Towards a 3D culture of mouse ovarian follicles

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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 full 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.
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). 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). 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).

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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 Co-star 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 stimulating 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., 2009). 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-luglucuronic acid units. Due to its biocompatibility, high affinity to water and handliness, 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**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Matrix preparation</td>
<td>Different sodium alginate concentrations may be used: 0.25%&lt;sup&gt;1-3&lt;/sup&gt;, 0.5%&lt;sup&gt;1&lt;/sup&gt;, 1%&lt;sup&gt;1&lt;/sup&gt;, 1.5%&lt;sup&gt;1-4&lt;/sup&gt;, 1.9%&lt;sup&gt;5&lt;/sup&gt;, 3%&lt;sup&gt;6&lt;/sup&gt;.</td>
</tr>
<tr>
<td>Dissolve 1% sodium alginate in deionized water, purity 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.</td>
<td></td>
</tr>
<tr>
<td>2. Follie isolation</td>
<td>- Fibrin-alginate matrix: this protocol includes several differences compared to the standard procedure described on the left column. Refer to original papers for details&lt;sup&gt;2&lt;/sup&gt;.</td>
</tr>
<tr>
<td>Isolate preantral follicles (two-layered or multilayered secondary follicles from 12/16 day-old mice, respectively&lt;sup&gt;7&lt;/sup&gt;) by puncturing the ovaries with a sterile needle under a stereomicroscope in Leibovitz-15 (L-15) medium with 1% FCS at 37°C.</td>
<td></td>
</tr>
<tr>
<td>Maintain follicles in α-MEM, 1% FCS at 37°C, 5% CO&lt;sub&gt;2&lt;/sub&gt; in air for 2 hr.</td>
<td></td>
</tr>
<tr>
<td>3. Transfer of follicles into the matrix</td>
<td>- Encapsulation solution may be supplemented with NaCl&lt;sup&gt;1&lt;/sup&gt;.</td>
</tr>
<tr>
<td>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&lt;sub&gt;2&lt;/sub&gt; encapsulation solution.</td>
<td>- 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&lt;sub&gt;2&lt;/sub&gt; 150 mM NaCl&lt;sub&gt;2&lt;/sub&gt;.</td>
</tr>
<tr>
<td>4. Follieic maturation</td>
<td>- Different supplements and concentrations are used for α-MEM&lt;sup&gt;3&lt;/sup&gt;: 0.3% BSA, 0.1% fetuin, 100 ng/ml hFSH, 10 ng/ml EGF.</td>
</tr>
<tr>
<td>Culture follicles in 100 µl α-MEM with 10 µl/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&lt;sub&gt;2&lt;/sub&gt; in air, 7 pH for 10-12 days. Change half of the medium volume every two days.</td>
<td>- The protocol that follows is routinely used in our laboratory.</td>
</tr>
</tbody>
</table>
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/nanogels; Xu et al., 2012) or, in gene therapy, as a scaffold to enclose and deliver plasmid DNA to the diseased tissue (Gojini 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**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. Matrix preparation</strong></td>
<td>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. Activates 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 occurs in 3-4 min.</td>
</tr>
<tr>
<td><strong>2. Follicle isolation</strong></td>
<td>Collect secondary preantral follicles (&lt; 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.</td>
</tr>
<tr>
<td><strong>3. Transfer of follicles into the matrix</strong></td>
<td>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.</td>
</tr>
<tr>
<td><strong>4. Follicle maturation</strong></td>
<td>Transfer single HA-embedded follicles in 800 µl α-MEM with 5% FBS, 100 mIU/ml FSH, 10 mIU/ml LH, ITS (insulin) in a 24-well plate. Culture follicles for 12 days at 37°C with 6% CO₂ in air. Change half of the medium volume every 24 hr.</td>
</tr>
<tr>
<td><strong>5. Maturation to MI</strong></td>
<td>Culture for 18-20 hr in α-MEM (see point 4) supplemented with 1.5 IU/ml hCG and 5 ng/ml EGF.</td>
</tr>
<tr>
<td><strong>6. Removal of follicles from the matrix</strong></td>
<td>With the help of a tuberculin syringe, remove follicles from HA hydrogel exposing for few min to 10 U/ml hyaluronidase.</td>
</tr>
<tr>
<td><strong>7. Fertilization</strong></td>
<td>Not tested yet.</td>
</tr>
</tbody>
</table>

References: 1) the protocol described is from a single study (Desai et al., 2012).
References: 1) Lutolf and Hubbell, 2003; 2) Shikanov et al., 2009; 3) Cortvrindt et al., 1996.

**TABLE 4**

**PROTOCOL FOR THE CULTURE OF MOUSE FOLLICLES IN MATRIGEL™**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Matrix preparation</td>
<td>Liquefy growth factor-reduced (GFR) Matrigel™ (BD Biosciences) at 4°C and dilute 1:1 in culture medium. Transfer 200 µl Matrigel in a 8-well-format chamber slides.</td>
</tr>
<tr>
<td>2. Follicle isolation</td>
<td>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.</td>
</tr>
<tr>
<td>3. Transfer of follicles into the matrix</td>
<td>Transfer follicles individually into the 8-well-format chamber slides with liquefied Matrigel and incubate at 37°C for 30 min to allow polymerization.</td>
</tr>
<tr>
<td>4. Follicle maturation</td>
<td>Add 100 µl α-MEM on the top of Matrigel and culture for 7 days at 37°C, 5% CO₂ in air with or without 30 ng/ml activin-A.</td>
</tr>
<tr>
<td>5. Maturation to MIll</td>
<td>- 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.</td>
</tr>
</tbody>
</table>

**Notes**

- Not tested yet.

**TABLE 5**

**PROTOCOL FOR THE CULTURE OF MOUSE FOLLICLES IN A PEG HYDROGEL**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Matrix preparation</td>
<td>Dissolve PEG tetravinyl sulfone and plasmin sensitive peptides at a reactive group stoichiometric ratio of 1:1.1 (PEG:peptides) in isotonic HEPES buffer at 7.4 pH and mix at 1:1 volumetric ratio.</td>
</tr>
<tr>
<td>2. Follicle isolation</td>
<td>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 µg/ml streptomycin.</td>
</tr>
<tr>
<td>3. Transfer of follicles into the matrix</td>
<td>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.</td>
</tr>
<tr>
<td>4. Follicle maturation</td>
<td>Transfer the follicle-containing hydrogel in a 96 well plate and culture follicles in 150 µl α-MEM with 3% BSA, 10 ml/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% CO₂ in air for 10 days. Change half of the media volume every two days.</td>
</tr>
<tr>
<td>5. Removal of follicles from the matrix</td>
<td>Mechanically retrieve follicles at the end of the culture.</td>
</tr>
<tr>
<td>6. Maturation to MIll</td>
<td>Culture follicles in α-MEM with 10% FCS, 1.5 IU/ml hCG, 5 ng/ml EGF at 37°C, 5% CO₂ in air for 16 hr.</td>
</tr>
<tr>
<td>7. Fertilization</td>
<td>Not tested yet.</td>
</tr>
</tbody>
</table>

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

(ECM-HA). After 12 days of culture, estradiol secretion perfollicle 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 MI 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 prompted 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,
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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