In vitro follicle culture in the context of IVF

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Abstract

The currently available assisted reproduction techniques for fertility preservation (i.e. in vitro maturation (IVM) and in vitro fertilization) are insufficient as stand-alone procedures as only few reproductive cells can be conserved with these techniques. Oocytes in primordial follicles are well suited to survive the cryopreservation procedure and of use as valuable starting material for fertilization, on the condition that these could be grown up to fully matured oocytes. Our understanding of the biological mechanisms directing primordial follicle activation has increased over the last years and this knowledge has paved the way toward clinical applications. New multistep in vitro systems are making use of purified precursor cells and extracellular matrix components and by applying bio-printing technologies, an adequate follicular niche can be built. IVM of human oocytes is clinically applied in patients with polycystic ovary/polycystic ovary syndrome; related knowhow could become useful for fertility preservation and for patients with maturation failure and follicle-stimulating hormone resistance. The expectations from the research on human ovarian tissue and immature oocytes cultures, in combination with the improved vitrification methods, are high as these technologies can offer realistic potential for fertility preservation.

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Introduction

For several decades, researchers have been interested in culturing gametes to better understand the intricate processes regulating the genesis of mature oocytes within the complexity of the female gonad. The ovary contains at all times follicles in different stages of growth, which are most often in the process of becoming atretic as soon as they are visible by ultrasound. A reduction of the complexity in understanding the growth of follicles can be achieved by synchronizing and monitoring in vitro the cohort to be studied, by using the latest sophisticated biochemical and microscopy technologies. Not only has the scientific curiosity sparked research in folliculogenesis and oogenesis, there is currently an increasing amount of patients suffering from childhood cancers, who require gamete-devastating chemo- and/or radio-therapies. These children, as well as adults in their reproductive years, could directly benefit from the possibility to culture gonadal tissue on the condition that it had been safely cryopreserved before chemotherapy. Ovarian tissue cryopreservation pioneered by several labs in Europe, USA and Australia has already proven to be a good option, as of today a pregnancy rate of nearly 30% has been reported after the use of autologous transplantation of the thawed tissue (Donnez & Dolmans 2015). For some categories of patients, in whom auto-transplantation or oocyte/embryo freezing cannot be performed for practical or safety reasons (given the risk related to cancer relapse and exposure to high hormone doses in in vitro fertilization (IVF) cycles i.e.), the only alternative to restore ovarian function could be the in vitro culture approach that allows implanting back just the oocyte, after the cure of cancer. However, the ‘in vitro road’ promises to be long and tedious.

The class of follicles with the most interesting implication for oncofertility is early stage follicles. Primary and primordial follicles are the most abundant follicles present in females of all ages, and they possess intrinsic capability to withstand cytotoxic therapies and survive cryopreservation better than growing follicles (Laronda et al. 2014, Tagler et al. 2014). Although accomplished in mouse and lamb, (Muruvi et al. 2005, Hornick et al. 2013) isolation and culture of primordial follicles are difficult tasks in primates, due to the small size, the limited connections between the granulosa cells and oocytes and the complex extracellular matrix environment in which they develop (Woodruff & Shea 2011). Moreover, finding a reliable and safe method for the synchronous activation of the primordial follicle class in vitro still remains challenging. Obtaining human ovarian tissue for the experimental setup of a reproducible follicle culture system is often complicated because the donor tissue is seldom of
a good initial quality. Therefore, the valuable data obtained in different animal models by researchers in developmental biology and veterinary field have to be considered inspirational for the work in humans. In the last two decades, several groups could prove that early preantral/secondary follicles can be grown up to a stage where the oocyte can re-initiate meiosis, although still at a low final yield, in isolated follicle culture systems in mice (Cortvrindt & Smits 2001, Xu et al. 2006, 2009a, Hornick et al. 2013), large domestic animals (Songsasen et al. 2011, Silva et al. 2015), non-human primates (Xu et al. 2009b, 2010, 2011a,b, 2013, 2017) and in human (Teller et al. 2008, Xu et al. 2009a, Laronda et al. 2014, Xiao et al. 2015, Yin et al. 2016, Abir et al. 2017). More sophisticated in vitro systems are being designed using the most recent knowledge from the omics, bio-printing technologies and microfluidics. Integration of these technologies should provide us with additional tools to better reproduce the follicular niche. A more optimistic message can be given for the in vitro maturation (IVM) of human oocytes, where cumulus oocyte complexes (COCs) are retrieved from small (2–12 mm) antral follicles. ‘IVM’ of ovarian oocytes from mouse, sheep, cow, pig, rhesus monkey and human was pioneered and successfully achieved in 1965 by the physiologist, Nobel Prize Winner 2010, Sir Robert Edwards (Edwards 1965a). Oocyte IVM, largely employed in animal breeding, can represent a potential alternative to follicle culture for cancer patients and a valid assisted reproductive technique for women unable to receive or access ovarian stimulation treatment, due to ovarian hypersensitivity risk, elevated cost or other social reasons. R G Edwards had the capacity to anticipate potential applications and benefits of IVM, as documented in his historical publications (Edwards 1965a,b). So far, IVM has been clinically applied in patients with polycystic ovary (PCO)/polycystic ovary syndrome (PCOS), but other indications can now be surely added, including fertility preservation, oocyte maturation failure and follicle-stimulating hormone (FSH) resistance. Increased knowledge obtained only in the last decade on how the meiotic arrest and progression in the antral follicle is regulated has inspired major ways for improvement of culture methods. This review summarizes the work of several ovarian follicular biology laboratories and proposes a selection of the most significant studies addressing the in vitro follicle culture challenges and potential applications within human IVF. Some of the most recent advances of human IVM are also discussed. The review of these data increases our understanding of the mechanisms involved in oocyte’s growth and final maturation and could suggest the refinements that have still to be made to further optimize the in vitro culture systems in view of fertilization and embryo culture in human.

**Proof-of-principle studies on early in vitro folliculogenesis using animal models**

Eppig and O’Brien pioneered in vitro follicle culture about two decades ago and obtained the first live birth in the mouse model from in vitro-grown primordial follicles (Eppig & O’Brien 1996). By making use of a 2-step culture system, individual follicles obtained by enzymatic dissection of whole neonatal ovary, were grown up to metaphase II oocyte. (Eppig & O’Brien 1996). This in vitro production system was subsequently perfected in 2003 and led to the generation of a mouse colony, showing the reproducibility of primordial follicle culture in this model and eliminating the initial skepticism that in vitro culture would result in abnormal live offspring (O’Brien et al. 2003). The achievement of normal progeny from the culture of early stage oocytes was taken further when Japanese biologists described an inventive method to obtain live births and healthy pups starting from primordial germ cells (PGCs) (Morohaku et al. 2016), and more recently from IPS (induced pluripotent stem) cells following in vitro generation of the entire oogenesis cycle (Hikabe et al. 2016). These very complex and time-consuming experiments not only inspired the work in other species, but fundamentally proved that earliest stages of oocytes, even before PGC colonize the genital ridge, can represent a novel source of female gametes.

Large animal models as bovine, ovine, caprine, porcine and non-human primates such as rhesus macaque (Macaca mulatta), baboon (Papio anubis) and cynomolgus (Macaca fascicularis), have also been used to investigate the mechanisms behind follicle activation and generation of fertilizable oocytes. The whole organ culture approach successfully used in rodents was replaced by ovarian cortical strips culture (Hovatta et al. 1997, 1999). In most large mammals, follicles form during fetal life; hence, the strategy to use primordial follicles collected during the last trimester of gestation from baboon and bovine (Wandji et al. 1996, 1997) seemed appropriate. It was observed that culturing ovarian cortex from fetal baboon and fetal or adult bovine in serum-free medium supplemented with Insulin-Transferrin-Selenium (ITS), induced a massive activation of primordial follicles already after 2 days (Wandji et al. 1996, 1997). This uncontrolled spontaneous growth of primordial follicles when cultured in vitro was intriguing and elicited several hypotheses. An initial hypothesis that the absence of ovarian medulla could be responsible for primordial follicle activation in bovine could not be proven. Using the bovine model, the involvement of TGFα in the regulation of primary follicle development was demonstrated (Derrar et al. 2000). Later, it was suggested that Insulin and Kit Ligand could also play a role as primordial follicle activators (Muruvi & Fortune 2009).
Using the caprine model, Silva et al. (2004) cultured ovarian cortex to study the potential role of FSH and epidermal growth factor (EGF) in the follicle activation process. Spontaneous activation nevertheless occurred during in vitro culture and FSH and EGF induced an increase in size of oocyte and follicle but had no effect on wholesale activation. (Silva et al. 2004). Overall, the data obtained by the large mammals models were pivotal to show that in vitro follicular activation could be successfully achieved, despite the majority of activated follicles could not progress in a correctly differentiated manner or survive. On the other hand, several studies demonstrated that preantral follicle culture could lead to the production of embryos in several species: ovine (Arunakumari et al. 2010), caprine (Magalhães et al. 2011), porcine (Wu et al. 2001), bovine (Gupta et al. 2008), clearly indicating that follicle culture success strongly correlates with the stage of follicle and the size of oocyte at the beginning of the culture.

Finally, a different approach in follicle culture, represented by a 3D alginate encapsulation culture system capable to support in vitro follicle growth from secondary to the antral stage, was described in rhesus macaque (Xu et al. 2010, 2011a). Interestingly, the in vitro-grown oocytes could re-initiate meiosis, but their developmental capacity remained suboptimal, as proved after in vitro insemination by ICSI (Xu et al. 2010, 2011a). Similar results were also obtained in the same animal model, starting from the primary follicle stage (Xu et al. 2013).

**Significant progress made in the human model**

Relevant insights in follicle activation mechanisms were also obtained from research in the human model. Pioneering studies took inspiration from the complex structural organization of the ovary and suggested that its dynamic environment and the availability of nutrients, hormones and growth factors could play a major role in primordial follicle activation and growth. Basing their work on this assumption, Picton and Gosden adopted the strategy to grow early follicles in situ in cortical slices, to resemble the ovary in vivo, instead of isolating follicles for in vitro culture (Picton et al. 1999). This study suggested that the activation of human primordial follicles was not gonadotropin dependent, as was later confirmed in bovine (Silva et al. 2004) but rather supported by the ovarian cellular environment. In a similar approach, ovarian cortical tissue, donated by 20 women following gynecological laparoscopies, was first cultured in Matrigel (extracellular matrix), to initiate the growth of the primordial and primary follicles and then divided into two parts to either be cultured further as slices or used for enzymatic or mechanical partial isolation of the follicles (Hovatta et al. 1999). This study confirmed that the most appropriate method to culture human follicles was non-isolated, but within small tissue slices. Overall, these data showed the importance to tailor the culture strategies for in vitro growth of human follicles to the size of follicle; therefore, multistep culture systems to support each of the specific stages were developed (Telfer et al. 2008, Smits et al. 2010, Telfer & McLaughlin 2012, Telfer & Zelinski 2013). The aims of the multistep approach were to sustain homogenous primordial follicle activation and initiation of early follicular growth, support the growth and differentiation of early preantral to antral stage and provide the right maturation conditions of COCs. In 2008, using a two-step culture system without serum and matrix, Telfer et al. (2008) showed that human primordial follicles undergo activation and initiate growth within mechanically loosened cortical pieces, developing to multilaminar preantral (secondary) stages after only 6 days of culture (Telfer et al. 2008). The effectiveness of the stepwise approach was recently reiterated by McLaughlin et al. (2018) reporting the 1st mature human oocyte from a unilaminar follicle grown in a multistep culture system. In this study, human ovarian cortex strips were cultured in a serum-free system and multilaminar follicles were dissected and individually cultured in the presence of recombinant activin A, in non-attachment (3D) condition. COCs and adherent mural granulosa were also cultured in presence of FSH and activin and further matured in vitro, resulting in a metaphase II oocyte (McLaughlin et al. 2018).

In view of a potential applicability in fertility preservation treatments, both approaches of cortical strips and isolated preantral follicles were taken in consideration. The most challenging aspect of the in vitro follicle culture, the activation step, was further investigated by Li et al. (2010) who proposed a protocol, developed in the mouse model, for chemical activation of dormant human primordial follicles by using ovarian cortical fragments from cancer patients treated with the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) inhibitor dipotassium bisperoxo (picolinato) oxovanadate (V) dihydrate (bpV(pic)) (Li et al. 2010). Short-term in vitro activation of dormant ovarian follicles after stimulation of the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) – protein kinase B (AKT) pathway and transplanted under the mouse kidney capsula led to the growth of a large amount of mature female germ cells. This method has been promptly translated into the clinical practice for fertility treatment of women with ovarian insufficiency (Kawamura et al. 2013).

A slightly different approach aimed at using ovarian tissue cortex pieces for isolation of preantral follicles, to further culture in vitro and transplant without the risk of reintroducing malignant cells (Dolmans et al. 2007). The protocol based on enzymatic digestion and gradient purification allowed the isolation of a large population of primordial follicles, with good viability and morphology (Dolmans et al. 2006). To increase
the chances of success for patients in need of fertility preservation treatments, recent research focused on identifying alternative sources for gamete collection rather than the ovarian cortex. It was suggested that the ovarian medulla could also be considered a source of follicles for fertility restoration in oncology patients and a protocol for enzymatic (Liberase, collagenase, deoxiribonuclease) isolation of viable preantral follicles from the ovarian medulla was recently proposed. The culture outcomes of these follicles looked promising, as they reached antral stage and had steroidogenic activity and anti-Müllerian hormone (AMH) secretion measured in their spent medium (Yin et al. 2016). Further steps have been taken in the direction of in vitro differentiation of human germ cells: an in vitro culture system capable to support the differentiation of human embryonic stem cells into ovarian follicle-like cells (FLCs) has been successfully applied (Jung et al. 2017). This approach could offer future perspectives for fertility restoration in patients with distinct pathologies like ovarian dysgenesis (e.g.: Turner Syndrome).

**Critical points**

Although major steps have been taken over the last two decades, improvements and refinements are still needed before in vitro folliculogenesis can be considered for clinical use. After achieving activation and initial growth in the cortical culture systems, in large mammals, only a small fraction of the primary follicles showed progress to the secondary stage, using suitable factors to support follicular growth and differentiation (reviewed by Smitz et al. 2010 and De Vos et al. 2014). For the primate model extended culture periods (e.g.: 5 weeks) were required when starting-off from secondary follicles (Ting et al. 2015). In non-human primates and in humans, the best results with this approach were obtained using expandable matrices in 3D systems. However, only a low number of oocytes was capable of resuming meiosis upon ovulation trigger (Xu et al. 2013, Ting et al. 2015). Overall, fertilization efficiency and developmental capacity of oocytes obtained from in vitro follicle culture is still extremely low, and no blastocyst production was achieved so far (Xu et al. 2010, 2011a,b, 2013). One of the reasons for the limited success of this technology can be found in the lack of more appropriate and physiological culture systems. Poor outcomes of early in vitro growth may indeed reflect suboptimal culture conditions. To this purpose, the supplementation of stage-specific growth factors may be crucial in maintaining physical interactions and preventing cellular stress, formation of reactive oxygen species, accumulation of waste compounds and so forth. Early preantral stages require more profound molecular and biochemical characterization to better define the environmental conditions able to support follicle growth up to the antral stage. The identification of one or more biomarkers that indicate the optimal time for ovulation trigger and fertilization (Sánchez et al. 2012) could represent a valuable tool for reproducing in vitro the grade of maturity of the oocyte embedded in the cultured follicle (e.g.: related to the arrest of transcription, nuclear maturity of the oocyte, etc.). Secondly, obtaining a synchronous activation of primordial follicles during tissue culture as seen in early work on both bovine and baboon (Wandji et al. 1996, 1997) as well as on human (Hovatta et al. 1997, 1999, Téllier et al. 2008) is a critical point for the success of this technique because of the specific needs of the follicles throughout their development from primary to early cavitary stages. The use of chemicals for in vitro activation followed by follicle culture might be a viable strategy only when the safety could be guaranteed. These pharmacologic manipulations might induce, besides a synchronous activation, other parallel metabolic effects in intracellular and intercellular processes. Preliminary work indicated that the inhibition of PTEN with dipotassium bisperoxo (5-hydroxypyridine-2-carboxyl) oxovanadate (bp(V)HOpic) initiated follicle growth and development to the secondary stage, but had a negative impact on the subsequent survival of isolated secondary follicles (McLaughlin et al. 2014).

Over the last few years, special efforts have been made to identify the role of the main hormones involved in regulating follicular growth in vivo, and how they modulate the current in vitro culture systems. In the rhesus macaque in vitro model, estradiol (E2) was identified as an essential factor with positive effects on follicle survival, growth, differentiation and oocyte health in a steroid-depleted milieu (Ting et al. 2015). AMH was shown to induce follicle growth and differentiation, oocyte maturation and steroid secretion in a matrix-free 3D culture system (Xu et al. 2017). In non-human primates, a dynamic exposure of secondary follicles to local secreted factors in the 3D alginate encapsulation system, followed by the inclusion of gonadotropins upon reaching the antral growth stage, showed to be the more physiological condition (Baba et al. 2017). Despite some mature oocytes have been obtained, their developmental competence remained low as no blastocyst formation in non-human primates and no live births after embryo transfer from IVG oocytes in large animals have been reported so far. A strategy to increase the quality of oocytes derived from long-term follicle culture could be obtained by introducing a ‘capacitation’ culture step preceding the final IVM stimulus. Making use of physiologic meiotic inhibitors as the C-type natriuretic peptide (CNP) (Zhang et al. 2010, Kawamura et al. 2011, Tsuji et al. 2012), the completion of the oocyte cytoplasmic maturation could be supported before polar body extrusion, leading to a higher developmental competence of these oocytes. Proof-of-concept studies using CNP within the IVM context starting from small- and middle-size antral...
Control of follicular activation and early growth

In order to overcome some of the above mentioned limitations, extensive work has been performed during the last decade to elucidate the main signaling pathways underlying follicular activation and early growth. Primordial follicles, enclosing meiosis-arrested oocytes, remain dormant in the mammalian ovary for periods of time as long as 50 years in human (Faddy & Gosden 1996). Only part of these will be activated and recruited in the growing pool to generate mature oocytes (Lass et al. 1997). The flattened granulosa cells, the somatic component of the primordial follicles, are essential for the survival of the resting oocyte. A characteristic sign indicating the recruitment of a primordial follicle into the growing pool is the change in shape of the granulosa cells from flat to cuboidal, followed by growth of the oocyte (Hirshfield, 1991). The activation of mammalian target of rapamycin complex 1 (mTORC1) – KIT LIGAND (KITL) signaling pathway in pre-granulosa cells (p-GCs) of primordial follicles triggers the awakening of dormant oocytes and follows through with the process of follicular activation via PI3K signaling in oocytes (Zhang et al. 2014) (Fig. 1). The mechanisms and most studied pathways regulating primordial follicle activation have been extensively reviewed (Adhikari & Liu 2009, Sánchez & Smitz 2012, Hsueh et al. 2015, Zhang & Liu 2015) and are briefly summarized below.

The PI3K/PTEN-Akt-mTORC1-S6k-rps6 cascade

One of the better described mechanisms involved in follicle activation is the PI3K-PTEN-AKT-FOXO3 pathway (Zhang & Liu 2015). The PI3K signaling pathway comprises a chain of molecules (kinases, phosphatases and transcription factors) mediating important cellular processes like proliferation, apoptosis, survival, growth, migration and metabolic activity (Cantley 2002, Chalhoub & Baker 2009) and acts on primordial follicle awakening via the oocyte (Reddy et al. 2005, 2008) (Fig. 1). The follicular activation mechanism involves the binding of KIT LIGAND to the tyrosine kinase receptor (c-KIT) on the oocyte, which leads to formation and activation of the PI3K complex. This further induces the activation and the nuclear translocation of AKT, that inhibits the activity of the transcriptional factor forkhead box O3 (FOXO3) responsible for keeping the oocyte in a dormant state (Reddy et al. 2009). PTEN acts as a negative regulator of PI3K (Simpson & Parsons 2001, Zhang et al. 2015a,b, Franciosi et al. 2014, Wei et al. 2015, Zhong et al. 2016, Romero et al. 2016) and human (Sánchez et al. 2017) models were effective and safe. Additional advances in the field could be achieved by identifying suitable biomarkers and crucial parameters for the biochemical monitoring of the in vitro follicle development process.

![Figure 1](image_url)
Cantley 2002). It has been shown that the modulation of PTEN activity via pharmacological inhibitors or PI3K activators during in vitro treatment of mouse ovaries and human cortical strips increased in vitro primordial follicle activation and led to successful growth of metaphase II oocytes, after xenotransplantation of the human ovarian tissue and hormonal stimulation (Li et al. 2010). Transplantation of the treated mouse grafts under the kidney capsules of ovariciomized mice, stimulated with gonadotropins, resulted in mature oocytes and healthy fertile pups after IVF (Li et al. 2010). Furthermore, AKT induces cell growth by activating the mammalian target of rapamycin (mTOR) (Adhikari & Liu 2010). mTOR, a serine/threonine kinase forms two distinct multiproteic complexes, mTOR complex 1 (mTORC1) and 2 (mTORC2) (Laplan & Sabatini 2012) involved in regulation of cell growth and proliferation (Adhikari & Liu 2009; Fig. 1). Mouse work indicated that combined treatment of mTOR and AKT activators on prepuberal ovaries, followed by transplantation, generated a higher graft weight compared to AKT stimulators only treatment (Cheng et al. 2015). Sun et al. (2015) showed that mTOR activators (phosphatidic acid, propranolol) induced awakening of pridordial follicles. When human ovarian cortex was briefly exposed to a combination of mTOR and PI3K pharmacological activators and cultured for 6 days, the yield in secondary stage follicles was significantly higher than when exposed to these stimulators individually (Sun et al. 2015).

The Hippo signaling pathway: another pathway proven to regulate onset of follicle growth

The Hippo signaling pathway plays a crucial role in growth regulation and normal organ size maintenance in all metazoan species (Pan 2007). This pathway was reviewed by Hsueh et al. (2015) in the context of ovarian physiology and physiopathology, describing its role in limiting early follicle development (Hsueh et al. 2015). Work in mouse from Kawamura et al. (2013) proved that fragmentation of ovaries promotes follicle growth. This effect was correlated with Hippo pathway disruption due to increased actin polymerization (conversion of G-Actin to F-Actin), which results in accumulation of yes-associated protein (YAP) in the nucleus. The transcription factor YAP upregulates the expression of CCN growth factors that modulate follicle growth and oocyte maturation (Kawamura et al. 2013) (Fig. 2).

Potential application in the clinic

The combination of the Hippo signaling disruption by tissue fragmentation and the pharmacological PI3K pathway stimulation in human ovarian cortical pieces with early preantral and secondary follicles, led to follicular growth up to pre-ovulatory stage within 4 weeks of xenotransplantation in immune-deficient mice (Kawamura et al. 2013).

These results were further translated into the clinical set up for infertility treatment of patients with primary ovarian insufficiency (POI) by Kawamura et al. (2013). Thawed ovarian tissue was fragmented for Hippo pathway disruption to support follicular growth and further on, treated with pharmacological activators of the PI3K pathway to induce follicular awakening, followed by autologous transplantation. Within 2–3 weeks, follicular growth was detected and first live births were reported (Kawamura et al. 2013, Suzuki et al. 2016) (Fig. 3).

Future perspectives

There are several follicle culture systems that have paved the way for growing oocytes as reviewed earlier (Smitz et al. 2010). Initial work to obtain oocyte growth within the cortical tissue strips seemed successful (Hovatta et al. 1997). However, passed this step, the tissue strip environment seemingly inhibited further growth. This led to the recommendation to isolate the growth-initiated follicles once these reached the secondary stage (Telfer et al. 2008). Further culture of isolated follicles...
In vitro growth and maturation of oocytes

In vitro treatment of ovarian cortical pieces with PI3K activators/PTEN inhibitors, induces primordial follicle growth by upregulating AKT signaling in granulosa cells of preantral follicles (ccc). Ovarian fragmentation promotes follicular growth by inducing actin polymerization and Hippo signaling disruption. This downregulates YAP phosphorylation, followed by an increase in nuclear levels of YAP, and an elevated expression of CCN growth factors and BIRC apoptosis inhibitors (Kawamura et al. 2013). Tissue fragmentation and AKT stimulation followed by auto-transplantation in primary ovarian failure patients, resulted in preovulatory follicles in a few weeks (Kawamura et al. 2013, Suzuki et al. 2015, Zhai et al. 2016). This method generated mature oocytes for IVF-ET for some of the POI patients (Kawamura et al. 2013). After IVF-ET, clinical pregnancy and live births were reported (Kawamura et al. 2013, Suzuki et al. 2015, Zhai et al. 2016). AKT, Protein Kinase B; BIRC apoptosis inhibitors, Baculoviral Inhibitors of apoptosis Repeats-Containing proteins; CCN growth factors: the acronym derived from three of its members: CCN1 (cysteine-rich angiogenic protein 61 or), CCN2 (connective tissue growth factor), and CCN3 (nephroblastoma overexpressed); ET, embryo transfer; IVF, in vitro fertilization; mTOR, mammalian target of rapamycin; PI3K, Phosphatidylinositol-4,5-bisphosphate 3-kinase; POI, primary ovarian insufficiency; PTEN, phosphatase and tensin homolog deleted on chromosome 10; YAP, Yes-associated protein.

Secondary follicles was carried out in small inserts with or without extracellular matrix (ECM) or in plastic well plates (Smits et al. 2010). The early preantral follicle culture is strongly influenced by the composition and architecture of its supporting tissue. This generated the need to develop extracellular matrices and biomaterials that could imitate the ovarian physiologic milieu for optimal follicle development.

Several extracellular matrices natural (collagen, synthetic (alginate) have been used in the attempt to keep the 3D structure during culture (West et al. 2007). The 3D culture techniques have advanced over the past few years, offering a wide range of options such as embedding cells in extracellular matrices (natural and synthetic), printing 3D scaffolds, 3D bio printing of biocompatible materials and cells, decellularization/recellularization of tissues and whole organs and also the use of more sophisticated microfluidics technology (Laronda et al. 2015, 2017). Choosing the promising way of follicle encapsulation in extracellular matrices, imposes physiological requirements according to the class of follicles to be cultured. For example, primordial and primary follicles take great advantage from biomaterials with different physical properties (e.g.: stiffness, structure and composition, porosity) compared to secondary follicles. In a primate model, the early preantral follicles required a rigid environment for optimal survival and growth in vitro, corresponding better to the characteristics of the natural ovarian cortex areas containing these classes of follicles (Hornick et al. 2012). Over the last decade, bioengineering provided many alternatives for composing ECMs with different characteristics in order to meet the requirements of the cells to culture. Such an example is the 3D printing of biomaterials and cells into complex functional structures and tissues, as a result of pluridisciplinary work involving cell biology, medicine, physics alongside bioengineering and biomaterial science. (Murphy & Atala 2014). The 3D bio printing has been applied in regenerative medicine, due to its great potential in in vitro manufacturing tissues and organs (Bajaj et al. 2014) aiming to eliminate the shortage of organs suitable for transplantation. A more physiological alternative is represented by the use of natural scaffolds obtained by separation of the extracellular matrix (ECM) from its residing cells (whole organ decellularization), which can preserve the original constitution and architecture of the tissue or organ (Gilpin & Yang 2017). Laronda et al. (2015), used this technique and successfully decellularized a mouse ovary and then re-cellularized it with granulosa, theca and germ cells. These transplanted ovaries induced puberty in sterilized mice (Laronda et al. 2015). This research is now continued by using 3D printed scaffolds, which would support the maintenance of the spherical follicle structure and of the gamete-somatic cells connections, for oocyte growth and maturation (Laronda et al. 2017). The 3D print microporous hydrogel scaffold was designed to meet the requirements of follicle growth and expansion with defined rigidity, advancing angles (impacting on survival rates) and porosity (allowing vascularization and ovulation). Following transplantation to sterilized mice of the follicle-seeded scaffolds, the ovarian function was fully regained and fertility restoration was proved by live birth of healthy pups after natural mating (Laronda et al. 2017). Cells and tissue culture can benefit from using micro- and nano-fluidics technologies as well, as these allow configuration of different elaborated systems, which could integrate, automate and synchronize specific steps of the process, in accordance with the physiologic needs of the cells. As an illustration in our field, Xiao et al. (2017) have taken these technologies to the next step in exploring the tissue–tissue interactions in the context of female reproductive system (Xiao et al. 2017). They have integrated tissues from the female reproductive tract and peripheral organs (mouse ovary and human fallopian tube, ectocervix, liver) into a microfluidic culture system (Evatar) and successfully recapitulated the human 28-day menstrual cycle in vitro (Xiao et al. 2017).
The final step of an in vitro-grown follicle: inducing meiosis in vitro

IVM has been successfully used in animal models, e.g. porcine (Hirao et al. 1994) and bovine (Van den Hurk et al. 2000, Hirao et al. 2013, Dieci et al. 2016), where large amounts of COCs can be obtained from small and mid-size antral follicles of slaughterhouse ovaries and maturation rates are relatively high (Pavlov et al. 1992, Merton et al. 2003). In these species, IVM has become the preferred method for livestock breeding, embryonic stem cell technologies, cloning and transgenic animal production. IVM was introduced by Trounson et al. (1994) as an assisted reproduction technique (ART) for women with PCOS because it reduces gonadotrophin-induced ovarian hyperstimulation risk, but it could represent a valuable technique for fertility preservation, together with or as an alternative to follicle culture. Since its establishment as a clinical practice, IVM has been characterized by incongruent outcomes in part due to intrinsic low developmental competence of immature oocytes retrieved from small and mid-size antral follicles, in part attributed to the heterogeneity of protocols and technical approaches across fertility centers (Son & Tan 2010). The IVM methodologies currently available target the cohort of follicles between 6 and 12 mm, and most clinicians opt for injecting human chorionic gonadotropin (HCG) in order to improve COCs recovery rate and to obtain germinal vesicle (GV) oocytes of a sufficiently progressed developmental competence (Son et al. 2005). However, the diversity in follicle sizes present at the moment of HCG injection results in the retrieval of oocytes at diverse maturation stages, with consequent complications in fertilization procedures for the lab (Son et al. 2008, Son & Tan 2010) and outcomes interpretation. Additionally, the HCG trigger may lead to increased apoptosis incidence in those follicles that have not yet a fully upregulated aromatase complex (Hillier 1993). Literature review on clinical IVM in PCOS patients concluded that implantation rates per COC retrieved from 10 to 12 mm follicles post-HCG trigger can vary from 5.5 to 34.5% (median 10.9%) (Son & Tan 2010). Therefore, due to the large variability in outcomes, constraints in the embryology laboratory and lower efficiency in ongoing pregnancy rates (OPRs) compared to IVF (Walls et al. 2015), IVM has not become a widely adopted ART. Nevertheless, a few IVF teams recognized that with modifications in the practice, IVM could have a future. A potential strategy for improving IVM applicability and outcomes consists in retrieving homogeneous pools of COCs at smaller diameters, to avoid being confronted with an excessive progression of inter-follicular selection (Gougeon 1986). In order to obtain COCs of an equal (GV) maturation stage for culture, is necessary to omit the HCG injection before oocyte retrieval (De Vos et al. 2011, Walls et al. 2015). This therapeutic choice preferentially leads to the retrieval of unexpanded COCs from follicles of significantly smaller diameters (2–8 mm). Sánchez et al. (2015) indicated that only half of the COCs retrieved under this condition have a condensed chromatin configuration (peri-nucleolar chromatin rim) and is transcriptionally silent, major features associated with acquisition of meiotic and developmental competencies in different animal species (Zuccotti et al. 2002, Lodde et al. 2007, Tan et al. 2009, Luciano et al. 2012, Labrecque et al. 2015). Consequently, maximally 50% of the retrieved oocytes from 2 to 8 mm follicles has the potential to extrude the first polar body (PB) after 30 h and yielded, with this protocol, 41% cumulative OPR per IVM cycle (unpublished results UZ Brussel). Comparable results were previously reported in a retrospective case-controlled study from Perth with a similar COC retrieval strategy in PCOS patients (Walls et al. 2015). Latter study also showed that the number of usable blastocysts formed in IVM was only 65% of what regular intracytoplasmic sperm injection (ICSI) obtained. The clinical data indicate that the major challenge within the IVM systems is to overcome the asynchrony in oocyte maturity (nuclear and cytoplasmic) of the COC from 2 to 8 mm follicles, before exposing them to a positive meiotic trigger. Indeed, removing oocytes from mid-size antral follicles leads to a ‘precocious’ re-initiation of nuclear maturation, which disturbs the ‘capacitation’ process during which oocytes acquire the cytoplasmic machinery that supports preimplantation development (Hyttel et al. 1997, Dieleman et al. 2002, Gilchrist & Thompson 2007). Hence, premature resumption of meiosis must be prohibited, while synchronization of nuclear and cytoplasmic maturation should be favored. In order to achieve this goal, a pre-maturation step during which meiotic arrest is imposed via modulation of the cyclic adenosine monophosphate (cAMP) signaling pathway has been introduced before the IVM culture in animal and human models. This approach was the base of the simulated physiological oocyte maturation (SPOM) system (Albuz et al. 2010). Applying the SPOM protocol, IVM was extended by 6 h in bovine and 4 h in mouse and COCs were firstly treated with 3-isobutyl-1-methylxanthine (IBMX) during pre-maturation and then cultured in the presence of an oocyte-specific phosphodiesterase inhibitor, while simultaneously induced to mature with FSH to mimic oocyte maturation in vivo (Albuz et al. 2010). The combination of pre-maturation in the presence of IBMX and IVM culture increased blastocyst yield in bovine (Luciano et al. 1999, Albuz et al. 2010) and Day 3 human embryos generated from COCs exposed to IBMX during the collection phase preceding IVM, did not show increased incidence of chromosomal abnormalities compared with regular IVF embryos (Spits et al. 2015). However, maturation rate was nearly 50%, as observed in the IVM systems without hCG trigger (UZ Brussel, unpublished data). In the same perspective, prior research made use
of specific phosphodiesterase type 3 inhibitors (PDE3-I, cilostamide, Org 9935) to maintain human oocytes under temporary meiotic arrest in vitro and promote developmental competence (Nogueira et al. 2003, Shu et al. 2008, Vanhoutte et al. 2008, 2009). Observing the COC during the pre-maturation phase, it was concluded that the biggest challenges in pre-maturation setups were to maintain a functional connection between oocytes and cumulus cells and then to relieve the induced meiotic arrest by a positive meiotic stimulus, as is the case in vivo. The transzonal projections (TZPs) mediate the accumulation of transcripts and proteins into the ooplasm (Macaulay et al. 2014, 2016), which is crucial for oocyte competence acquisition (Gilchrist et al. 2008, Luciano et al. 2011, Lodde et al. 2013).

Abundant work in several animal models (Zhang et al. 2011, Franciosi et al. 2014, Wei et al. 2015, Zhang et al. 2015a,b, Zhong et al. 2016), inspired by initial work from the Eppig-Lab and the Conti-Lab, showed that CNP can be used during pre-maturation culture (PMC) to maintain immature oocytes under meiotic arrest. An overview of the main approaches taken to prevent meiosis resumption in GV oocytes during PMC culture is presented in Fig. 4. CNP is known as the natural oocyte maturation inhibitor present in the follicle (Zhang et al. 2010, Kawamura et al. 2011, Tsuji et al. 2012). A schematic representation of the mechanism of action of CNP in maintaining the meiotic arrest, compared with SPOM is showed in Fig. 5. Remarkably, a PMC combining CNP with E2, FSH and growth differentiation factor 9 (GDF9) enhanced maturation, developmental potential of low competence COCs obtained from unstimulated prepubertal mice, as evidenced by the improved blastocyst yield (Romero et al. 2016) and differently from cilostamide, preserved the integrity of TZPs (Fig. 6A and B). In a recent study involving sibling oocytes from a group of 15 infertile women with PCOS and addressed to prospectively evaluate the effects of a new IVM culture method on oocyte developmental competence, Sánchez et al. (2017) showed that PMC in the presence of CNP followed by IVM with FSH and amphiregulin (AREG) increases oocyte maturation potential compared to the clinical IVM protocol and leads to a higher availability of Day 3 embryos and good-quality blastocysts for single embryo transfer. Additionally, COCs preserved cumulus-oocyte connections after 24 or 46 h of PMC (Fig. 6C and D) and the blastocysts obtained after PMC+IVM did not show increased aneuploidy rates as compared to blastocysts from age-matched infertile patients from conventional ART (Sánchez et al. 2017).

In case a woman would need urgent chemotherapy or radiotherapy, which would put her fertility at risk, aspiration of the contents of the small (2–8 mm) or medium (9–12 mm) follicles present on default in the ovary, could provide valuable COC, without having to stimulate or operate her. In this perspective, improved

![Image](https://via.placeholder.com/150)
**Figure 5** Overview of the main pathway involved in oocyte meiotic arrest (based on Zhao et al. 2010) and different strategies developed for the optimization of in vitro maturation. Cyclic nucleotides cAMP and cGMP are crucial to the maintenance of meiotic arrest. High levels of cAMP activate protein kinase A (PKA) (Bornsøelager et al. 1986) that, via downstream effectors, inhibits the protein complex CDK1/cyclin B (or meiosis-promoting factor, MPF), a positive regulator of meiotic progression (Jones 2004). Cyclic AMP in oocytes is generated downstream of GPR3 and GPR12, regulators of G proteins controlling adenylyl cyclase (ADCY) (Edwards 1965; Mehlmann et al. 2004) while cGMP, originates in granulosa cells from the cumulus cell-specific guanylate cyclase NPR2 activity via CNP signaling. Before the LH-surge, cGMP is transferred via gap junctions to the oocyte where it inhibits PDE activity (Norris et al. 2009, Vaccari et al. 2009). E2, enhances CNP receptor signaling / coordinates NPR2 expression in cumulus cells and oocyte-secreted factors promote NPR2 mRNA accumulation in cumulus cells (Zhang et al. 2010). After the LH-surge PDE, an oocyte-specific phosphodiesterase PDE3A, becomes activated to decrease cAMP levels in oocytes and initiates pathways governing meiotic resumption (Richard et al. 2001). When COCs are aspirated from the follicles, CNP signaling pathway is interrupted. Inability to sustain oocyte cAMP levels leads to precocious resumption of meiosis and loss of synchrony between oocyte maturation and ovulation. Therefore, the granulosa cell ligand CNP and its receptor NPR2 in cumulus cells prevent precocious meiotic maturation, which is critical for maturation and ovulation synchrony and for normal female fertility. First pre-IVM systems (Nogueira et al. 2003, Vanhoutte et al. 2008, 2009) invoked pharmacological control of meiosis arrest (PDE inhibitors, cistostamide, milrinone). In 2010, a novel in vitro SPOM system was proposed by Gilchrist’s lab. This IVM system, as described by Albuz et al. (2010) entailed a pre-IVM phase of 1–2 h in presence of IBMX and forskolin to elevate COC/oocyte cAMP and an IVM phase containing a type-3 PDE inhibitor with simultaneous hormonal-induced oocyte maturation and extended IVM interval. ADCY, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; CC, cumulus cells; cGMP, cyclic guanosine monophosphate; CNP, C-type natriuretic peptide; E2, estradiol; GC, granulosa cells; GPR 3/12, G-Protein-Coupled Receptor 3/12; Gs, G-proteins; GTP, Guanosine triphosphate; IBMX, 3-Isobutyl-1-methylxanthine; NPR2, natriuretic peptide receptor 2; OSFs, oocyte-secreted factors; PDE, phosphodiesterase; PDEi, phosphodiesterase inhibitor; SPOM, Simulated Physiological Oocyte Maturation.

**Conclusions**

In vitro follicle growth is an attractive strategy for fertility preservation as it does not require hormone stimulation of the patient, is suitable for both reproductive-age women and prepubertal girls and carries a very low risk of reintroducing cancer cells into the patient. The most successful follicle culture strategy to date in humans, which produced meiotically mature oocytes from in vitro-cultured follicles implies embedding preantral follicles in a matrix. In order to optimize the activation step of primordial follicles and after that the yield in meiotically competent oocytes from cultured tissue, a stage-dependent modulation of the matrix composition and the provision of stage-dependent biochemical growth factor supplementation will require more consideration. Access to recently characterized factors secreted by the oocyte in purified form, to purified ECM substances, which can be bioprinted, together with the assays for biomarkers which could indicate the culture needs throughout the long in vitro growth period, could improve quantity and quality in oocyte output.
In vitro growth and maturation of oocytes occurs in a very dynamic and transient fashion. Work on growth (IVG) of oocytes has shown that the development of oocytes, the transient surge in follicular somatic cell cAMP is important for regulating oocyte maturation in our understanding of the fundamental mechanisms of physiological culture systems able to support in a coordinated fashion the final development of oocytes according to follicles size, is mandatory to improve IVM outcomes and extend IVM clinical applicability.

Many advances have been made over the last 15 years in our understanding of the fundamental mechanisms regulating oocyte maturation in vivo and in vitro. Work from several researchers clarified that CNP and cGMP are responsible for cAMP-mediated meiotic arrest and how the ovulatory gonadotrophin surge reverses these processes. In the Graafian follicle in vivo, the transient surge in follicular somatic cell cAMP is important for subsequent oocyte developmental competence, as some IVM systems in routine would not recapitulate this cAMP surge in vitro, leading to the lower efficiency of IVM compared to clinical IVF. Next-generation IVM systems are in preclinical testing. These systems aim to (1) first prevent spontaneous meiotic resumption at oocyte collection using either phosphodiesterase inhibitors or CNP, subsequently (2) artificially increase the cAMP levels in COCs and/or finally (3) induce meiotic resumption using FSH, LH or EGF-like peptides. Such more sophisticated IVM systems typically lead to increased numbers of MI1 oocytes and to improvements of blastocyst yield. This research has implications for improving the efficacy and clinical uptake of clinical IVM and fertility preservation. Additional refinements of the culture conditions including, for instance, oocyte-secreted factors (OSFs) or cytokines supplementation and appropriate scaffolds for 3D culture to replace the complex environment of the maturing oocyte, are expected to further improve current IVM outcomes. The coupling of small-follicle IVM technologies after tissue transplantation or in vitro growth (IVG) of follicle strategies might ultimately improve pregnancy outcomes for cancer patients. The IVM technology would also avoid the costly hormonal stimulation phase, by retrieving COCs from small antral follicles for in vitro culture. The technology would simplify treatment for infertile women and decrease considerably cost, risk and burden and would open access to treatment also in the less economical advanced regions of our world.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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