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Towards a 3D culture of mouse ovarian follicles

MARTINA BELLI[1,#], GIULIA VIGONE[1,#], VALERIA MERICO[1], CARLO ALBERTO REDI[1], MAURIZIO ZUCCOTTI*[,2] and SILVIA GARAGNA*[,1,3,4]

[1]Laboratorio di Biologia dello Sviluppo, Dipartimento di Biologia e Biotecnologie 'Lazzaro Spallanzani', Università degli Studi di Pavia, Pavia, [2]Dipartimento di Scienze Biomediche, Biotecnologiche e Traslazionali, Università degli Studi di Parma, [3]Centro di Ingegneria Tissutale, Universita' degli Studi di Pavia, Pavia and [4]Centro di Eccellenza in Biologia Applicata, Università degli Studi di Pavia, Pavia, Italy.

ABSTRACT The ovarian follicle has a three-dimensional (3D) structure in which the oocyte is surrounded by tightly connected follicle cells that mediate the action of external signals and nourish the gamete during its maturation. Thus, the maintenance of follicle organization during the whole growth process is crucial for the correct acquisition of developmental competence. In recent years, much attention has been given to *in vitro* culture systems capable of maintaining follicle architecture. With the aim of providing a quick reference guide, in this review we will summarize the techniques developed for the 3D culture of mouse follicles.

KEY WORDS: *3D culture, ovarian follicle, follicle organization, follicle architecture, matrix*

Introduction

In the past few years important improvements have been achieved in the *in vitro* culture of mammalian ovarian follicles. Some laboratories have even been able to culture follicles from the primordial/primary stage up to their complete maturation, acquisition of fertilizability and developmental competence (Eppig and O'Brien 1996). As thoroughly described earlier (Desai *et al.,* 2010), despite the many upgrades suggested throughout the years, a single standard protocol has not yet been agreed, not even with a model species like the mouse. The difficulties and challenges lay in the peculiar features of the follicle: a small "organ" within the ovary that possesses a unique vascular system built up around a 3D structure in which the oocyte is surrounded by companion, tightly connected, follicle cells. The oocyte growth is strictly dependent on autocrine and paracrine bidirectional signaling, the latter through gap junctions and transzonal projections, between the germinal and the somatic components of the follicle (Matzuk *et al.,* 2002; Luciano *et al.,* 2011). Specifically, follicle cells mediate the action of external signals and nourish the oocyte during its maturation. Due to the key role of this cross talk, the maintenance of the follicle three-dimensionality during the whole growth process is crucial for the correct acquisition of the developmental competence.

Follicles have been cultured under 2D or 3D systems, both trying to reproduce the complex bidirectional stimuli (e.g., exchange of nutrients, soluble and insoluble signals and hormones) and both with advantages and drawbacks.

Historically, 2D systems were the first to be developed with remarkable results, including the growth of primary follicles to complete maturation, fertilizability and full developmental competence (Eppig and Schroeder 1989; Cortvrindt *et al.,* 1996; Eppig and O'Brien 1996). These methods comprise the culture in multi-well plates, microdrops, gel-coated dishes or membranes coated with extracellular matrix (ECM) proteins. Follicles are cultured on a 2D surface that only partly maintains the spatial configuration of the follicle, letting the follicle cells expand at the bottom of the dish, with the consequent partial loss of the oocyte-follicle cells interactions. For this reason, in recent years, much attention has moved to the use of matrices and culture systems capable of maintaining the follicle 3D architecture. Although this approach is still in its infancy and requires much improvement, some important goals have been achieved. The purpose of this review is to give state-of-the-art of 3D methods that have been developed for the culture of mouse follicles, with the aim of providing an essential reference guide to those who are approaching these techniques with this species.

Abbreviations used in this paper: 3D, three dimensional; ECM, extracellular matrix; HA, hyaluronic acid; PEG, polyethylene glycol; PVA, polyvinyl alcohol.

***Address correspondence to:** Maurizio Zuccotti. Dipartimento di Scienze Biomediche, Biotecnologiche e Traslazionali, Università degli Studi di Parma, Via Volturno 39, I-43125, Parma, Italy. e-mail: maurizio.zuccotti@unipr.it or Silvia Garagna. Laboratorio di Biologia dello Sviluppo, Dipartimento di Biologia e Biotecnologie 'Lazzaro Spallanzani', Università degli Studi di Pavia, Via Ferrata 9, I-27100, Pavia, Italy. e-mail: silvia.garagna@unipv.it

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TABLE 1

PROTOCOL FOR THE CULTURE OF MOUSE FOLLICLES IN A COLLAGEN MATRIX

Procedure	Notes
1. Matrix preparation Dilute a 1% collagen solution in PBS at 4°C in the maturation medium and adjust pH to 7.4. See notes for different collagen sources.	*Sources of collagen:* - in-lab extracted[1,2] or commercial[3] rat tail collagen (type I) (pure or diluted in PBS); - bovine dermal collagen[4]; - Costar Transwell-COL membrane inserts treated with equimolar mixture of Types I and III collagen from bovine placentae[5].
2. Follicle isolation Isolate primary follicles from a 8-11[1]/10[2]/12[5] day-old female in isolation medium (see notes) supplemented with collagenase[1,2,5]. Or puncture the ovary of a 24-28[4] or 49-56[3] day-old female with a sterile needle and collect preantral or antral follicles, respectively.	*Follicle isolation media:* - Leibovitz-glutamax medium with supplements and phosphodiesterase 3 inhibitor[3]; - Hepes-buffered medium M199 with supplements[1]; - Eagle's MEM with supplements, IBMX and FSH[5] - Eagle's MEM with Hank's salts and Hepes with supplements and FSH[4]; - M2 medium with FCS and dbcAMP[2].
3. Transfer of follicles into the matrix Using a sterile micropipette, transfer follicles (the number varies in different studies from 1 to 50[2-4]), with the minimum possible quantity of medium, in a multiwell-plate containing a 1% collagen solution. Incubate at 37°C for 10-20 min to induce gelatinization. Then, overlay with the appropriate follicle maturation medium (see point 4).	- To avoid follicle loss, some authors indicate the need for a double gel encapsulation[1,2]; - Double-layer collagen matrix: follicles may be deposited at the interface between a lower and an upper layer[4]; - Two-steps culture: follicles cultured in a collagen matrix followed by IVM in suspension[3].
4. Follicle maturation Depending on the follicle maturation stage, culture for 6-14 days at 37°C, 5% CO_2, 5% O_2, 90% N_2. Medium is changed every 1[3,5] -3[1,2] days.	*Follicle maturation media:* - α-MEM with glutamax and supplements[3]; - Bicarbonate-buffered medium M199 with supplements[1]; - Eagle's MEM with supplements[5] - α-MEM with supplements [4]; - MEM with FCS[2].
5. Removal of follicles from the matrix Replace the follicle maturation medium with the same medium containing collagenase I for 10 min at 37°C. If needed pipette gently to denude oocytes.	
6. Maturation to MII Transfer the matured follicles or denuded oocytes in maturation medium and incubate for 15 hr at 37°C, 5% CO_2, 5% O_2, 90% N_2.	- The protocol reported is routinely used in our laboratory for the culture of follicles and it is taken from references[6,7]. Briefly, 30-40 follicles are matured in pre-equilibrated 500 µl bicarbonate-buffered α-MEM supplemented with 50 mIU/ml FSH, 10 ng/ml EGF, antibiotic, 3 mg/ml BSA and 1 mg/ml fetuin, at 37°C, 5% CO_2 in air for 18 hr.
7. Fertilization Incubate cumulus cells-enclosed or cumulus cells-free MII oocytes in IVF medium containing 2x10[6] sperm under mineral oil at 37°C, 5% CO_2 for 2-3 hr; wash away sperm and transfer the oocytes in M16 medium[8].	- The fertilization protocol reported is routinely used in our laboratory, but other procedures may be adopted[9].
8. Preimplantation development Incubate the inseminated oocytes at 37°C, 5% CO_2 in air[8].	- The protocol for *in vitro* preimplantation development reported is routinely used in our laboratory, but other procedures may be adopted[9].

References: 1) Torrance *et al.,* 1989; 2) Carrol *et al.,* 1991; 3) Vanhoutte *et al.,* 2009; 4) Gomes *et al.,* 1999; 5) Eppig and Schroeder 1989; 6) Yeo *et al.,* 2008; 7) Albuz *et al.,* 2010; 8) Zuccotti *et al.,* 2002; 9) Nagy *et al.,* 2003.

Matrices

Most of the culture systems designed for 3D ovarian follicle growth imply the use of a matrix. Follicle encapsulation in such matrices allows a spherical growth of the follicle, with its surrounding follicle cells expanding in all directions. This system also aims at maintaining trophic factors, produced and released by granulosa cells, in the vicinity of the oocyte, thus avoiding their dispersion in the culture medium and conserving the nourishing role of these cells. In spite of the achievements obtained, it is not yet clear which is the biocompatible material best for its physical and chemical properties (e.g., toxicity, permeability, viscosity, elasticity, stiffness and handiness). There are several types of matrices, either natural (agar/agarose, alginate, hyaluronic acid (HA), collagen, fibrin, Matrigel) or synthetic [polyethylene glycol (PEG), polyvinyl alcohol (PVA), polylactic acid (PLA), polyglycolic acid (PGA)], that are commonly used. Natural polymers are more biocompatible and bioactive; synthetic polymers are standard in composition and predictable in their action/degradation. Among these, we detailed the use of collagen, alginate, HA, Matrigel and PEG matrices, giving, within a Table, a step-by-step summary of the methods that have produced the best results in terms of follicle growth, oocyte maturation and, when tested, fertilization and developmental competence. Specific references detailing the procedures are quoted within Tables 1-5.

Collagen matrix

Collagen is a biomolecule of the ECM with properties of flexibility and elasticity, organized in a triple helix structure, involved in several aspects of the cell biology, including cell attachment and adhesion. It is commonly used in tissue or cell culture as a gel/matrix and, recently, it has been employed in the culture and growth of mammalian ovarian follicles (Table 1) (Gomes *et al.,* 1999; Vanhoutte *et al.,* 2009; Sharma *et al.,* 2009). The mechanical properties of this biomaterial allow the maintenance of the 3D architecture of the encapsulated follicle, preventing cumulus cells migration and preserving both peri- and intrafollicular ECM compartments (Gomes *et al.,* 1999). Also, being a natural component of the ECM, the collagen matrix might favor intercellular communications compared to cultures in suspension or in adhesion on collagen-coated petri-dishes (Abakushina *et al.,* 2011). Another positive property of the collagen matrix is its transparency that allows the operator to observe, using a phase contrast inverted microscope, the follicle throughout its growing phases (Torrance *et al.,* 1989).

Three are the main protocols that have been developed and are still under testing: i) a collagen matrix, ii) a collagen matrix supplemented with ECM proteins such as fibronectin, laminin and RGD sequence (Arginine-Glycine-Aspartic Acid) and iii) collagen microdrops (mainly used in species other than the mouse; Sharma *et al.,* 2009). Table 1 gives a summary of the most critical steps of the procedures used for the preparation of this matrix, the culture of follicles, the fertilization and development of the eggs obtained.

The culture of mouse follicles in a collagen matrix dates back to the late '80s, when preantral follicles were grown, over a period of 14 days, from a unilaminar to a multilaminar structure (Torrance *et al.,* 1989). The need for a metabolic coupling between

follicle and granulosa cells was also evidenced in a study by Eppig and Schroeder (1989), in which preantral follicles (< two layers follicle cells) from 12-day-old mice were cultured on Costar Transwell-COL membrane inserts treated with an equimolar mixture of type I and III collagen from bovine placenta, with the addition in the culture medium of isobutylmethylxantine (IBMX; to block meiotic resumption) and follicle stimulanting hormone (FSH). The culture protocol applied was capable of maintaining a metabolic coupling with a minimal migration of the granulosa cells from the cumulus. These two studies and those that followed, represented the basis for further improvements in the mouse, but also in other species such as bovine (Abakushina *et al.,* 2011), buffalo (Sharma *et al.,* 2009) and human (Combelles *et al.,* 2005; Vanhoutte *et al.,* 2009). For example, supplementing the medium with molecules that inhibited/delayed meiotic resumption substantially improved follicle growth, oocyte maturation and developmental competence to blastocyst [e.g., dbcAMP (Carroll *et al.,* 1991) or phosphodiesterase 3 inhibitor (Vanhoutte *et al.,* 2009)]. Furthermore, the addition of FSH resulted in a substantial improvement of the preantral follicle culture (Gomes *et al.,* 1999). Some protocols envisage a two-step procedure consisting of a 3D prematuration culture in collagen matrix, followed by the transfer of cumulus-enclosed oocytes to an *in vitro* maturation medium (Vanhoutte *et al.,* 2009).

Whilst the advantages described above have encouraged the use of collagen matrices in follicle culture, a number of disadvantages have emerged. The preparation of a collagen scaffold has not yet been standardized and may vary from time to time and from lab to lab; also, when loading the gel and during its polymerization, follicles are submitted to a dramatic change in temperature that may cause damages; moreover, a critical step occurs when follicles are freed from the collagen for further culture or analyses. These drawbacks have prompted several groups to explore the use of an alginate matrix as an alternative (see below).

Calcium alginate matrix

Alginate is a linear polysaccharide, produced by brown algae, made of β-$_D$-mannuronic and α-$_L$-glucuronic acid units. Due to its biocompatibility, high affinity to water and handiness, alginate has been used for the culture of a variety of cell types, including human (Sidhu *et al.,* 2012) and murine (Maguire *et al.,* 2006) embryonic stem cells, induced pluripotent stem cells (Wei *et al.,* 2012), fibroblasts (Bohari *et al.,* 2011) and, although still in its early days, it is the most commonly used biomaterial for ovarian follicle culture.

Table 2 gives a summary of the most critical steps of the procedures used for the preparation of this matrix, the culture of follicles, the fertilization and development of the eggs obtained. Follicles are first placed singularly into microdrops of alginate and then transferred in a calcium solution whereby calcium ions form interchain ionic bridges that transform aqueous alginate into gel. This process ends with the encapsulation of single follicles within transparent beads that are easy to handle and allow microscopic observation of both the oocyte growth and granulosa cells proliferation throughout the culture period. The use of this matrix has several advantages. Since cell-matrix interactions are marginal, the encapsulated follicle can be easily released by a calcium-chelating agent, thus allowing a step-by-step analysis of the cellular and molecular characteristics that the oocyte and its companion follicle cells acquire during follicle maturation,

TABLE 2

PROTOCOL FOR THE CULTURE OF MOUSE FOLLICLES IN AN ALGINATE MATRIX

Procedure	Notes
1. Matrix preparation Dissolve 1% sodium alginate in deionized water, purify by charcoal stripping to improve the purity of alginate and sterile through 0.22 µm filter. Reconstitute sodium alginate in 1X PBS to the chosen concentration.	- Different sodium alginate concentrations may be used: 0.25%[1-3], 0.5%[1], 1%[1], 1.5%[1,4], 1.9%[5], 3%[6]. - Fibrin-alginate matrix: this protocol includes several differences compared to the standard procedure described on the left column. Refer to original papers for details[2,7].
2. Follicle isolation Isolate preantral follicles (two-layered or multilayered secondary follicles from 12/16 day-old mice, respectively[3,4,8]) by puncturing the ovaries with a sterile needle under a stereomicroscope in Leibovitz-15 (L-15) medium with 1% FCS at 37°C. Maintain follicles in α-MEM, 1% FCS at 37°C, 5% CO_2 in air for 2 hr.	- BSA[3-5,8] may be used instead of FCS.
3. Transfer of follicles into the matrix Using a hand-pulled micropipette, transfer single follicles into 2-3 µl droplets of alginate on a polypropylene mesh (100 µm); then, immerge the mesh into 50 mM $CaCl_2$ encapsulation solution.	- Encapsulation solution may be supplemented with NaCl[1]. - Alternatively, to form the beads, after the transfer of follicles in alginate, the mixture is loaded into a 1 ml syringe and extruded through droplets directly into a warmed beaker of 50 mM $CaCl_2$ 150 mM NaCl[5].
4. Follicle maturation Culture follicles in 100 µl α-MEM with 10 mIU/ml rFSH, 1mg/ml fetuin, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium, 3% BSA for each bead and incubate at 37°C, 5% CO_2 in air, 7 pH for 10-12 days. Change half of the medium volume every two days.	
5. Removal of follicles from the matrix Release follicles in 100 µl L-15 medium with 10 U/ml alginate lyase at 37°C for 30 min.	- Alternatively, release the follicles by adding 20 mM sterile EGTA to the medium and incubate at 37°C, 5% O_2, 5% CO_2, 90% N_2 for 5 min[5].
6. Maturation to MII Transfer follicles into α-MEM with 10% FCS, 1.5 IU/ml hCG, 5 ng/ml EGF at 37°C, 5% CO_2 in air for 16-18 hr.	- Different supplements and concentrations are used for α-MEM[5]: 0.3% BSA, 0.1% fetuin, 100 ng/ml hFSH, 10 ng/ml EGF. - The protocol that follows is routinely used in our laboratory for the culture of follicles and it is taken from references[9,10]. Briefly, 30-40 follicles are matured in pre-equilibrated 500 µl bicarbonate-buffered α-MEM supplemented with 50 mIU/ml FSH, 10 ng/ml EGF, antibiotic, 3 mg/ml BSA and 1 mg/ml fetuin, at 37°C, 5% CO_2 in air for 18 hr.
7. Fertilization Incubate cumulus cells-enclosed or cumulus cells-free MII oocytes in IVF medium containing $2x10^6$ sperm under mineral oil at 37°C, 5% CO_2 for 2-3 hr; wash away sperm and transfer the oocytes in M16 medium[11].	- The fertilization protocol reported is routinely used in our laboratory, but other procedures may be adopted[12].
8. Preimplantation development Incubate the inseminated oocytes at 37°C, 5% CO_2 in air[11].	- The protocol for *in vitro* preimplantation development reported is routinely used in our laboratory, but other procedures may be adopted[12].

References: 1) Xu *et al.,* 2006; 2) Jin *et al.,* 2010; 3) Mainigi *et al.,* 2011; 4) Kreeger *et al.,* 2006; 5) Pangas *et al.,* 2003; 6) West *et al.,* 2007; 7) Shikanov *et al.,* 2009; 8) Parrish *et al.,* 2011; 9) Yeo *et al.,* 2008; 10) Albuz *et al.,* 2010; 11) Zuccotti *et al.,* 2002; 12) Nagy *et al.,* 2003.

including the ability of resuming meiosis (Pangas *et al.,* 2003). An additional important feature is the possibility of determining the pores size of the alginate matrix (Rowley *et al.,* 1999), thus enabling a control on the diffusion of test macromolecules such as proteins and hormones. Although different alginate concentrations have been tested (from 0.25% to 3%), the most encouraging results in terms of follicle growth, oocyte meiotic resumption and developmental competence, were obtained with the lowest (Xu *et al.,* 2006; West *et al.,* 2007). Whilst the addition of FSH is common to most culture protocols [either in the culture medium only (Kreeger *et al.,* 2006; Xu *et al.,* 2006; Shikanov *et al.,* 2009; Mainigi *et al.,* 2011; Parrish *et al.,* 2011) or to both the alginate matrix and the culture medium (Heise *et al.,* 2005)], other procedures envisage the addition of ECM proteins such as collagen, laminin and fibronectin known to interact with follicles, regulate their growth and increase the overall maturation rate (Kreeger *et al.,* 2006). An interpenetrating fibrin-alginate matrix, produced by the simultaneous or sequential polymerization of the two polymers to form a combined network, has recently been tested. When this was compared to alginate alone, follicle survival and growth rates showed no differences; instead, the rate of meiotically competent oocytes at the end of the culture period was significantly higher (Shikanov *et al.,* 2009).

Though encouraging, these studies still leave a number of relevant questions to be addressed and problems to be solved. As highlighted by a very recent study, oocytes matured with this protocol show defects in spindle formation, chromosome alignment, abnormal cortical granule biogenesis and failure to extrude the first polar body (Mainigi *et al.,* 2011).

Hyaluronan hydrogel

The glycosaminoglycan HA is a component of the extracellular matrix, especially of the soft connective tissue, involved in the maintenance of the matrix structure (space filler), the homeostasis of the extracellular space and the steric interactions with other components of the matrix, thus forming a network of macromolecules that interacts with the surrounding cells (Laurent and Fraser 1992). For many years, the use of HA has found numerous applications as a drug delivery vehicle (hydrogels particles/microgels/nanogels; Xu *et al.,* 2012) or, in gene therapy, as a scaffold to enclose and deliver plasmid DNA to the diseased tissue (Gojgini *et al.,* 2011).

Its characteristics of plasticity and viscosity, together with its biocompatibility, make HA a good candidate as a biomaterial for the *in vitro* culture of many cell types and tissues. Moreover, HA can be easily dissolved in water (HA hydrogel) and, when needed, it is quickly degraded with the help of enzymes (e.g., hyaluronidase) (Isayeva *et al.,* 2010). Once HA is turned into a hydrogel, its optical transparency allows the observation of the cultured cells inside; also, its physical features enable this material to be molded into many different and functional shapes, thus permitting the operator to adjust the culture conditions to specific cell types and to obtain a gel with the pursued characteristics of morphology, stiffness and bioactivity (Desai *et al.,* 2012). It is for its bioactivity that a number of researchers prefer HA hydrogel to inert synthetic biomaterials such as PEG (Xu *et al.,* 2012).

HA hydrogel has been used, as a tissue-engineered scaffold, for the culture of cells such as mesenchymal stem cells to produce cartilage (Erickson *et al.,* 2012), haematopoietic stem cells (Demange *et al.,* 2012), human adipose-derived stem cells to differentiate into corneal stroma (Espandar *et al.,* 2012), endothelial progenitor cells (Camci-Unal *et al.,* 2012) and human embryonic stem cells (Gerecht *et al.,* 2007).

As for the *in vitro* culture of ovarian follicles, a recent novel method describes, with promising results, the culture of mouse fresh or vitrified preantral follicles embedded in a 3D scaffold appropriately shaped to form a cylindrical bead (Desai *et al.,* 2012). Table 3 gives a summary of the most critical steps of the procedures used for the preparation of this matrix and the culture of follicles. Compared to other shapes tested, the cylindrical bead allows the maintenance of a better 3D environment, a decreased follicle extrusion and, when using a 3 mg/ml HA concentration, an improved transparency and easiness of observation. HA hydrogel has been used alone or together with ECM components

TABLE 3

PROTOCOL FOR THE CULTURE OF MOUSE FOLLICLES IN A HYALURONAN HYDROGEL[1]

Procedure	Notes
1. Matrix preparation Prepare a 3 mg/ml tyramine-based HA in PBS or Global medium™ (LifeGlobal). Add Matrigel™ (BD Bioscience) (ratio 1:9) on ice to form ECM-HA. Activate the HA-gel by adding 5 µl horse radish peroxidase to 500 µl aliquots of HA; then expose 25 µl of activated HA to 1 µl 0.03% hydrogen peroxide. Gelification occours in 3-4 min.	
2. Follicle isolation Collect secondary preantral follicles (~ 120 µm in diameter) in pre-warmed L-15 medium with 0.1% collagenase on a laminar flow bench top at 37°C for 90 min. Change medium every 30 min. Release follicles from the stroma using pipette tips of decreasing diameter and eventually a glass micropipette. Repeat the collagenase digestion with the undigested tissue. Wash repeatedly the isolated follicles before culture.	
3. Transfer of follicles into the matrix Transfer a group of 8-10 follicles in a 25 µl drop of activated HA. Join this drop with a 1 µl drop of 0.03% hydrogen peroxide. Use two 21G needles with a 90° angle-bent edge to shape the beads to a cylindrical form[1].	- Three different methods were tested: drops, microcapillary plugs and cylindrical beads. The latter allowed the maintenance of a better 3D environment and decreased follicle extrusion.
4. Follicle maturation Transfer single HA-embedded follicles in 800 µl α-MEM with 5% FBS, 100 mIU/ml FSH, 10 mIU/ml LH, ITS (Invitrogen) in a 24-well plate. Culture follicles for 12 days at 37°C with 6% CO_2 in air. Change half of the medium volume every 24 hr.	
5. Maturation to MII Culture for 18-20 hr in α-MEM (see point 4) supplemented with 1.5 IU/ml hCG and 5 ng/ml EGF.	
6. Removal of follicles from the matrix With the help of a tuberculin syringe, remove follicles from HA hydrogel exposing for few min to 10 U/ml hyaluronidase.	
7. Fertilization Not tested yet.	

References: 1) the protocol described is from a single study (Desai *et al.,* 2012).

TABLE 4

PROTOCOL FOR THE CULTURE OF MOUSE FOLLICLES IN MATRIGEL™ (1)

Procedure	Notes
1. Matrix preparation Liquefy growth factor-reduced (GFR) Matrigel™ (BD Bioscience) at 4°C and dilute 1:1 in culture medium. Transfer 200 µl Matrigel in a 8-well-format chamber slides.	
2. Follicle isolation Digest pieces of ovaries in Hepes-buffered DMEM-F12 (supplemented with 5% BSA) with collagenase IA, DNase I for 30 min at 37°C. Isolate preantral follicles with 28-30G needles under a stereomicroscope.	
3. Transfer of follicles into the matrix Transfer follicles individually into the 8-well-format chamber slides with liquefied Matrigel and incubate at 37°C for 30 min to allow polymerization.	
4. Follicle maturation Add 100 µl α-MEM on the top of Matrigel and culture for 7 days at 37°C, 5% CO_2 in air with or without 30 ng/ml activin-A.	- α-MEM (serum-free) supplemented with: 100 mIU/ml recombinant FSH, 3 mg/ml BSA, ITS+3 (insulin 10 µg/ml, transferrin 5.5 µg/ml, selenite 5 ng/ml; Invitrogen) and 100 U/ml penicillin-G, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin-B.
5. Maturation to MII Not tested yet.	

References: 1) the protocol described is from a single study (Oktem and Oktay 2007).

(ECM-HA). After 12 days of culture, estradiol secretion *per* follicle was significantly higher in ECM-HA compared to HA or control. Although the rate of germinal vesicle breakdown (GVBD) in HA and ECM-HA was significantly higher compared to control samples (α-MEM without encapsulation), the rate of MII oocytes obtained remained higher in control, indicating the need for further studies.

Matrigel

ECM components are often used, added to the culture medium or to the matrix, in cell/tissue 3D cultures: they promote cell survival, proliferation and differentiation. Every component can be added singularly (fibronectin, laminin, collagen) or as a complex (e.g., Matrigel). Matrigel is an extract of the Engelbreth-Holm-Swarm mouse sarcoma, rich in proteins of the ECM, that *in vitro* can mimic the basal lamina function, thus acting as a substrate that sustain the viability and the growth of the cells in culture. It is made of laminin (the major component), collagen type IV, heparin sulfate proteoglycan, entactin and growth factors (e.g., TGF-β and EGF) (Oktem and Oktay 2007; Zhu *et al.,* 2012). Matrigel has been used for the *in vitro* culture of many cell types, including murine (with the addition of activin-A, a member of the TGF-β superfamily; Oktem and Oktay 2007) and human (Hovatta *et al.,* 1997, 1999; Xu *et al.,* 2009) ovarian follicles, promoting their multilayer development and antral space formation. Table 4 gives a summary of the most critical steps of the procedures used for the preparation of this matrix and the culture of follicles.

Synthetic matrices

The matrices described above derive from natural polymers that cross-link or self-assemble into hydrophilic structures called hydrogels, which have the advantage of being highly biocompatible and bioactive. Nevertheless, the difficulty in modifying these natural biomaterials to obtain desired physical properties has prompt tissue engineers to design novel synthetic matrices with well-defined mechanical and degradation properties (for a review see Tibbitt *et al.,* 2009). To date, PEG hydrogel is one of the best tested in the culture of ovarian follicles (Shikanov *et al.,* 2011). A major advantage in the use of PEG hydrogel is the biochemical nature of this molecule that, following follicle encapsulation, is slowly degraded by proteases that the growing follicle secretes during culture. This local gel degradation generates the space needed for follicle volumetric expansion (up to 17-fold), while maintaining the global integrity and overcoming the growth-limiting compressive forces exerted by other types of matrices. PEG, which was previously shown to maintain the viability of encapsulated cells (Bryant and Anseth 2002), enables, through 10 days of culture,

TABLE 5

PROTOCOL FOR THE CULTURE OF MOUSE FOLLICLES IN A PEG HYDROGEL

Procedure	Notes
1. Matrix preparation Dissolve PEG tetravinyl sulfone and plasmin sensitive peptides at a reactive group stoichometric ratio of 1:1.1 (PEG:peptides) in isotonic HEPES buffer at 7.4 pH and mix at 1:1 volumetric ratio[1].	- The preparation of this hydrogel involves a sequence of many steps, for details see references[1,2].
2. Follicle isolation Isolate preantral follicles from 14/15 day-old mice by puncturing the ovaries with a sterile needle in L-15 medium with 10% FCS, 100 U/ml penicillin and 100 mg/ml streptomycin[3].	
3. Transfer of follicles into the matrix Add a single follicle to each 5 µl gel and cast between parafilm-coated glass slides. Let the gel cross-link in a humidifier incubator at 37°C for 5 min.	
4. Follicle maturation Transfer the follicle-containing hydrogel in a 96 well plate and culture follicles in 150 µl α-MEM with 3% BSA, 10 mIU/ml rFSH, 1 mg/ml bovine fetuin, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium and incubate at 37°C, 5% CO_2 in air for 10 days. Change half of the media volume every two days.	
5. Removal of follicles from the matrix Mechanically retrieve follicles at the end of the culture.	
6. Maturation to MII Culture follicles in α-MEM with 10% FCS, 1.5 IU/ml hCG, 5 ng/ml EGF at 37°C, 5% CO_2 in air for 16 hr.	
7. Fertilization Not tested yet.	

References: 1) Lutolf and Hubbell, 2003; 2) Shikanov *et al.*, 2009; 3) Cortvrindt *et al.*, 1996.

the survival and growth of follicles from the secondary to the fully-grown antral stage. These antral oocytes are capable of resuming meiosis and reach the MII phase (Shikanov *et al.,* 2011). Table 5 gives a summary of the most critical steps of the procedures used for the preparation of this matrix and the culture of follicles.

Another synthetic hydrogel, with similar features, is PVA that maintains its mechanical properties and cell viability for several days of culture (Alves *et al.,* 2012), although it remains yet untested on ovarian follicles.

Non-matrices

Recent attempts have been made to maintain the 3D organization by culturing follicles in inverted microdrop suspension (Wycherley *et al.,* 2004; Nation and Selwood 2009), rotating wall vessels (Rowghani *et al.,* 2004), orbiting test tubes (Rowghani *et al.,* 2004; Heise *et al.,* 2005, 2009) or in roller bottle systems (Nation and Selwood 2009). In an inverted microdrop suspension system, mouse follicles were cultured for up to 6 days in 96-well round-bottomed plates that allowed the maximum oxygen access and nutrients supply at the media/gas interface and supported growth better than upright cultures (Wycherley *et al.,* 2004). Similar results were achieved culturing marsupial primary follicles up to the antral stage (Nation and Selwood 2009).

Orbiting test tubes and rotating wall vessels techniques maintain follicles in suspension throughout the culture period by agitating the medium, thus preventing the cells from adhering to the culture plate and flattening (Heise *et al.,* 2005, 2009). Rotating wall vessels resulted in the damage of rat follicles, which, instead, showed an overall increase in their growth rate when cultured in orbiting test tubes compared to conventional culture systems (Rowghani *et al.,* 2004).

Conclusions

The making and development of an organ *in vitro* is one of the main challenges of future research that will involve the collaborative work of biologists, biotechnologists, bioengineers, physicists, chemists and physicians. Among the different organs that are under study, the ovary has a peculiarity. Each ovarian follicle, for its anatomo-histological characteristics and its surrounding own proper blood vessels network, may be considered as a distinct "small organ" that can be isolated since its early stages of development and whose maturation can be achieved separately from the ovary. The main worry is the maintenance of the 3D architecture throughout the whole culture period together with the multitude of physical and chemical relationships occurring between the oocyte and the surrounding follicle cells.

In this review we have shown that, although still in its early years, the 3D *in vitro* culture of mouse ovarian follicles has made important methodological advancements. Biocompatible matrices used before for the culture of various cell types have been tested and proved to be amenable to encapsulate the follicle, allow its observation during growth and the exchange of gas and metabolites. Clearly, as underlined above, a great deal of work still remains to be done, both with the use of the known biomaterials and with the development of new ones. The results obtained are encouraging and will help to improve our understanding of the bidirectional relationship between the female gamete and its companion somatic cells.

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