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 (Telfer *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 mechanism(s) 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 TGF α 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, Smitz 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 (picolinate) 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, deoxyribonuclease) 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 matrixes 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, Telfer *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 cavitory 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 (bpV(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

follicles in both animal (Zhang *et al.* 2011, 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.

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,

Figure 1 *In vitro* follicular activation and initial growth. Increased mTORC1 activity in p-GCs leads to upregulation of KITL production, which binds its KIT/tyrosine kinase receptor on the oocyte (Zhang *et al.* 2014) and triggers the formation and activation the PI3K complex. This stimulates conversion of PIP2 to PIP3, which in high levels drives the nuclear translocation and phosphorylation of AKT. Activated AKT triggers nuclear export and suppression of FOXO3 transcription factor activity, to promote follicle activation (Reddy *et al.* 2009; Sánchez & Smitz, 2012). Deletion of the *Foxo3a* in female mice led to infertility due to global follicular activation (Castrillon 2003). PTEN acts as a regulator of PI3K by converting PIP3 into PIP2 (Simpson & Parsons 2001, Cantley 2002). Its absence in the oocytes of a knock-out mouse upregulated PI3K activity, triggering premature activation and primary ovarian failure as seen in the *Foxo3*-null mutant ovaries phenotype (John *et al.* 2008, Reddy *et al.* 2008). AKT induces cell growth and proliferation via mTOR activity. mTOR, a serine/threonine kinase, is the catalytic subunit of two distinct multiprotein complexes, mTORC1 and mTORC2. AKT induces activation of mTORC1 by inhibiting TSC2, a suppressor of mTOR (Adhikari & Liu 2010). mTORC1 regulates protein translation by phosphorylation of downstream molecules, 4E-BP1 (not shown) and S6K1, which triggers activation of rpS6 (Reddy *et al.* 2009). Deletion of mTOR-negative regulators *Tsc1* or *Tsc2* from mouse oocytes, leads to primary ovarian failure (POF) due to increased mTORC1 activity in the oocytes triggering follicular over-activation and depletion (Adhikari *et al.* 2009, 2010). 4E-BP1, eukaryotic translation initiation factor 4E binding protein 1; AKT, Protein Kinase B; FOXO3/FOXO3a, Forkhead box O3; KITL, KIT Ligand; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2; p-GCs, pre-granulosa cells; PI3K, Phosphatidylinositol-4,5-bisphosphate 3-kinase; PIP2, Phosphatidylinositol (4,5)-bisphosphate; PIP3, phosphatidylinositol (3,4,5) triphosphate; POF, primary ovarian failure; PTEN, phosphatase and tensin homolog deleted on chromosome 10; rpS6, ribosomal protein S6; S6K1, ribosomal protein S6 kinase beta-1; TSC1, Tuberous sclerosis protein 1 (tuberin); TSC2, Tuberous sclerosis protein 2 (hamartin).

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 ovariectomized 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 multiprotein complexes, mTOR complex 1 (mTORC1) and 2 (mTORC2) (Laplante & 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 prepubertal 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 primordial 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

Figure 2 The Hippo pathway disruption (based on Hsueh *et al.*, 2015). It plays an essential role in maintaining normal organ size and comprises a series of negative growth regulators that trigger phosphorylation and inactivation of the key signaling effectors, YAP/TAZ. Actin polymerization (conversion of G-actin to F-actin) following tissue fragmentation is positively correlated with Hippo signaling disruption (Kawamura *et al.*, 2013). A decline in YAP phosphorylation leads to increase in YAP nuclear levels. Nuclear YAP works together with TEAD transcriptional factors to upregulate the expression of BIRC apoptosis inhibitors and CCN growth factors (Pan 2007), which support cell growth, survival and proliferation (Holbourn *et al.* 2008). BIRC apoptosis inhibitors: Baculoviral inhibitors of apoptosis repeat-containing proteins; CCN growth factors: acronym derived from three of its members: CCN1 (cysteine-rich angiogenic protein 61 or), CCN2 (connective tissue growth factor) and CCN3 (nephroblastoma overexpressed); F-actin, filamentous; G-actin, globular actin; LATS1/2, mammalian homologs of *Drosophila* Warts; MOB1A/B, mammalian homologs of *Drosophila* Mats; MST1/2, mammalian ; homologs of *Drosophila* Hippo; SAV1, homolog of *Drosophila* Salvador; TAZ, transcriptional coactivator with PDZ-binding motif; TEAD transcriptional factors: Transcription factors containing the TEA/ATTS DNA binding domain; YAP, Yes-associated protein.

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.* 2015, Zhai *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

Figure 3 PI3K/AKT/mTOR pathway and Hippo pathway disruption in ovarian failure patients. *In vitro* treatment of ovarian cortical pieces with PI3K activators/PTEN inhibitors, induces primordial follicle awakening via PI3K/AKT/mTOR signaling in the oocyte and supports 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 (Smitz *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 matrixes and biomaterials that could imitate the ovarian physiologic milieu for optimal follicle development.

Several extracellular matrixes 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 matrixes (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 matrixes, 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 (Pavlok *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, Vanhoute *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 shown 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

Figure 4 Capacitation of the oocyte and pre-maturation strategies. Not all the oocytes are competent to develop into embryos. Removal of the cumulus-oocyte-complex from the follicle results in spontaneous meiotic resumption with consequent induction of major asynchrony between nuclear and cytoplasmic maturation. A 'pre-maturation' culture strategy, with the aim to improve oocyte competence and *in vitro* maturation (IVM) efficiency, is illustrated here. Meiotic arrest can be achieved by using pharmacological agents during pre-maturation to provide oocytes with sufficient time to synchronize the maturation of the nucleus and cytoplasm. Making use of cAMP analogs or modifiers of PDEs and/or adenylate cyclase activity to delay or inhibit germinal vesicle break down, resulted in enhanced maturation potential and increased blastocyst. The concept of oocyte 'capacitation' initially proposed by Hyttel *et al.* (1997) is indicating a potential that the oocyte should acquire during the growth phase and that includes modulations of organelles, ultrastructural modifications and transcription which allow the oocyte to attain meiotic and full developmental competence. This feature was not totally preserved by the pharmacological approach but it has been achieved by supplying CNP (Zhang *et al.* 2010), during the pre-maturation culture (Franciosi *et al.* 2014, Sánchez *et al.* 2017). The figure shows the image of a human COCs just after the pick-up and at the end of 24 h pre-maturation in presence of CNP. A functional communication between oocyte and somatic cells during the 'capacitation' period offers a window to intervene with different factors as cumulus cells regulators, OSFs (GDF9 and BMP15 in first option), nutrients and metabolites needed during later development, bridging in few hours a process that would otherwise require several days. BMP15, bone morphogenetic protein 15; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; CNP, C-type natriuretic peptide; FGFs, fibroblast growth factors; GDF9, growth differentiation factor 9; GV, germinal vesicle; GVBD, germinal vesicle break down; PDEi, phosphodiesterase inhibitor.

IVM technologies may represent a valid alternative to follicle culture applications within IVF context. The proof of concept has already been provided (Segers *et al.* 2015). By further improving the understanding of gametogenesis and consequently selecting the most appropriate culture system per maturation stage/follicle size, the burden related with ART could realistically be shifted from treating the patient(s) to treating her gamete(s) into the clinical embryology lab (Fig. 7).

Figure 5 Overview of the main pathway involved in oocyte meiotic arrest (based on Zhang *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) (Bornslaeger *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 Gs proteins controlling adenyl cyclase (ADCY) (Edwards 1965b, 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, Vanhoute *et al.* 2008, 2009) invoked pharmacological control of meiosis arrest (PDE inhibitors, cilostamide, milnirone). 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, adenyl 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

Figure 6 Immunofluorescent labeling of representative mouse (upper panel) and human (lower panel) cumulus oocyte complexes after pre-maturation culture. Cumulus-oocyte regulation is exerted via transzonal projections (TZPs), specialized extensions of cumulus cells and gap junctions, therefore the maintenance of TZPs integrity is a key factor for the acquisition of oocyte developmental competence (Albertini *et al.* 2001). F-actin staining (actin green) labels the cytoskeleton of cumulus cells and oocytes and allows the visualization of well-preserved TZPs at the oocyte-cumulus cell interface (A, C, D white arrows). Red arrows indicate the areas in which the contact between cumulus cells and oocyte is lost (B). In panels A (CNP, 48 h) and B (cilostamide, 48 h) two different approaches for keeping the meiotic arrest are compared in the mouse model. Differently from cilostamide, CNP preserves TZPs continuity around the oocyte (white arrows). Bottom panels depict surrounded human oocytes after 24 h (C) and 46 h (D) pre-maturation culture (PMC) in presence of CNP. Original magnification, mouse COCs (A, B) $\times 40$, zoom 2.0. Scale bar: 20 μm ; human COCs (C, D) $\times 40$, zoom 2.5. Scale bar: 50 μm .

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.

Figure 7 Selection of the proper culture system to support low competence oocytes deriving from small antral follicles based on the dynamic view of the follicle growth within the human ovary (modified from Gougeon (1986), permission asked, pending). By integrating 'morphometrical' and endocrinological data, as well as biological effects of different molecules synthesized by the human follicle, Gougeon proposed in 1986 a detailed model of follicle growth within the human ovary. Folliculogenesis starts when resting follicles enter the growth phase. Several months are required for a new growing follicle to reach the preantral stage (0.15 mm), then 70 additional days to reach the size of 2 mm. Early growing follicle growth is regulated by interactions between FSH and local factors produced by theca, granulosa cells and oocyte. In the late luteal phase, follicles reaching 2 mm diameter, enter the selection stage and become sensitive to cyclic changes of FSH (Gougeon stage V). When compared to the entire oogenesis, maturation *in vivo* occurs in a very short temporal window, yet substantial processes as regulation of inhibition/induction of meiosis, physical and biochemical interaction with cumulus cells, chromatin condensation, organelle redistribution and cytoskeletal changes have to take place before the oocyte can reach full developmental competence in a follicle of minimally 17 mm diameter. Differently from IVF whose target are pre-ovulatory follicle(s) (>15 mm) which underwent already selection and *in vivo* maturation, IVM recruits oocytes from small antral follicles, just after the selection window. The oocyte is apparently able to recapitulate the same process *in vitro*, however some steps may occur abnormally, compromising oocyte quality/viability and maturation outcome. The majority of standard IVM systems target follicles between 6 and 12 mm. CAPA IVM technology introduces a pre-maturation step that enables low developmental competence oocytes retrieved from follicles between 2 and 8 mm to develop into viable excellent quality blastocysts (Sánchez *et al.* 2017). The development 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 MII 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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