphase. Subsequently, the oocytes reach MII stage and complete maturation (76).

Accordingly, synchronization of nuclear and cytoplasmic maturation processes within the oocyte could be achieved through controlling COC cAMP level. Experiments showed that artificial regulation of meiotic resumption by cAMP elevating agents improved subsequent human oocyte developmental competence (77, 78). Combined treatment of cilostamide (PDE3 specific inhibitor) and forskolin (adenylate cyclase activator) positively influences the IVM oocyte developmental competence (79). Using CNP in the medium increased the meiotic maturation rate of IVM human oocytes (80). Some other factors also have been proved to promote oocyte maturation or benefit subsequent processes (Table 3). The addition of GDF9 had no effect on maturation rate, while it significantly enhanced fertilization, embryo formation, and viability rates of blastocysts (82). And the growth hormone (GH) was applied to improve the IVM maturation rate of human oocytes (81). More specifically, melatonin had no significant

effect on the maturation rate of oocytes, while it increased the blastocyst formation rate after fertilization from 24.5 to 49.3%, by protecting mitochondrial function (75). High-glucose concentrations altered DNA methylation levels of Peg3 and adiponectin in human IVM oocytes (83).

## CONCLUSION AND PROSPECTS

Based on the animal experiments and the basic human follicle IVC system established, the concept of achieving MII oocytes from human primordial follicles through IVC to obtain offspring after *in vitro* fertilization (IVF) seems promising. However, currently, the clinical use of human follicular IVC systems is still limited by low MII rates, ambiguous fertilization capacities, and unknown safety. Based on the information provided by this review, future studies may focus on the following: (1) IVA: clarifying the safe concentration and safe exposure duration of existing activators, or further exploring the drug-free activation; (2) IVG: exploring the optimal method and timing of human follicle isolation, and developing the 3-dimension system, combined with bioengineering technology, such as microfluidic technology (84, 85), to simulate the *in vivo* follicular development environment; (3) IVM: establishing a whole-course culture system to improve the maturation rate and developmental potential of IVG-derived oocytes, in addition to studying this stage alone; (4) to explore the fertilization ability

and embryo developmental potential of mature oocytes obtained by IVC within ethical agreements.

In conclusion, IVC is likely to provide an ideal option for the fertility preservation of young cancer patients and for the infertility treatment of patients with POI or POF in the future. However, before this technology is applied to clinical practice, a large number of studies are required to optimize each stage of follicular development *in vitro*, establishing a relatively unified, standard, efficient and safe culture system.

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## AUTHOR CONTRIBUTIONS

All authors have contributed to the preparation of this manuscript, read, and approved the manuscript.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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