Multiparameter assessment of mouse oogenesis during follicular growth \textit{in vitro}

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Comparison of oocyte development within the follicle \textit{in vitro} and \textit{in vivo} has a major impact on research into ovarian physiology and clinical practice. Despite major differences in ovarian physiology between rodents and humans, mice provide a useful model for studies of the endocrine and paracrine mechanisms controlling follicular development. In this study, early preantral follicles were isolated from 12-day-old mice and cultured individually in microdrops under oil during 6, 9 or 12 days. Taking into account previous observations, several oocyte criteria (diameter, chromatin configuration, transcriptional activity, intracytoplasmic calcium signalling and ability to undergo meiosis) were assessed to check that the development pattern of oocytes during follicle growth \textit{in vitro} was similar to that already observed for oocytes developing \textit{in vivo}, and that they reached the fertilizable oocyte stage. Results indicate that, during the 12-day-culture period, the oocytes grew until 74.3±4.2 μm, they became transcriptionally quiescent with a surrounded nucleolus (SN) chromatin organization, 50% of them exhibited regular calcium signals and 73.4% of them resumed meiosis. These data demonstrate that the protocol used generates oocytes with characteristics similar to oocytes allowed to mature fully \textit{in vitro} and that it could be useful to set up the experimental culture of human ovarian follicles.

\textbf{Key words:} calcium/chromatin/folliculogenesis/transcription

\section*{Introduction}

Ovarian tissue cryopreservation is increasingly being offered as a means of preserving the fertility of cancer patients before sterilizing treatments, as the ovarian cortex is the unique source of oocytes (Poir et al., 2002). Autologous grafting has been successfully carried out after ovarian cortex cryopreservation (Donnez et al., 2004; Meirou et al., 2005), but this procedure carries a risk of transmission of the malignant disease (Shaw et al., 1996). The development of early preantral follicles \textit{in vitro} such that the oocytes they contain become competent for maturation, fertilization and embryonic development would be of benefit to many patients with cryopreserved ovarian tissue. So, such a procedure remains a major challenge. Only in mice has this technique been demonstrated to lead to correct development of the oocyte. However, only a few publications have reported the production of live offspring from mouse follicles cultured \textit{in vitro} (Eppig and Schroeder, 1989; Spears et al., 1994). A simplified system of culture in microdrops under oil has been developed (Cortvindt et al., 1996), but little is known about oocyte quality after follicular growth in culture. It has been clearly demonstrated that technical aspects of the culture process may greatly perturb the imprinting process during oocyte maturation \textit{in vitro} (Kerjean et al., 2003), possibly by modifying gene expression and/or epigenetic marks. For example, culture conditions can lead to expression of the inactivation of the \textit{Xist} gene in the placenta of fetal male mice, where no such expression should be observed (Mann et al., 2004). In addition, the maternally imprinted \textit{H19} gene has been shown to be expressed from both alleles in blastocysts cultured \textit{in vitro} (Doherty et al., 2000), and this biallelic expression persists in extraembryonic tissue (Mann et al., 2004). Several criteria for evaluating the competence of mouse oocytes to be fertilized have been identified for \textit{in vivo} oogenesis. Oocytes isolated from preantral follicles are unable to undergo germinal vesicle breakdown (GVBD) \textit{in vitro}, but the rate of GVBD steadily increases with oocyte diameter (Eppig et al., 1994; Lefevre et al., 1997). During oogenesis, the acquisition of meiotic competence is associated with a gradual increase in ability to perform spontaneous Ca\textsuperscript{2+} oscillations not seen in incompetent oocytes (Lefevre et al., 1997). In fully grown oocytes, meiosis resumption has been shown to be related to spontaneous cytoplasmic InsP\textsubscript{3}-dependent calcium oscillations (Carroll et al., 1994; Lefevre et al., 1995; Cotichino and Fleming, 1998; Pesty et al., 1998). Meiotic competence and ability to develop after fertilization are also correlated with chromatin configuration (Debey et al., 1993; Zuccotti et al., 1998). In fact, two principal nuclear DNA configurations have been described in fully grown oocytes, differing essentially in the presence of a rim of condensed chromatin around the nucleolus [surrounded nucleolus (SN)] or a dispersed chromatin within the nucleus [non-surrounded nucleolus (NSN)]. Several observations suggest that the SN configuration is a more advanced stage, towards ovulation than the NSN configuration (Bouniol-Baly et al., 2004).

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under an atmosphere containing 5% CO₂ in air. Ten follicles were cultured per

Aldrich), at 37°C, 100-μIU/ml recombinant FSH (Organon, Puteaux, France). Follicles were cul-

Mannheim, Meylan, France), 5-

results show that these criteria may provide valuable information

about the quality of the retrieved oocytes. We investigated whether these features were also found in oocytes growing in preantral follicle cultures in vitro, by assessing oocyte size, chromatin configuration, transcriptional activity and intracytoplasmic calcium signalling after various periods of follicular culture. The ability of the oocyte to undergo meiosis and to reach the ovulatory met-

aphase II stage was also assessed at the end of the culture period. The

Figure 1. (A) Early preantral follicles (100 and 130 μm) with two granulosa

cell layers (as shown here by Sytox-Green chromatin labelling) were selected. (B) Granulosa cell mass formed after 12 days of culture. Black arrows indicate

the border of this mass; empty arrow indicates theca cell monolayer; indicates the antral-like cavity appearing between day 9 and day 10 (Scale bar: 100 μm). After retrieval from the follicles, the oocyte size, excluding zona pellucida, was also measured by determining the mean of two perpendicular diameters measured under an inverted Zeiss confocal microscope (×40) (Carl Zeiss S.A. Le Pecq, France).

Fluorescence detection of transcriptional activity and chromatin configuration

Transcription was assayed by measuring BrUTP incorporation into nascent RNAs, using an immunofluorescence method first developed by Wansiak et al. (1993) and adapted to mouse oocytes and embryos (Boumiol et al., 1995). Briefly, 100 nM BrUTP (Sigma) in 2 mM PIPES buffer (pH 7.5), 140 mM KCl was introduced into the oocyte cytoplasm by microinjection (5–10 pl). The oocytes were cultured for 10–20 min in M2 medium supplemented with dbcAMP, under oil, at 37°C. They were then rinsed, fixed by incubation in 4%

parafomaldehyde (PFA) in phosphate-buffered saline (PBS) for 30 min at 37°C, permeabilized by incubation for 20 min in 0.5% Triton X-100 and incubated in 2% bovine serum albumin (BSA, Sigma-Aldrich) in PBS. The oocytes were then incubated overnight at 4°C with the primary antibody—a mouse monoclonal antibody (IgG) raised against BrUTP but also recognizing some of the secondary antibody, a fluorescein isothiocyanate (FITC)-conju-

gated donkey anti-mouse IgG (H + L) antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA; 1:400 in PBS + 2% BSA). They were rinsed several times in PBS and incubated for 1 h at room tempera-

ture with the secondary antibody, a fluorescein isothiocyanate (FITC)-conju-

gated donkey anti-mouse IgG (H + L) antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA; 1:400 in PBS + 2% BSA).

Chromatin configuration was investigated by rinsing the oocytes, labelling them with 2 μg/ml Hoechst 33342 (Riedel de Haen, Germany) and mounting them on slides in Citifluor (Citifluor Products, Canterbury, UK). The oocytes were examined with a Zeiss (Carl Zeiss, Oberkochen, Germany) inverted microscope equipped for epifluorescence. Images were recorded with a CCD-camera (Photometrics, Tucson, AZ, USA; type KAF 1400 12-bit range) cooled to 10°C and analyzed with IPLAB spectrum imaging software (Vysis, France).

Materials and methods

Follicle culture

Female F1 hybrid (C57BL/ICBA) 12-day-old mice were used. At this age, the ovaries contain mostly primordial and early preantral follicles, with one or two layers of granulosa cells and some theca cells (Cortvrindt et al., 1996). Mice were killed by cervical dislocation, without anesthesia, to prevent carbon dioxide asphyxiation toxicity to the follicles/oocytes. The ovaries were removed and immediately transferred to dissection medium, consisting of L-15 Leibovitz Glutamax (Gibco-BRL, Cergy-Pontoise, France) supplemented with 10% fetal calf serum [(FCS), Gibco-BRL], 100 IU/ml penicillin (Sigma-Aldrich, Saint Quentin Fallavier, France) and 100 μg/ml streptomycin (Sigma-Aldrich). Follicles were isolated by mechanical dissection under a stereomicroscope, at ×10 magnification, using 27-gauge needles to ensure that follicular structure remained intact. Isolated follicles were selected according to the following criteria (Figure 1): (i) intact follicle with one or two layers of granulosa cells and some adhering theca cells; (ii) visible, round and central oocyte and (iii) folli-
cle diameter between 100 and 130 μm. Follicles were rinsed three times in dis-

section medium and put in culture according to the open system developed by Cortvrindt et al. (1996). The culture medium consisted of α-MEM Glutamax (Gibco-BRL) supplemented with 5% FCS, 10-μg/ml transferrin (Boehringer Mannheim, Meylan, France), 5-μg/ml insulin (Boehringer Mannheim) and 100-μl/ml recombinant FSH (Organon, Puteaux, France). Follicles were cul-

tured individually in 20 μl microdrops, under detoxified mineral oil (Sigma-

Aldrich), at 37°C, in medium equilibrated overnight before the start of culture, under an atmosphere containing 5% CO₂ in air. Ten follicles were cultured per plate (60 mm Ø, Falcon tissue-culture-treated Petri dishes, VWR, France). Follicles were cultured for 6, 9 or 12 days, with the first day of culture designated as day 1. At the end of the culture period, the oocytes were retrieved from follicles either by rupture of the follicle wall with needles at day 6 (three experiments) or by repeated aspirations into a micropipette at

day-12 (four experiments). Then, oocytes were randomly distributed to study either chromatin configuration and transcriptional activity or intracytoplasmic calcium signalling. Maturation achievement of the oocytes was studied on five other 12-day-culture experiments

Measurement of follicle and oocyte diameters

Follicles were observed under an inverted microscope (Nikon, France). Only the intact follicles, that is, with a central oocyte surrounded by granulosa cell mass and peripheral spindle-shaped theca cell monolayer, were taken into account. For each follicle, two perpendicular diameters were measured using a calibrated ocular micrometer, at a magnification of ×200, before culture, and then on days 6, 9 and 12 of culture. Follicle diameter measurements took into account the granulosa cell mass with a spherical structure and the oocyte. Spindle-

shaped theca cells originating from the follicle theca and attached to the dish were not included in the measurements.

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1999; De la Fuente and Eppig, 2001). Transcriptional status is also correlated with chromatin configuration. An analysis of transcriptional activity in the mouse, based on BrUTP incorporation by RNA polymerases I and II, revealed an absence of transcription in SN-type oocytes and an active transcription in NSN-type oocytes (Boumiol-Baly et al., 1999; De la Fuente and Eppig, 2001). Similarly, in human GV oocytes, transcription stops as soon as condensed chromatin begins to wrap around the nucleolus-like body (also known as the ‘karyosphere’) (Parfenov et al., 1989, 2000; Miyara et al., 2003).

After retrieval from the follicles, the oocyte size, excluding zona pellucida, was also measured by determining the mean of two perpendicular diameters measured under an inverted Zeiss confocal microscope (×40) (Carl Zeiss S.A. Le Pecq, France).

A. before culture

B. at day-12

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Confocal microscopy analysis of calcium flux

Immediately after their recovery, oocytes were loaded with fluo-3/AM at 37°C in the dark for 30 min, washed and analysed in M2 culture medium (Sigma). A 1 mM stock solution of the cell-permeant fluorescent Ca2+ indicator fluo-3/acetoxymethyl-ester (Fluo3/AM; Molecular Probes Inc., Eugene, OR, USA) was prepared in 8% Pluronic F-127 in dimethylsulphoxide (DMSO) (Molecular Probes Inc.) and stored at –20°C. Immediately before use, the stock solution was dissolved to a final concentration of 5 μM in M2 culture medium. The loaded oocytes were placed on a coverslip, in a drop of medium, and covered with mineral oil (Sigma). They were then observed on the heated stage of an inverted microscope, to monitor cytosolic Ca2+ levels, as previously described (Pesty et al., 1998).

Fluorescence was measured with a LSM 5 Pascal Zeiss confocal microscope. Kinetic determinations were made with Zeiss time-series measurement software. Time-lapse calcium imaging studies were carried out, with oocytes viewed together through a ×20 objective (Plan Achromat ×2000.75), in a single optical plane through the GV.

Maturation of oocytes grown in vitro

On day 12 of culture, intact follicles with a diameter ≥500 μm were transferred to a 30 μl droplet of α-MEM GlutaMAX medium supplemented with 5% FCS, 10 μg/ml transferrin, 5 μg/ml insulin, 100 mM/L recombinant FSH, 1.5 IU/ml hCG and 5 ng/ml epidermal growth factor (EGF) (Boehringer Mannheim). After 17–20 h of culture in this droplet, the oocytes were mechanically denuded by a gentle pipetting through a narrow mouth glass pipette, and their nuclear maturation was assessed by the extrusion of the first polar body.

Results

Follicle and oocyte growth

On day 1, just after dissection of the ovary and before culture, the selected early preantral follicles had diameters of 100–130 μm (Figure 1). On day 6, the mean diameter of the studied follicles was 167.8 ± 29.5 μm and increased to 582.2 ± 108.7 μm by day 12, which means a factor five multiplication of the follicle size (Figures 1 and 2A). The oocytes growing within their follicles in vitro reached final sizes similar to prevulatory oocytes recovered in vivo: mean oocyte diameter of 42.6 ± 0.7 μm at the beginning of the experiment increased regularly reaching 74.3 ± 4.2 μm at the end of the culture period (Figure 2B). Moreover, between days 9 and 10, an antral-like cavity appeared inside the granulosa cell mass.

Chromatin configuration and transcriptional activity

Three types of chromatin configurations were observed (Figure 3): (i) chromatin distributed diffusely around the nucleolus (NSN type for non-surrounded nucleolus); (ii) chromatin compacted as a ring around the nucleolus (SN type for surrounded nucleolus) and (iii) intermediary stages pNSN (partly NSN) and pSN (partly SN) as already described (Bouniol-Baly et al., 1999) with part of the chromatin beginning to make a ring around the nucleolus and part still dispersed.

After 6 days in culture, most of the studied oocytes (n = 11/15) exhibited a NSN type of chromatin and were also transcriptionally active. By contrast, after 12 days in culture, most of the oocytes (n = 12/15) exhibited a SN type of chromatin and were transcriptionally quiescent. Interestingly, day 9 appeared as a pivotal time of culture. Indeed, around this period, chromatin configuration as well as transcriptional activity were progressively modified: 34.5% (n = 10/29) of the oocytes analysed were still of the NSN type and transcriptionally active; 37.9% (n = 11/29) were already of the SN type and transcriptionally quiescent, whereas 27.5% (n = 8/29) exhibited both an intermediate chromatin configuration (pNSN or pSN) and still a slight transcriptional activity.

Acquisition of intracytoplasmic calcium signalling

Following their release from the follicles after 6, 9 or 12 days of culture, the oocytes were loaded with a specific fluorochrome (Fluo-3) and observed by confocal microscopy. After 6 days in culture, 97.0% of the oocytes (n = 33/34) displayed no calcium signalling (Figure 4A), except one displaying irregular calcium signalling (Figure 4B). After 9 days in culture, 26% of the oocytes (n = 5/19) displayed fairly regular calcium peaks (Figure 4C), while no or irregular calcium signal were detected for the others. Finally, after 12 days in culture, 50% of the observed oocytes (n = 24/48) displayed calcium signals, which were regularly repeated and similar to the spontaneous calcium oscillations of fully mature oocytes in 45.8% (n = 11/24) of cases (Figure 4D).
Intrafollicular oocyte maturation

After 12 days in culture, the majority of follicles \( n = 272/350; 76\% \) remained with an intact structure. These follicles were transferred to maturation medium supplemented with hCG and EGF. After 17–20 h, an ovulation-like process occurred, and almost all the follicles spontaneously released their oocytes. GVBD occurred in 85.6% of the oocytes, and 73.4% of the oocytes reached metaphase II.

Discussion

Folliculogenesis and meiotic maturation are known to be strictly time-dependent processes (for review, see Smitz and Cortvrindt, 2002). The selection of follicles after mechanical isolation should ideally yield a very homogeneous population of follicles, making it possible to obtain sufficient numbers of competent oocytes after culture. We have previously evaluated different culture systems for a narrow class of intact early preantral follicles, 100–130 \( \mu m \) in diameter, retrieved from 12-day-old prepuberal mice (Martins et al., 2002; Mousset-Simeon et al., 2005). Finally, the liquid-phase model was chosen—an open culture system developed by Cortvrindt et al. (1996)—which scored best in our evaluations. Nutrients, hormones and gases are more available in such an open structure than in a closed system (for review, see Smitz and Cortvrindt, 2002) increasing oocyte survival rate. This open culture model was therefore considered highly suitable for this study focusing on oocyte development.

Our data confirm that follicles grew in vitro, with a rapid granulosa cell proliferation observed between days 1 and 9. Follicle growth slowed slightly after day 9, but oocytes continued to develop, reaching a diameter similar to that of fully grown oocytes in vivo. During the culture period, chromatin organization and transcriptional activity of the oocytes were correlated, as observed in vivo. At the start, all oocytes displayed the NSN chromatin organization and an active transcription. To the contrary, after 12 days in culture, all oocytes displayed the SN-type organization of chromatin, and transcription was inactive. Interestingly,
activity may not be strictly controlled by the maturing oocyte; companion granulosa cells may also play an active role in modulating transcriptional activity in the oocyte, as shown by previous studies (De la Fuente and Eppig, 2001; De la Fuente et al., 2004). The timing of transcriptional silencing has been identified as a key element for the progression of meiosis and a successful embryonic development (De la Fuente and Eppig, 2001; De la Fuente et al., 2004). These observations raise the interesting possibility that transcription may be involved in functions other than meiotic maturation in the mammalian oocyte. One possible such function is maternal mRNA storage during oocyte maturation for later recruitment in major genome activation during early embryonic development.

A progressive setting up of the calcium machinery was also observed. The oocytes retrieved from follicles cultured for 6 days remained silent in terms of calcium signalling. By day 9, isolated spikes were identified in a few oocytes and a more pronounced signalling was detected on day 12, with a series of fairly regular spontaneous calcium oscillations. These results are consistent with previous work showing that immature oocytes recovered from preantral follicles with 2–3 cell layers retrieved from prepubertal mice display no intracellular calcium signalling (Lefevre et al., 1997; Gomes et al., 1999). They also demonstrate the progressive acquisition of calcium signalling during in vitro oogenesis, as observed in vivo (Lefevre et al., 1997). The appearance of intracellular calcium oscillations is one of the first steps in meiosis resumption in mature oocytes, occurring just before GVBD (Carroll et al., 1994; Homa, 1995; Lefevre et al., 1995); they depend clearly on the phosphoinositide pathway (Coticchio and Fleming, 1998; Pesty et al., 1998). As follicles cultured in vitro for 12 days are equivalent to the antral follicles found in 24-day-old mouse ovaries—this time-point corresponding to the first wave of meiotic maturation leading to ovulation—a high frequency of oocytes displaying calcium oscillations, equivalent to that obtained for mature oocytes, was expected. However, the lower frequency actually obtained may be accounted for by our previous data. Indeed, during folliculogenesis in vivo, phosphoinositide machinery establishes progressively because, at the beginning of the process, oocytes retrieved from preantral follicles do not respond to exogenous InsP3 whereas oocytes retrieved a few days later did (Lefevre et al., 1997). It is also noticeable that the age of the female affects oocyte characteristics: whereas none of the oocytes retrieved from preantral follicles from prepuberal mice displayed calcium signalling, some calcium spikes were observed in about half the oocytes retrieved from preantral follicles of adult mouse ovaries (Gomes et al., 1999). Moreover, gonadotrophin stimulation further increases the numbers of oocytes displaying calcium oscillations in both cases (Gomes et al., 1999) or showing condensed chromatin and a lack of transcription (De la Fuente and Eppig, 2001) as females pass from being juvenile to being adults. Indeed, calcium oscillations have been studied essentially in fully mature oocytes recovered from adult female mice after hormone stimulation. Thus, the low frequency of oocytes with regular calcium oscillations obtained from the cultured follicles in this study is therefore perfectly consistent with the regular physiological control occurring during the life of the animal (Smitz and Cortvriendt, 2002). Moreover, when the oocyte–granulosa cell complexes were stimulated with FSH at the end of the culture period, a high percentage of oocytes was observed that had extruded a polar body, in a process resembling ovulation, suggesting that these oocytes had probably begun to display calcium oscillations before ovulation.

All together, these observations indicate that there are important similarities between oocytes allowed to mature fully in vivo and cultured oocyte–granulosa cell complexes. However, as the follicles were cultured individually, certain intraovarian control mechanisms may not operate in this system, increasing the proportion of oocytes with

Figure 4. Oocytes were loaded with the specific cell-permeant calcium probe Fluo-3/AM. Three major patterns of calcium signalling are observed in oocytes during maturation in vitro: (A) a basal signal, as observed in all oocytes on day 6; (B) isolated calcium transients or (C) irregular calcium oscillations, as observed on day 9; (D) regular calcium oscillations, as observed on day 12.

a transition period was observed at around day 9, when a third of the oocytes had an intermediate chromatin organization and some transcriptional activity. These results are consistent with those reported for oocytes in vivo, suggesting that the SN configuration corresponds to a more advanced stage of oocyte development, closer to ovulation than the NSN configuration (Bouniol-Baly et al., 1999; De la Fuente and Eppig, 2001; Miyara et al., 2003; De la Fuente, 2006). Moreover, this chromatin remodelling and the subsequent decrease in transcription...
compact chromatin surrounding the nucleolus and transcriptional silencing. Indeed, the individual culture of follicles prevents all the interfollicular interactions intervening the development of follicles, both positively and negatively, during folliculogenesis in vivo. Further studies could investigate the contribution of crosstalk between follicle cells and oocytes to the progression of oogenesis, taking into account other factors, such as EGF-like agents (Ashkenazi et al., 2005), GDF-9 (Dragovic et al., 2005), BMP-6 and BMP-15 (Hussein et al., 2005) or Akt/Pi3K (Hoshino et al., 2004).

Our understanding of the way in which the oocyte develops within its follicle, both during follicular culture in vitro and during oogenesis in vivo, has a major impact on research into ovarian physiology as well as clinical practice. Advances in this understanding may enable us to improve in vitro procedures, making it possible to obtain ‘high-quality’ fertilizable oocytes able to develop to term. Mice constitute a relevant model for the identification of endocrine and local mechanisms controlling follicular development, and the results acquired with this model could be applied to the experimental human follicle cultures. Once finalized, this human ovarian follicular culture system would greatly increase the treatment options open to infertile couples, by avoiding the need for intensive ovarian stimulation in patients with diseases such as polycystic ovary syndrome and for young cancer patients, by cryopreserving ovarian tissue for subsequent follicle culture.

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References


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