Int. J. Dev. Biol. 59: 211-216 (2015)
doi: 10.1387/ijdb.150172mz

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

3D culture of ovarian follicles: a system towards their engineering?

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ABSTRACT Infertility in women is a health priority. Designing a robust culture protocol capable of attaining complete follicle growth is an exciting challenge, for its potential clinical applications, but also as a model to observe and closely study the sequence of molecular events that lie behind the intricate relationship existing between the oocyte and surrounding follicle cells. Here, we describe the procedures used to maintain the ovarian follicle 3D architecture employing a variety of *in vitro* systems and several types of matrices. Collagen and alginate are the matrices that led to better results, including proof-of-concept of full-term development. Pioneer in its kind, these studies underlie the drawbacks encountered and the need for a culture system that allows more quantitative analyses and predictions, projecting the culture of the ovarian follicle into the realm of tissue engineering.

KEY WORDS: *Ovarian follicle, 3D culture, matrix, collagen, alginate, tissue engineering*

Introduction

Women infertility is a health priority that has several causes including X-chromosome abnormalities, autosomal genetic pathologies, environmental hazards, bilateral ovariectomy, and, above all, it may be the result of cytotoxic therapies required for malignant and autoimmune diseases. Impressively, more than one-third of women (including children, teens and young women) diagnosed with cancer and exposed to chemo/radio therapy are under the risk of developing ovarian failure, sterility and early menopause as common alongside effects (Anchan and Ginsburg, 2010). In numbers, this translates into a ~ 100.000/year or ~ 1.000.000/year women patients that, in countries like Italy or in the USA respectively, are treated for cancer and may develop infertility. Chemotherapy and radiation irreversibly damage the ovary inducing stromal fibrosis, loss of early follicles resulting in premature ovarian failure. Improvements in the diagnosis and treatment of childhood, adolescent and adult tumours has led to a marked amelioration of the recovery and survival rates, raising the need for developing strategies of fertility preservation that precede the therapeutic intervention and that neither delay nor interfere with the protocol required for the patient's survival.

An increasing number of women are offered the option of cryopreserving their ovarian tissue prior to the therapeutic treatment, although, at this time, this opportunity envisages the transplantation of the frozen-thawed tissue, a complex protocol that is not appropriate to all patients and that may lead to the reintroduction of cancer cells, as for patients diagnosed with leukemia (Meirow *et al.,* 2008).

Considering the important improvements that have been made in the development of *in vitro* protocols of follicle maturation, the culture of ovarian follicles isolated from stored ovarian tissue may become a valid alternative. Thus, designing a robust culture pro-

Abbreviations used in this paper: 3D, three dimension.

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Accepted: 28 May 2015.

ISSN: Online 1696-3547, Print 0214-6282

tocol capable of attaining complete follicle growth, ideally from the tiny primordial to the fully-grown follicle, is an exciting challenge for its potential clinical applications. Also, a culture system able to maintain the three-dimensional (3D) follicle architecture represents a model to observe and closely study the sequence of molecular events that lay behind the intricate biochemical and biophysical relationship existing between oocyte and surrounding follicle cells, an integrity of connections that is necessary during follicle growth to the acquisition of the oocyte developmental competence (Li and Albertini, 2013).

The main culture procedures adopted envisage the growth of follicles either with their somatic component adhering to the bottom of the Petri dish or embedded within a supporting scaffold (i.e., matrix) or a sequential combination of the two. When follicles are cultured in the absence of a matrix, although still partially keeping their 3D organisation, their somatic component tends to flatten and spread on the under surface. Whilst this latter procedure remains the most widely employed and has a history of success, several other culture systems that use matrices throughout the culture period have been experimented with promising results.

In this short review, we describe the main matrix culture systems designed to maintain the 3D architecture of the ovarian follicle, the principal results obtained and drawbacks encountered. Also, we underlay the need, at this stage, for a culture system that allows more quantitative analyses and predictions, an objective that could be achieved through a multidisciplinary approach.

Follicle growth in matrix culture systems

Introducing a third dimension in a cell culture procedure requires the design of scaffolds for supporting the organisation of the tissue that cells are contributing to build up and, furthermore, a complex that regulates the exchange of nutrients and waste products. A variety of culture setups have been developed aimed at maintaining the architecture of the follicle, with the somatic component growing in all directions around the oocyte. Most of these systems imply the use of a biomaterials to create a matrix in which the follicle is either embedded or encapsulated and sustained throughout its growth (Fig. 1). Different biomaterials, either natural or synthetic, have been tested, each with advantages and drawbacks.

Natural matrices have the advantage of being highly biocompatible and bioactive (i.e., they enter into a functional contact with cells in culture), but have the disadvantage that their mechanical and physical properties are difficult to model and present a high variability between batches. On the contrary, synthetic matrices are more standard in composition and can be designed with the desired properties to meet each specific demand, although the apparent lack of bioactivity restricts their use to mere scaffolds for cell culture. Two are the main natural matrices tested, collagen and alginate.

Collagen is a natural component of the extracellular matrix (ECM), very flexible and elastic, involved in many cellular processes and, historically, the first experimented biomaterial. Several culture protocols have been developed using either a layer of sole collagen matrix, a collagen matrix enriched with ECM proteins (i.e., fibronectin and laminin) and RGD (arginine-glycine-aspartate) sequences, or collagen microdrops. Since its first use in the late '80s, collagen was proved capable of sustaining follicle growth, follicle cells proliferation and oocyte maturation in both 2D (Eppig and Schroeder, 1989) and 3D (Torrance *et al.,* 1989) culture protocols.

Torrance and co-workers first introduced the idea of maintaining the 3D architecture during follicle culture: two-layers preantral follicles, isolated from 10-day-old mice, were embedded in a collagen-gel solution and cultured for fourteen days until they developed to a multilaminar complex, although without an antrum (Torrance *et al.,* 1989). An improvement was achieved by transferring preantral oocytes surrounded by two-three layers of granulosa cells onto a film of collagen already polymerised and covered with a second coating of collagen solution (Gomes *et al.,* 1999). When the latter polymerised, the follicles were cultured for further six days. Compared to a bi-dimensional culture that developed into a flattened follicle with distorted morphology, follicles cultured in collagen grew preserving a better architecture, more homogenous equatorial diameter and spherical shape, a basal lamina integrity and showed a reduced follicle cells loss.

Using the same embedding procedure, a further refinement was obtained using a two-steps culture protocol for both murine and human immature small antral follicles (Vanhoutte *et al.,* 2009). Initially, follicles were pre-matured in collagen for 24 hr in the presence of phosphodiesterase 3-inhibitor that prevented meiotic resumption and allowed completion of cytoplasmic maturation: then, they were matured with a standard *in vitro* maturation (IVM) protocol. At the end of this period, trans-zonal projections with active gap junctions were observed, suggesting the maintenance of a functional communication between oocyte and the somatic companion cells. These oocytes attained meiotic competence and, once fertilised, completed preimplantation development (*in vitro vs. in vivo* grown follicles mouse blastocysts, 43% *vs.* 70%; human blastocysts, 55.6% *vs.* 55.8%) (Vanhoutte *et al.,* 2009).

A turning point was reached using a three-step culture protocol that led to the birth of live mouse offspring (Mochida *et al.,* 2013). Primary and early secondary follicles isolated from 6-day-old mouse ovaries were first cultured for nine days embedded in a collagen gel, then they were moved on a collagen-coated membrane (Transwell-COL) and cultured for further eight days. Depending on the original size, 9.9% (60-70 μm in diameter), 33.5% (70-80 μm) and 61.6% (80-95 μm) follicles with an antral-like cavity were harvested on day seventeen of culture and matured to metaphase II (MII) in IVM medium (~18%). Fertilised MII oocytes reached the 4-cell stage (30.3%) and, when transferred to pseudo-pregnant females, two developed to term. Although the number of MII oocytes that reached full term was low, this study consolidates the concept that the culture of very early follicles can be successfully obtained in a 3D culture system.

The 3D culture of human follicles has been intermittently tested with interesting, although yet preliminary, outcomes. In a pilot morphological study, primordial/primary follicles, isolated from fresh or frozen–thawed ovarian tissue, were embedded in a collagen gel and cultured for 24 hr obtaining an increased number of somatic cells and a larger follicle size (Abir et at., 1999). In a further work, human follicle cell-free oocytes arrested at the germinal vesicle (GV) or metaphase I (MI) were embedded in a 3D collagen matrix together with their isolated follicle cells and matured until they reached the MII stage (Combelles *et al.,* 2005). While the maturation rate to MII was similar to that of control denuded oocytes, those co-cultured in the 3D system displayed increased MAPK activity, although identical maturation-promoting factor (MPF) levels (Combelles *et al.,* 2005).

A collagen-based demineralised bone matrix was used to grow follicle cells isolated from antral oocytes (> 70 μm in diameter) (Ma *et al.,* 2007). After 24 hr culture, the isolated oocytes were added to the scaffold and cultured for further 14 hr. The porous texture allowed cumulus cells proliferation and facilitated the diffusion of nutrients. In particular, the pattern of distribution of the cortical granules, the levels of MPF and the zona pellucida hardening were similar to those of *in vivo* matured oocytes. Moreover, when compared to control denuded oocytes matured in microdrops, good maturation (MII 93.4% ctrl *vs.* 93.3% in microdrops) and developmental (blastocysts 79.5% ctrl *vs.* 30.9% in microdrops) rates were obtained.

In summary, although these studies have achieved key results, including full-term development, a robust protocol that allows consistent outcomes is not yet available and three main drawbacks of the use of a collagen matrix have emerged. First, its preparation has not yet been standardised, since collagen is frequently prepared directly in the laboratory thus producing utmost variability. Second, the temperature, above 37° C, required to maintain collagen at the liquid phase at the time when follicles are embedded and, third, at the end of the culture period collagen is treated with collagenase to release the enclosed follicles, hence undertaking the risk of digesting also the endogenous collagen present in the basal lamina.

Alginate, another natural matrix tested, is a polysaccharide with properties of biocompatibility and permeability, which can be moulded into round shaped beads filled with cells. When the cell-alginate solution gets in contact with a calcium solution, beads are formed. Thus, single ovarian follicles can be encapsulated in alginate beads and grown in a 3D environment. To date, different groups have succeeded in culturing two- or multi-layered follicles of 100-130 μm and 150-180 μm in diameter, respectively, in a matrix of alginate alone (Pangas *et al.,* 2003; Heise *et al.,* 2005; Xu *et al.,* 2006a,b; West *et al.,* 2007; Parrish *et al.,* 2011; Tagler *et al.,* 2013) or combined with fibrin (Jin *et al.,* 2010; Shikanov *et al.,* 2009, 2011b) or ECM proteins (Kreeger *et al.,* 2006). These studies achieved important results, including the recording of the luteinisation and ovulation events (Skory *et al.*, 2015) and the birth of live and fertile mice (Xu *et al.,* 2006a). In this latter work, single multi-layered secondary follicles, isolated from 16-day-old female mice, were encapsulated into beads of 1.5% alginate and matured in 100 μl α-MEM for eight days until they reached the antral stage. The granulosa cells proliferated and actively secreted hormones such as estradiol, androstenedione and progesterone, which increased linearly during culture as expected for the *in vivo* counterpart. The resulting cumulus-oocyte-complexes were enzymatically released with alginate lyase, the antral oocytes denuded and further cultured for 16 hr until they reached the MII phase (70.9%). Upon *in vitro* fertilisation, 68.2% reached the 1-cell stage and, when twenty of these were transferred to pseudopregnant females, four reached full-term development. This work proves that encapsulation inside an alginate bead allows follicle growth in diameter and acquisition of morphological and functional features of antral follicles grown within the ovary. The oocytes however resume meiosis and acquire developmental competence with lower rates compared to controls. To address this problem, Mainigi and colleagues cultured early secondary follicles encapsulated into 0.25% alginate beads up to the antral stage (Mainigi *et al.,* 2011). They confirmed that the alginate culture sustains follicle growth and oocyte maturation, but oocytes were seriously compromised, with disorganised and misaligned meiotic spindles, a non-uniform distribution of cortical granules and undermined fertilisation and developmental competence.

In all the studies described above, oocytes were co-cultured with their companion cumulus cells, without the surrounding theca cells that enclose the ovarian follicle and, if present *in vitro*, would help the maintenance of a compact spherical shape. With this in mind, Sittadjody and colleagues encapsulated granulosa cells in single alginate beads that were then further coated with a layer of poly-L-ornithine and mixed with theca cells suspended in alginate and encapsulated again in alginate using a micro-fluidic device (Sit-

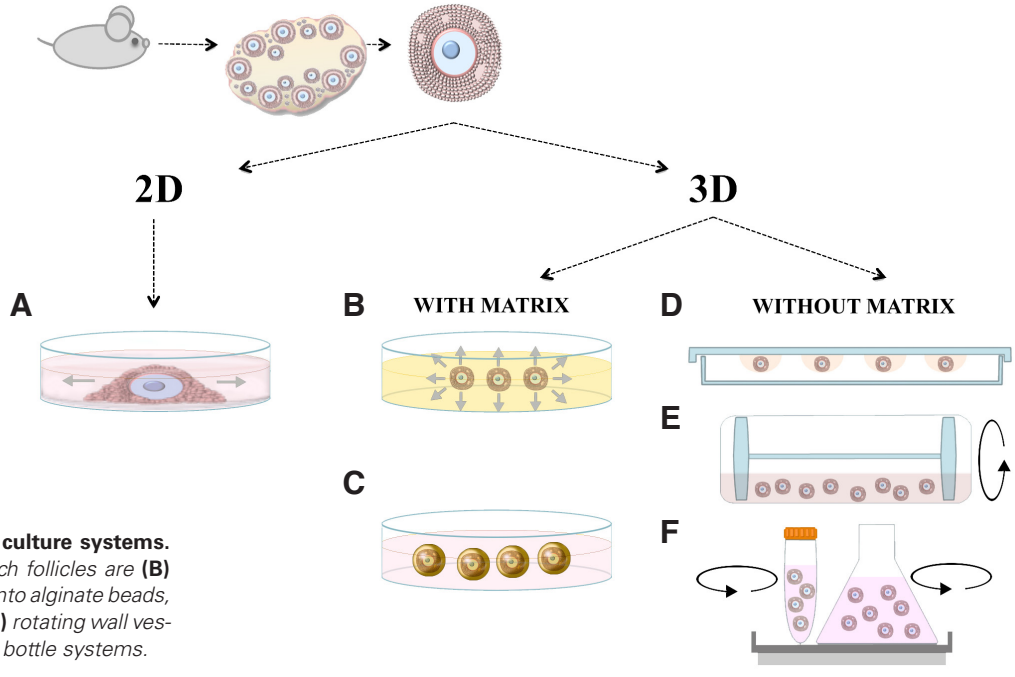

Fig. 1. Summary of the main follicle culture systems. **(A)** *2D culture;* **(B-F)** *3D culture in which follicles are* **(B)** *embedded in a gel matrix,* **(C)** *enclosed into alginate beads, suspended in* **(D)***hanging microdrops,* **(E)** *rotating wall vessels, and* **(F)** *orbiting test tubes or roller bottle systems.*

tadjody *et al.,* 2013). The encapsulated cells displayed endocrine functions during 30 days of culture secreting 17-beta-estradiol, activin and inhibin in response to FSH and LH.

Besides rodents, the alginate culture system has been tested in other species, including nonhuman primates and humans. *Rhesus* monkey secondary follicles were cultured encapsulated in alginate beads for up to 30 days maintaining their growth rate and steroidogenic function. Higher survival and growth rates were obtained by using 0.5-2% alginate concentration, higher compared to that used for the mouse, suggesting that different physical environments may be required for the growth of follicles from different species (Xu *et al.,* 2009a,b; Hornick *et al.,* 2012). Alginate hydrogels proved to sustain also the growth of human small preatral follicles maintaining their viability (Amorim *et al.,* 2009) and steroidogenic function (Xu *et al.,* 2009a).

Other biomaterials, both natural and synthetic, have been tested although none of them supported full-term development and are hereafter only briefly listed (for a more detailed review see Belli *et al.,* 2012) hyaluronic acid (Desai *et al.,* 2012), Matrigel (Hovatta *et al.,* 1997, 1999; Oktem and Oktay, 2007: Xu *et al.,* 2009a; Zhu *et al.,* 2012), agarose (Huanmin and Yong, 2000; Mousset-Simeón *et al.,* 2005; Fujihara *et al.,* 2012) and polyethylene glycol (Shikanov *et al.,* 2011a). In addition, a number of other techniques aimed at keeping the follicle 3D organization have been experimented, including hanging microdrops (Wycherley *et al.,* 2004; Nation and Selwood, 2009), orbiting test tubes (Rowghani *et al.*, 2004; Heise *et al.*, 2005, 2009), rotating wall vessels (Rowghani *et al.*, 2004 or roller bottle systems (Nation and Selwood, 2009) (Fig. 1), although with limited results.

Conclusions and future directions

The maintenance of the ovarian follicle 3D architecture, with its somatic epithelium surrounding the growing oocyte, has been attempted with a variety of procedures using several type of matrices with collagen and alginate being those most used and those that led to better results, including proof-of-concept of full-term development. Pioneer in its kind, these studies have projected the culture of the ovarian follicle into the realm of tissue engineering, a research field that has already acquired solid experimental principles and a systems biology view in the production of other tissues (Lanza *et al.*, 2007, Guilak *et al.*, 2014). To this regard, the future build out of a more robust 3D follicle culture protocol will necessarily need to take into account of those biophysical cues that, *in vitro*, act upon the follicle structure such as shear stress, compressive and tensional forces and the geometry of the culture microenvironment. Though never tested during follicle culture, these forces have been shown to be critical for the 3D organisation of other developmental processes and tissues, by inducing - *via* mechano-transduction - alterations to a specific cell status of force equilibrium (*tensegrity*) and triggering biochemical responses that lead to changes to the transcriptional profile and functional features (Wozniak and Chen, 2009; Miller and Davidson, 2013). A hurdle to the measure of these physical cues on ovarian follicles could be their small size, although, to this regard, significant technological improvements have been very recently made that allow their analysis even directly on the single cell. For example, a cantilever of an atomic force microscope has been used to quantitate the responses of a cultured cell to stretching and compressive forces locally applied (Watanabe-Nakayama *et al.,* 2011) or to study the hardness (indicator of tensegrity) of single bacteria (Nakanishi *et al.,* 2012). Also, a microfluidic mass sensor has been developed to measure the density (mass/volume) of single erythrocytes, allowing their selection into classes depending on their response to specific drug treatments (Grover *et al.*, 2011). The possibility of taking measurements of these physical cues combined with the analysis of markers of the follicle developmental competence, such as the expression of specific transcripts, transcriptional programmes and epigenetics requirements could help the establishment of a more robust culture system. To this end, the use of integrative bioinformatics tools has recently provided new insights into the transcriptional and epigenomics programmes that act behind oocyte maturation and the process of folliculogenesis. Whole-genome transcriptional profiles are already available for human, mouse and cow oocytes (Adjaye, 2005; Huang and Wells, 2010: Assou *et al.*, 2011; Diedrichs *et al.*, 2012; Labreque and Sirard, 2014) and cumulus cells (Assou *et al.*, 2008; van Montfoort *et al.*, 2008; Ouandaogo *et al.*, 2011; Vigone *et al.*, 2013; Fragouli *et al.*, 2014; Xu *et al.*, 2014). Also, the epigenetic signature (i.e., DNA methylation and histone acetylation and methylation) of the female gamete has been described either for specific genome sequences using the traditional bisulfite conversion and immunofluorescence assays (Endo *et al.,* 2005; Yeo *et al.,* 2005; Hou *et al.,* 2008; Kageyama *et al.,* 2007; Liu *et al.,* 2004; Racedo *et al.,* 2009; Zuccotti *et al.,* 2011; Denomme *et al.,* 2012; Qiao *et al.,* 2010; Pan *et al.,* 2012; De La Fuente *et al.,* 2004; Akiyama *et al.,* 2004; Spinaci *et al.,* 2004) or, for the whole genome, employing the reduced representation bisulfite sequencing (RRBS), a technique that couples the classical bisulfite conversion with the next generation sequencing technology (Smallwood *et al.,* 2011: Tomizawa *et al.,* 2012).

In summary, it is our belief that the future of *in vitro* 3D follicle culture lays in the cooperation of reproductive biologists with other research fields to build up a sound systems-biology approach towards a culture system that allows quantitative analyses and predictions. A more standardised culture protocol will allow to address, more precisely, issues that still remain open, such us the understanding of how oocytes and their surrounding cells are hierarchically organised and linked at both the cellular and molecular levels, what are the functional networks involved in this interaction, what is the role that mechano-transduction plays during follicle growth and how the follicle and its somatic and germ cell components react to the biophysical forces present in the culture system.

Acknowledgements

We thank the following organizations for support: MZ, FIL-University of Parma: SG and VM, FAR University of Pavia: WW and JA, the German Federal Ministry of Education and Research (BMBF grant number 0315717A) as partner of the ERASysBioPlus initiative supported under the EU ERA-NET Plus scheme in FP7 and Medical Faculty of Heinrich Heine University, Duesseldorf: FM and RB, Fondazione Cariplo (grant 2008–2006), the Italian Ministry of Health ITALBIONET project: LF, INAIL 2010 Grant.

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