Preantral follicle culture as a novel in vitro assay in reproductive toxicology testing in mammalian oocytes

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The most common genetic disorder in humans, trisomy, is caused predominantly by errors in chromosome segregation during oogenesis. Isolated mouse oocytes resuming meiosis and progressing to metaphase II in vitro have recently been used to assess targets, aneugenic potential and sensitivity of oocytes to chemical exposures. In order to extend in vitro maturation tests to earlier stages of oogenesis, an in vitro assay with mouse preantral follicle cultures has been established. It permits the identification of direct and also indirect effects of environmental chemicals on the somatic compartment, the follicle and theca cells, that may lead to disturbances of oocyte growth, maturation and chromosome segregation. Early preantral follicles from prepubertal female mice are cultured in microdroplets for 12 days under strictly controlled conditions. The follicle-enclosed oocytes resume maturation, develop to metaphase II and become in vitro ovulated within 16 h after a physiological ovulatory stimulus with recombinant human gonadotrophins and epidermal growth factor. These oocytes grown and matured in vitro possess normal barrel-shaped spindles with well-aligned chromosomes. Their chromosomes segregate with high fidelity during anaphase I. The model aneugen colchicine induced a meiotic arrest and aneuploidy in these in vitro grown, follicle-enclosed oocytes in a dose-dependent manner, comparable to in vivo tests. Therefore, preantral follicle culture appears to provide an effective and reliable method to assess the influences of environmental mutagens, pharmaceutical agents and potentially endocrine disrupting chemicals on the fidelity of female meiosis.

Introduction

Aneuploidy resulting in human trisomy is the most common genetic disorder affecting human beings, and predominantly arises during female gametogenesis, especially during first meiosis of oogenesis (Eichenlaub-Ritter, 1996, 2000, 2003; Hassold and Hunt, 2001; Plachot, 2001; Pellestor et al., 2002; Sandalinas et al., 2002). Advanced maternal age is established as an aetiologic factor in the genesis of aneuploidy in oocytes (Bond and Chandley, 1983; Hassold and Hunt, 2001). Age, environmental exposures or mutations may all interfere directly with spindle formation or indirectly with oocyte—somatic cell interactions and hormonally controlled processes during folliculogenesis and oogenesis that affect spindle formation and congression of chromosomes (Hodges et al., 2002; Hunt et al., 2003). Furthermore, they may cause premature depletion of the follicle pool by induction of follicular atresia (Matikainen et al., 2001; Tilly, 2001) or oocyte apoptosis (Reynaud and Driancourt, 2000; Blumenfeld, 2002; Gosden and Negano, 2002; Matikainen et al., 2002) and, thereby, lead to reduced fertility. It has been suggested that a depletion of the follicle pool may be critical for predisposing to trisomy (Eichenlaub-Ritter, 2000; Freeman et al., 2000; Kline et al., 2000; El-Toukhly et al., 2002; te Velde and Pearson, 2002).

The complex developmental program during oogenesis encompasses distinct phases of oocyte meiosis, follicular recruitment, granulosa cell proliferation and differentiation, as well as apoptosis and follicle selection in the adult female, and relies on timed and highly orchestrated interactions between the oocyte, the follicular compartment and the neuroregulatory axis and paracrine and autocrine hormonal stimuli (Eppig et al., 2002). Meiosis is initiated during fetal life and arrested after pachytenic in the diplotene stage (termed dictyate stage) for long periods. Mammalian females have a fixed number of oocytes at birth, which decreases dramatically with advancing age due to follicular atresia. Resumption of meiosis takes place in adult females under hormone control (Crisp, 1992) and only fully grown oocytes can respond to the maturational stimulus after they acquired maturational competence during preceding stages of oocyte growth and follicular development. The different stages of oogenesis and folliculogenesis each may exhibit specific sensitivities to environmental chemicals (Plowchalk et al., 1993; Eichenlaub-Ritter et al., 1996; Hoyer et al., 1996; Bolon et al., 1997; Bucci et al., 1997; Christian, 1997; Hirshfeld, 1997; Zacharewski, 1998; Müller et al., 1999; Hunt et al., 2003). Therefore, in in vivo experiments it is difficult to identify the target and mechanisms of chemical action contributing to aneuploidy induction in oocytes (for discussions see Mailhes, 1995; Hirshfeld, 1997). Mechanisms responsible for trisomy formation in humans are thus still poorly understood (Hassold and Hunt, 2001). The novel in vitro system of preantral follicle culture provides the possibility to vary culture parameters and, thereby, to gain insights into the mechanisms by which chemical exposure contributes to aneugenic effects in oocytes. In this assay, aneugenic insults can be determined qualitatively and quantitatively. It may provide an alternative methodology aimed at replacing and reducing laboratory animal use and promote animal welfare (Davila et al., 1998; Jackson, 1998; Ecobichon, 2001).

Fully grown, denuded mouse oocytes which spontaneously resume maturation in vitro after isolation from large antral follicles have been used as an assay system for some time. These oocytes have been applied to determine dose—response relationships after chemical exposure, to identify active

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metabolites, stage-specific sensitivities, mechanisms and targets for environmental chemicals affecting meiotic progression, spindle formation, chromosome behaviour and fidelity of chromosome segregation (see for example Eichenlaub-Ritter, 1994; Eichenlaub-Ritter and Betzendahl, 1995; Yin et al., 1998a,b; Sun et al., 2001). Such in vitro oocyte cultures have also been employed to compare thresholds of chemically induced aneuploidy between human lymphocytes and mouse oocytes dividing in vitro (Kirsch-Volders et al., 2003). However, oocytes actually acquire cytoplasmic and nuclear competence within the follicle prior to resumption of meiosis (Gilchrist et al., 1995). Evidence exists that disturbances during oocyte growth or at the beginning of meiotic resumption, when the oocyte is still metabolically coupled to companion follicle cells by gap junctions, may adversely influence its capacity to form a functional spindle and control chromosome behaviour and separation (see for example Hodges et al., 2002). In order to extend the in vitro oocyte test to earlier stages of oogenesis and to explore the potentially protective or synergistic effects of somatic cells in the follicle on the response of the oocyte to environmental chemicals, we established the preantral follicle culture method. It allows a combined analysis of the hormonal milieu, the morphology of the follicle, the shape of the spindle and the segregation of chromosomes in the oocyte.

The present report describes the preantral follicle culture in the presence of recombinant hormones (Cortvrindt and Smitz, 2002), as it can be used to study the influences of environmental chemicals, mutagens, pharmaceuticals and naturally occurring or synthetic chemicals on folliculogenesis and oocyte quality and genetic constitution. The pilot study shows that in vitro grown and matured oocytes from preantral follicles that progress to metaphase II possess euploid numbers of chromosomes. Furthermore, data are presented to demonstrate that a classical aneugen, colchicine, applied during the last stages of meiosis dose-dependently interferes with cell cycle progression and chromosome segregation, comparable to exposures in vivo (Tease and Fisher, 1986; Mailhes et al., 1988, 1990).

Materials and methods

Animals

All female mice used in this study were (C57Bl/6J × CBA/Ca) F1 hybrids raised in the Departments’ animal facility from mice originally obtained from Harlan Winkelmann (Borchen, Germany). They were housed in a temperature- and light-controlled room at 23±25°C, on a 12 light:12 dark (on 7 a.m., off 7 p.m.) light cycle and fed with pellet food and water ad libitum.

Experimental groups

For comparison of maturation and chromosomal constitution of oocytes matured in vivo and in vitro, oocytes from three experimental groups were analysed (Figure 1): (i) in vitro grown and in vitro matured oocytes obtained from preantral follicle cultures for a total of 13 days (designated in vitro grown, IVG) (10 prepubertal females); (ii) in vitro matured (IVM) oocytes obtained from large antral follicles, which grew in vivo within follicles and then spontaneously resumed maturation in vitro (12 females); (iii) an in vivo control (IVC) group consisting of oocytes, which grew and matured in vivo within their follicles and were isolated after ovulation in hormonally stimulated cycles (seven females) (Table II). For the colchicine experiment, preantral follicles were isolated from eight prepubertal females and randomly allocated to one control and two colchicine treatment groups.

Follicle collection for in vitro growth and maturation of oocytes (IVG)

Ovaries of prepubertal mice (aged 12–14 days) were aseptically removed from the animals after killing and placed in prewarmed isolation medium, consisting of L15 Leibovitz medium (Gibco-Invitrogen, Germany) supplemented with 10% heat-inactivated foetal calf serum (Gibco) and 100 IU/ml penicillin + 100 μg/ml streptomycin. The ovaries were mechanically dissected using fine hypodermic needles (26 gauge). Only follicles with two layers of granulosa
cells, a centrally located oocyte and a diameter in the range 100–130 μm, which were enclosed by an intact basal membrane and had some attached theca cells, were collected (Figure 2) (Cortvrindt et al., 1996). Mechanical dissection of the ovary and the early preantral follicles instead of enzymatic digestion was used to conserve all cell types and receptor systems of the ovarian follicle for culture. This technique yields about 30 to more good quality early preantral follicles per ovary and, accordingly, ~60–70 follicles per animal.

Follicle culture for IVG

After three washing steps in isolation medium and two washes in culture medium, the preantral follicles were cultured individually in 10 μl droplets of culture medium overlaid with mineral oil (Sigma, Deisenhofen, Germany) in 60 mm Petri dishes (Falcon, Germany) (20 droplets/dish) (Figure 1). The culture medium consisted of α-minimal essential medium (α-MEM) with glutamax; Gibco) enriched with 5% foetal calf serum (Gibco), ITS (5 g/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium; Sigma), 100 μU/ml recombinant follicle stimulating hormone (rFSH) (kindly donated by Serono, Munich, Germany) and 10 μU/ml recombinant luteinizing hormone (rLH) (kindly provided by Serono). After 24 h of culture, permitting the follicles to adapt, follicles were scored for the presence of theca cells, an intact basal membrane and an appropriate diameter of the follicle, assessed with an inverted microscope at a magnification of 320×. After 48 h of culture, 10 μl of culture medium was added to each droplet. Subsequent refreshments were conducted every other day by removing and replacing 10 μl of medium (Figures 1 and 2). Conditioned culture medium was collected and frozen for later analysis of oestradiol and progesterone production. The morphological characteristics of the follicles were recorded before each refreshment (Figure 2). On day 12 of culture, the follicles were replenished with medium containing recombinant human chorionic gonadotrophins (rHCG) (kindly donated by Serono) and recombinant epidermal growth factor (rEGF) (Promega, Madison, WI) as an in vitro ovulatory stimulus at a final concentration of 1.5 IU/ml rHCG and 5 ng/ml rEGF in the culture droplet (Figure 1). With these hormone concentrations, optimal nuclear maturation rates can be achieved (Smits et al., 1998).

Sampling of conditioned medium for oestradiol and progesterone measurements

The 10 μl samples of spent medium from surviving follicles were pooled at each refreshment. The steroids and metabolites, which are produced by the follicle and retained in the medium, can be analysed for further characterization of follicular development and oocyte quality. Two steroids were measured in the present study. Oestradiol secretion was monitored every second day from day 4 onwards up to the day of induction of in vitro ovulation. Progesterone concentration, an indicator of induction of luteinization, was quantified at and after the day of induction of in vitro ovulation (Cortvrindt et al., 1996).

Oestradiol was measured using a commercially available radioimmunoassay (Estradiol-2) from Clinical Assays (DiaSorin NV, Brussels, Belgium) after an appropriate dilution with steroid-free diluent that was parallel to the standard curve. The antibody was highly specific for 17β-oestradiol. The oestradiol assay had a measurement range of 4–500 ng/l, an analytical sensitivity of 4 ng/l and a total precision of <10% [per cent coefficient of variation (CV)].

Progesterone concentration was measured by a direct radioimmunoassay (PogCTRACIS Biointernational, Gif-Sur-Yvette, France). This assay possesses a measurement range of 0.1–60 μg/l, an analytical sensitivity of 40 ng/l and a CV of <10%.

Oocyte harvest and staining for analysis of maturation

In response to the in vitro ovulation stimulation, cumulus cells mucify (Figure 2), a process associated with loss of gap junctional communication between oocytes and granulosa cells and with production of hyaluronic acid in response to gonadotrophic stimulation (Vanderhyden et al., 1990; Zhuo and Kimata, 2001). After denudation of cumulus and corona cells by gently pipetting through a mouth-operated micropipette, the oocytes (Figure 2) were transferred individually into another Petri dish with 20 pre-warmed droplets (each 10 μl) of M2 medium containing 0.5 μg/ml Hoechst 33342 (Sigma) for vital chromosomal staining, covered with mineral oil and incubated at 37°C for 20 min. According to nuclear staining, the maturational status was assessed in the oocytes prior to spreading by analysing vital stained oocytes with an inverted Zeiss microscope (Axiovert 10) equipped with a 50 W mercury lamp and a heated stage. It was ensured that all somatic cells were removed prior to spreading. On the basis of the fluorescence patterns, the oocytes were assigned to different groups. Germinal vesicle (GV) oocytes were maturation incompetent and still arrested in the dictyate stage at the end of culture/maturation. Germinal vesicle breakdown (GVBD) oocytes had resolved their nuclear membrane and characteristically possessed one set of condensed chromosomes, but did not emit a polar body (PB). Due to the low power magnification at this stage of analysis of meiotic progression it was not possible to identify oocytes which had progressed to metaphase II without emitting a PB and had all chromosomes assembled on a common spindle (‘diploid metaphase II’) from truly meiosis I arrested oocytes. However, those GVBD oocytes with two sets of chromosomes, which were in anaphase I, could be detected and

### Table I. Development of mouse early preantral follicles in vitro and morphological characteristics of follicles

<table>
<thead>
<tr>
<th>No. of follicles isolated</th>
<th>Follicle diameter* (μm)</th>
<th>Follicles with theca cells (% of day 0)</th>
<th>Granulosa cell/oocyte apposition (% of day 0)</th>
<th>Follicle attachment (% of day 0)</th>
<th>Follicles with antral cavity (% of day 0)</th>
<th>Survival of follicles (% of day 0)</th>
<th>Muscification of follicles (% of day 0)</th>
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<tbody>
<tr>
<td>710</td>
<td>120 ± 10</td>
<td>591 (83.2)</td>
<td>654 (92.1)</td>
<td>602 (84.8)</td>
<td>596(83.9)</td>
<td>648(91.3)</td>
<td>572(80.6)</td>
</tr>
</tbody>
</table>

*Follicle diameter was measured ~12 h after isolation, to allow follicles to recover from mechanical stress during isolation procedure and to obtain a characteristic spherical morphology.

### Table II. Cytoplasmic and nuclear maturation of oocytes from different experimental groups

<table>
<thead>
<tr>
<th>N</th>
<th>Cytoplasmic maturation</th>
<th>Nuclear maturation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Diameter ± SD (μm)</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---------------------</td>
</tr>
<tr>
<td>IVG</td>
<td>10</td>
<td>662</td>
</tr>
<tr>
<td>IVM</td>
<td>12</td>
<td>409</td>
</tr>
<tr>
<td>IVC</td>
<td>7</td>
<td>270</td>
</tr>
</tbody>
</table>

<sup>a</sup>Significant difference to IVM; <sup>b</sup>P < 0.05.<br>
<sup>c</sup>Significant difference to IVC; <sup>d</sup>P < 0.01.<br>
<sup>e</sup>Significant difference to IVM and IVC oocytes; <sup>f</sup>P < 0.01.<br>
<sup>g</sup>Significant difference to IVM and IVC oocytes; <sup>h</sup>P < 0.001.
Figure 2. Stages of folliculogenesis and oocyte maturation in the preantral follicle culture (photos), parameters assessed for follicle and oocyte development and quality (blue bars) and period of chemical exposure (pink bar). (A) Preantral follicle on day 1 of culture with oocyte (black arrow), layers of granulosa cells (white arrow) and theca cells on the basal membrane (grey arrow) surrounding the preantral follicle. (B) Theca cells attaching onto the slide (black arrow) and oocyte within the follicular investment (star) on day 2 of culture. (C) Oocyte (black arrow) and proliferating follicle cells (white arrow) on day 6 of culture. (D) Follicular differentiation with cumulus follicle cells surrounding the oocyte (black arrow), translucent antral-like cavity (white arrow) and mural granulosa cells on the periphery of the follicle grown in vitro for 12 days. (E) ‘Ovulated’ oocyte–cumulus complex on day 13 with mucified cumulus follicle cells (white arrow) loosely attached to the mature oocyte (star) 16 h after stimulation by rHCG/rEGF. (F) Oocyte isolated after ‘in vitro ovulation’ from mature follicle with first polar body (black arrow) and thick extracellular matrix, the zona pellucida (blue arrow). Parameters at the bottom, which can be used to assess follicle and oocyte quality, are indicated in the bars below. The pink bar at the bottom indicates the period of colchicine exposure in the present experiment. The red arrows indicate the sampling of conditioned medium.
Figure 3. Chromosomes and spindles in oocytes from preantral follicle culture (IVG), from in vivo controls (IVC) or from in vitro matured oocytes (IVM). (a) Bivalent chromosomes in IVG meiosis I oocyte, which underwent GVBD but was incompetent to mature to metaphase II. (b) IVG oocyte in anaphase I of meiosis with two sets of dyads 16 h after stimulation with rHCG/rEGF. (c) Diploid IVG oocyte without polar body, which possesses 40 dyads, indicating asynchrony between nuclear maturation and cytokinesis. (d) Normal euploid IVG oocyte with 20 metaphase II chromosomes. (e) Anaphase II in IVC oocyte with two sets of chromatids. (f) Hyperploidy (22 chromosomes) in IVG oocyte from follicle culture treated with colchicine (5 nM). (g) Hyperploid IVM oocyte with 19 dyads and one bivalent (arrows). (h) Euploid IVG oocyte in metaphase II with 19 dyads and a pair of chromatids (arrows), which presumably underwent balanced predivision prior to anaphase II. (i) Image of normal barrel-shaped metaphase II spindle (left) and well-aligned chromosomes (right) in oocyte grown and matured in vitro in preantral follicle culture (IVG). (a–h) C-banded chromosomes. (i) Tubulin immunofluorescence (left) and DAPI stained chromosomes (right).
separated before spreading. Oocytes with PB had two sets of chromosomes and visibly undergone cytokinesis. Oocytes were pooled according to their meiotic stages and then GVBD and PB oocytes were spread separately for chromosomal analysis.

Collection of oocytes for IVF

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Fully grown, maturation-competent oocytes were obtained from age-matched (24–26 days of age) female F1 hybrids from the same litter as those used for follicle culture (Figure 1). The females were primed with 5 IU pregnant mare serum (PMSG) (Ovarelin, Intervet, Germany) to stimulate folliculogenesis. The females were killed 48 h later (at days 24–26) and the ovaries were isolated. The oocytes were mechanically released from large antral follicles and freed of cumulus cells by gently pipetting through a mouth-operated micropipette (Eichenlaub-Ritter and Betzendahl, 1995). The oocytes were transferred to a 4-well dish containing prewarmed M2 medium (Quinn et al., 1992). After 6 h of culture, the oocytes were analysed for meiotic stage under a stereomicroscope and classified as GV, GVBD or PB oocytes as described above. The oocytes were pooled based on the meiotic stage and spread for chromosomal analysis.

Collection of in vivo grown and matured control oocytes (IVC)

Age-matched (24–26 days of age) F1 female mice from the same litter as used for follicle culture (Figure 1) were stimulated with 5 IU PMSF by i.p. injection. After 48 h, 5 IU HCG (Predalon®; Organon, Oberschleissheim, Germany) was i.p. administered for induction of oocyte maturation. Another 16 h later, the females were killed and ovulated oocytes were collected from the ampullae of the oviducts. Cumulus cells were removed by gentle, brief digestion with hyaluronidase (300 IU/ml; Sigma). Oocytes were analysed for maturational status as described before and pooled for spreading.

Chromogenic exposure of IVG oocytes

To demonstrate the sensitivity of preantral follicle culture to aneugenic chemicals, an experiment with the known spindle poison colchicine was performed. Colchicine (Sigma Deisenhofen, Germany) was added to the culture medium during the last refreshment, when meiotic resumption was induced by addition of rHCG and rEGF. At this stage, oocytes are still in direct contact with the surrounding follicle cells. The threshold for aneuploidy induction by colchicine was previously determined to be 20 nM in human lymphocyte cultures using the micronucleus assay with cytochalasin B and fluorescence in situ hybridization (Elhajouji et al., 1997). An initial analysis using the in vitro mouse oocyte test showed that this high concentration caused a prominent meiotic block at meiosis I with hardly any oocyte emitting a PB (data not shown). Therefore, the considerably lower concentrations of 2.5 and 5.0 nM colchicine were used in the preantral follicle culture. Follicles were isolated from eight prepubertal females in three repetitive experiments. Follicles in each set were randomly allocated to plates for three experimental groups and cultured for 12 days. Fresh medium containing rHCG and rEGF for maturation induction and no colchicine (control) or 5 or 10 nM colchicine was added when the last refreshment at a final concentration of 2.5 and 5.0 nM, respectively, during the last 16 h of culture. Colchicine-exposed and control oocytes were analysed for meiotic progression before oocyte spreads were prepared for cytogenetic analysis.

Spreading and C-banding

The spreading procedure was performed according to the method described by Tarkovski (1966) with some modifications (Eichenlaub-Ritter and Betzendahl, 1995). In short, oocytes were treated with 0.75% hypotonic sodium citrate at room temperature for 5 min. The oocytes were then briefly fixed in ice-cold acetic acid/methanol (1:3) and three to maximally five oocytes were immediately afterwards dropped onto a clean, ice-cold slide in a room with 60–70% humidity and finally air dried. A micropipette was employed for oocyte transfer and the whole process was monitored under a stereomicroscope. The position of oocytes on the slide was marked with a diamond pencil. C-banding was performed as previously described (Eichenlaub-Ritter and Betzendahl, 1995).

Cytogenetic analysis

Cytogenetic analysis was performed with a phase contrast microscope (Zeiss) at 1000× final magnification. All oocytes were assigned to their meiotic stage based on their chromosomal configuration. GVBD oocytes possessed either bivalents, and accordingly reached meiosis I during maturation (Figure 3a), or possessed two sets of dyads (metaphase II chromosomes), which were indicative of anaphase I (Figure 3b). A third group of GVBD oocytes had a diploid set of dyads (at least 36 and up to 40 individual metaphase II chromosomes could be counted in metaphase II plates of oocytes without a PB in this setup) and were therefore recorded as ‘diploid’ metaphase II oocytes (Figure 3c). PB oocytes contained either one set of dyads according to metaphase I stage (Figure 3d) or two sets of chromatids when they became spontaneously activated and progressed to anaphase II during culture (Figure 3e).

The oocytes in which chromosomal numbers could be counted unambiguously were analysed further for ploidy. Those possessing >17 metaphase II chromosomes or up to 19 dyads plus one chromatid were included as hypoploids. Oocytes with 20 metaphase II chromosomes or the respective number of chromatids were euploid (Figure 3d). Oocytes with >20 metaphase II chromosomes or the respective number of dyads plus bivalents or chromatids were hyperploid (Figure 3f). True non-disjunction as a mechanism causing aneuploidy was separately assessed when a bivalent was found in a set of metaphase II chromosomes (Figure 3g). Premature separation of chromatids prior to anaphase II was also analysed. Presence of a pair of chromatids of equal size was interpreted as balanced pre-division (Figure 3h). Single chromatids were not encountered in untreated oocytes.

Statistical analysis

Statistical analysis was performed with the χ2-test with Yates correction or the test.

Anti-tubulin immunofluorescence

Extraction, fixation and reaction with-anti-tubulin antibody for spindle analysis was performed with a limited number of oocytes as previously described (Eichenlaub-Ritter and Betzendahl, 1995). A monoclonal mouse anti-tubulin (Sigma) was used as first and a fluorescein isothiocyanate-labelled polyclonal anti-mouse antibody as second antibody (Hu et al., 2001). Oocyte chromosomes were stained with DAPI (Sun et al., 2001) and images recorded with an Axioshot microscope (Zeiss, Germany), equipped with appropriate filters and a sensitive CCD camera (Sensicam, Kelheim, Germany).

Results

In vitro development of preantral follicles

Preantral follicles were selected according to size after their manual isolation and intactness of the follicle and the centrally located oocyte were assessed by stereomicroscope before further culture (Figure 2A and Table I). All follicles for one experiment were pooled and randomly allocated to culture dishes. In all, 710 follicles were initially obtained from 10 prepubertal mice (Tables I and II). The preantral follicles were subsequently cultured individually for 12 days at 37°C, 100% humidity and 5% CO2 in air (Figure 1) within a microdroplet of culture medium covered by mineral oil. The in vitro developmental characteristics of the follicles included in this pilot study are summarized in Table I. Of a total of 710 preantral follicles with an average diameter of 119.97 ± 9.95 μm, 83.2% were visibly associated with steroidogenic theca cells and 92.1% possessed a close follicle (granulosa) cell/oocyte apposition (Figure 2A), a good indicator of an intact follicle. At day 2 of culture, the great majority of the follicles had attached to the culture dish (Figure 2B). After the first days of culture, the basal membranes of most follicles were not totally surrounding the complete follicle anymore, due to the rapid proliferation and outgrowth of granulosa cells. Characteristically, layers of follicle cells were spread out on the bottom of the dish by day 6 of culture (Figure 2C). Accordingly, follicles in this model of preantral follicle culture were morphologically distinct from those developing in vivo, since the follicles lost their spherical shape with progressing days in culture. However, this possibly facilitated diffusion of oxygen and promoted development of the follicle (for discussions see Cortvrindt and Smitz, 1997; Hu et al., 2001) (Figure 2C). Normal further development of follicles was indicated by the differentiation of granulosa cells into cumulus granulosa cells, which were completely surrounding the oocyte and layers of mural follicle cells at the periphery of the follicle. From day 8 onwards, some follicles (8%) began to form a fluid-filled space, an antrum-like cavity (Figure 4), which could be visualized as a translucent area within the granulosa cell masses under the stereomicroscope (Figure 2D). During the following 4 days, the size of the follicles increased dramatically. At day 12 of

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culture, more than 80% of the cultured follicles reached the pre-ovulatory stage with a large antrum-like cavity (Figures 2D and 4). At the end of the culture, the survival rate was calculated by determining the percentage of viable follicles over the sum of isolated early preantral follicles (Table I). Follicle survival in the culture was considered positive as long as an oocyte remained surrounded by granulosa cells and attached to the dish. In the present study, the survival rate of follicles was >99% (Table I) and the vast majority of the surviving follicles had formed an antral-like cavity (Figure 2D). On day 12 of culture, resumption of maturation and ovulation in vitro were induced by addition of rHCG and rEGF (Figures 1 and 2). Accordingly, muci®cation with expansion of cumulus cells was observed 16 h after the in vitro ovulatory stimulus (Figure 2 E). The percentage of follicles with muci®cation was >80% in this study.

Oestradiol and progesterone secretion pro®les in preantral follicle culture
The follicle cultures were characterized by a consistent increase in the secretion of oestradiol as measured in the conditioned medium throughout the in vitro culture period from day 4 onwards (Figure 4, left y-axis). Progesterone secretion increased characteristically only after the in vitro ovulatory stimulus (Figure 4, right y-axis), from day 12 to day 13 of culture. Similar to oestradiol, there was also some variation between progesterone secretion in conditioned medium from different plates, e.g. between 0.38 and 1.05 µg/l at day 12 and 2.29 and 5.02 µg/l at day 13, respectively, but the pattern with a dramatic increase due to the ovulatory trigger was consistent between plates and experiments. Generally, the patterns of hormone secretion observed in preantral follicle culture were similar to those characteristic for in vivo follicle hormone secretion (Fehrenbach et al., 1998).

Meiotic maturation of oocytes from different groups
Full resumption of meiotic maturation of mammalian oocytes is de®ned as the transition from dictyate stage with meiosis arrest at the late G2 stage to the metaphase II stage, when oocytes possess a first PB, become arrested at meiosis II and will be ovulated in vivo. The resumption of meiosis and the resolution of the nucleus (GVBD), the earliest visible morphological indicator of the initiation of oocyte nuclear maturation, are induced in vivo by the intrinsic pre-ovulatory LH surge or the administration of HCG in stimulated cycles. In cultures, it relies on the removal of the oocyte from its follicular inhibitory environment for IVM or the addition of rHCG and rEGF in this preantral follicle culture assay. Accordingly, we assessed oocyte diameter and meiotic stages by microscopic analysis and vital staining of chromosomes after collection of the oocytes from the different experimental groups following stimulation of meiotic resumption and in vitro or in vivo maturation for 16 h (Figure 1). Most oocytes of all groups possessed a ®rst PB (Figure 2F). However, as previously already noticed (Hu et al., 2001), the diameters of the oocytes differed between the experimental groups. Oocytes from follicle culture (IVG) had slightly but signi®cantly (P < 0.05) smaller diameters as compared with those grown and matured in vivo (in vivo control, IVC) (Table II, left side). There was no statistically signi®cant difference between diameters of oocytes from the IVG and those from the in vitro matured group (IVM) (Table II) as expected.

The percentage of oocytes which were competent to mature fully to metaphase II differed between all experimental groups.

In the IVG group, 13.1% of oocytes were unable to resume maturation (GV) (Table II) and 11.8% became arrested after GVBD. Still, the large majority (75%) emitted a ®rst PB (Table II). About 97% of the control oocytes (IVC) from littermates stimulated hormonally to undergo folliculogenesis and maturation in vivo (IVC) extruded a ®rst PB, while 87% of the ones freed from large follicles and maturing in vitro without follicle cells (IVM) progressed to second meiosis (Table II). While the rate of maturation was lower in the IVG group, the yield of oocytes with PB per female was highest in the IVG group. On average, nearly 50 PB oocytes were obtained per female (497 from 10 females) in this group, but only 30 oocytes per female in the IVM group (357 from 12 females) and 37 oocytes per female in the IVC group (261 from seven females).

Nuclear maturation of oocytes from different groups
Only those oocytes resuming maturation with GVBD and PB were subjected to further cytogenetic analysis. The results of the initial analysis of nuclear meiotic progression are summarized in Table II (right side). Of 558 oocytes with GVBD or PB from the IVG group, only 10.2% (7.7% in metaphase I and 2.5% in anaphase I) were still in meiosis I after 16 h of maturation, while the majority (80.2% in metaphase II and 3.9% in anaphase II) had reached second meiosis. Nearly 95% of the IVM oocytes had metaphase II chromosomes, and only 5.5% were partially competent to undergo maturation and were still at meiosis I with bivalent chromosomes after 16 h of culture (Figure 3a and Table II, right side). A few oocytes in the IVC group were also ovulated in meiosis I (2.7%); anaphase I (0.4%) or progressed to anaphase II (2.7%) in the hormonally stimulated cycles, while 94.2% had normal metaphase II chromosomes. Only in the group of oocytes obtained from preantral follicle culture were 5.7% diploid metaphase II oocytes observed containing 40 instead of 20 metaphase II chromosomes (Table II, right side). This indicates some asynchrony between nuclear and cytoplasmic maturation events in this group. Still, when comparing the average numbers of oocytes with metaphase II chromosomes which
were obtained per female in the three groups, the yield was again highest in the IVG group (44.7 oocytes per female) in comparison with the IVM group (only 24.3 oocytes per animal) and the IVC group (34.7 oocytes per female). In conclusion, the preantral follicle culture appears to be an efficient method to obtain high numbers of fully grown, meiotically competent oocytes at metaphase II.

Chromosomal constitution of PB oocytes from different groups

In the analysis of the fidelity of chromosome segregation we did not include the diploid oocytes which did not emit a PB. The cytogenetic analysis of oocytes with PB in which the number of metaphase II chromosomes could be identified unambiguously is shown in Table III. Of 259, 257 and 195 oocytes analysed in the IVG, IVM and IVC groups, over 95% were euploid with 20 metaphase II chromosomes. Hypoploidy rates were low (between 2.1 and 3.8%) and did not differ significantly between the three groups. Only a single oocyte in the IVC group showed true non-disjunction, as characterized with 2\(\times\)20 metaphase II chromosomes; MI, metaphase I with bivalent chromosomes; MI, metaphase II; MI, anaphase II with 2\(\times\)20 chromosomes; Diploid oocyte, diploid metaphase II oocyte with separated homologues but without polar body.

In a separate set of experiments, follicles were exposed to colchicine at a concentration of 2.5 or 5 nM during the last 16 h of culture, when meiotic maturation was induced by rHCG and rEGF. Colchicine significantly (\(P < 0.05\)) interfered with the muci®cation of cumulus cells at the higher dose as compared with the control. The rate of muci®cation dropped from 82.3% in the control to 68.7 and 42.2% for 2.5 and 5 nM colchicine, respectively. Although colchicine had no significant effect on meiotic resumption with respect to the percentage of oocytes remaining in the dictyate stage with intact GV, oocyte maturation to meiosis II was dose dependently affected. Of the 112, 138 and 179 oocytes collected from preantral follicle culture in the control, 2.5 and 5.0 nM groups, 75.9, 64.5 and 52.5% matured to metaphase II stage, respectively (Table IV). The difference from the control was statistically signi®cant at 5.0 nM colchicine (\(P < 0.01\)). In accordance with expectation, most GVBD oocytes arrested at meiosis II possessed bivalent chromosomes and the percentage of oocytes with bivalents increased from 6.8% in the control to 16.8 and 28.3% in the 2.5 and 5.0 nM colchicine groups, respectively. Correspondingly, frequencies of oocytes containing metaphase II chromosomes increased dose dependently from 80.7% in the control to 70.3 and 57.5% in groups exposed to 2.5 and 5.0 nM colchicine, respectively. The difference was signi®cant for both treatment groups (\(P < 0.05\) in the 2.5 nM group; \(P < 0.01\) in the 5.0 nM group).

The results of the numerical chromosome analysis are also presented in Table IV. In all groups hypoploidy rate exceeded hyperploidy rate. Hypoploidy increased with increasing dose of colchicine, reaching signi®cant difference from the control in the 5 nM colchicine group. Colchicine exposure also dose

### Table III. Cytogenetic analysis of metaphase II oocytes from IVG, IVM, and IVC

<table>
<thead>
<tr>
<th>Ploidy</th>
<th>Aberrant chromosome behavior</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of oocytes</td>
<td>Hypoploid (%)</td>
</tr>
<tr>
<td>IVG</td>
<td>259</td>
</tr>
<tr>
<td>IVM</td>
<td>257</td>
</tr>
<tr>
<td>IVC</td>
<td>195</td>
</tr>
</tbody>
</table>

\(^a\)Includes only oocytes with \(\geq17\) metaphase II chromosomes.

\(^b\)Includes one oocyte with 19 MII chromosomes and 2 chromatids.

\(^c\)Oocyte with 19 MII chromosomes and 1 bivalent.

### Table IV. Cytosplasmic and nuclear maturation and chromosome constitution of oocytes grown and matured in preantral follicle culture in the absence (control) or presence of colchicine for the last 16 h

<table>
<thead>
<tr>
<th>Control</th>
<th>112 (12.5)</th>
<th>13 (11.6)</th>
<th>85 (75.9)</th>
<th>88 (6.8)</th>
<th>2 (2.3)</th>
<th>71 (80.7)</th>
<th>3 (3.4)</th>
<th>6 (6.8)</th>
<th>49 (3.6)</th>
<th>46 (3.9)</th>
<th>0 (0.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 nM colchicine</td>
<td>138 (13.8)</td>
<td>30 (21.7)</td>
<td>89 (64.5)</td>
<td>101 (16.8)(^a)</td>
<td>3 (2.9)</td>
<td>71 (70.3)</td>
<td>2 (2.0)</td>
<td>8 (7.9)</td>
<td>52 (6.1)</td>
<td>43 (82.7)</td>
<td>3 (5.8)</td>
</tr>
<tr>
<td>5.0 nM colchicine</td>
<td>179 (14.5)</td>
<td>39 (33.0)(^b)</td>
<td>94 (52.5)(^b)</td>
<td>106 (28.3)(^b)</td>
<td>2 (1.9)</td>
<td>61 (37.5)(^b)</td>
<td>3 (2.8)</td>
<td>10 (9.4)</td>
<td>45 (18.7)</td>
<td>30 (66.7)(^b)</td>
<td>7 (15.6)(^b)</td>
</tr>
</tbody>
</table>

\(n\), number of oocytes; GV, germinal vesicle; GVBD, germinal vesicle breakdown; PB, polar body; MI, metaphase I with bivalent chromosomes; AI, anaphase I with 2\(\times\)20 metaphase II chromosomes; MI, metaphase II; AII, anaphase II with 2\(\times\)20 chromatids; Diploid oocyte, diploid metaphase II oocyte with separated homologues but without polar body.

\(^a\)Significant difference to control; \(P < 0.05\).

\(^b\)Significant difference to control; \(P < 0.01\).

\(^c\)Including one oocyte with 19 MII chromosomes and 1 bivalent.

Influence of colchicine on oocyte maturation and chromosome distribution in preantral follicle culture

In a separate set of experiments, follicles were exposed to colchicine at a concentration of 2.5 or 5 nM during the last 16 h of culture, when meiotic maturation was induced by rHCG and rEGF. Colchicine significantly (\(P < 0.05\)) interfered with the muci®cation of cumulus cells at the higher dose as compared with the control. The rate of muci®cation dropped from 82.3% in the control to 68.7 and 42.2% for 2.5 and 5 nM colchicine, respectively. Although colchicine had no significant effect on meiotic resumption with respect to the percentage of oocytes remaining in the dictyate stage with intact GV, oocyte maturation to meiosis II was dose dependently affected. Of the 112, 138 and 179 oocytes collected from preantral follicle culture in the control, 2.5 and 5.0 nM groups, 75.9, 64.5 and 52.5% matured to metaphase II stage, respectively (Table IV). The difference from the control was statistically signi®cant at 5.0 nM colchicine (\(P < 0.01\)). In accordance with expectation, most GVBD oocytes arrested at meiosis II possessed bivalent chromosomes and the percentage of oocytes with bivalents increased from 6.8% in the control to 16.8 and 28.3% in the 2.5 and 5.0 nM colchicine groups, respectively. Correspondingly, frequencies of oocytes containing metaphase II chromosomes decreased dose dependently from 80.7% in the control to 70.3 and 57.5% in groups exposed to 2.5 and 5.0 nM colchicine, respectively. The difference was signi®cant for both treatment groups (\(P < 0.05\) in the 2.5 nM group; \(P < 0.01\) in the 5.0 nM group).
dependently increased hyperploidy rate, although the increase reached statistical significance only in the 5 nM group relative to the control ($P < 0.05$) (Figure 3f). We did not attempt to increase the numbers of oocytes in the three groups to define the lowest effective dose levels for aneuploidy induction in this pilot study. Already the limited number of oocytes clearly show a dose–response relationship which demonstrates the sensitivity of this assay and indicates its usefulness for aneuploidy screening. One oocyte with a single chromatid was observed in the highest treatment group, which might be derived from untimely segregation of chromatids at meiosis I instead of at anaphase II (predisdivision) (for a discussion see Sun et al., 2001). In conclusion, colchicine interfered dose dependently with meiotic progression to metaphase II and with fidelity of chromosome segregation in the follicle-enclosed, in vitro matured oocytes.

**Discussion**

**Methods of folliculogenesis and oocyte growth in vitro**

Mouse follicle cultures have been established to study factors essential for oocyte development during in vitro folliculogenesis (see for example Eppig and Schroeder, 1989; Nayudu, 1994; Spears et al., 1994; Cortvrindt et al., 1996; Eppig and O’Brien, 1996; Hartshorne, 1997; Van den Hurk et al., 1997; Hu et al., 2001). In the present study, early preantral follicles with a mean diameter of 119.97 ± 9.59 mm (stage 3b or 4) (Pedersen and Peters, 1968) were isolated from 12–14 day old mice and cultured for 13 days to provide ‘in vitro ovulated’ oocytes (Cortvrindt et al., 1996; Hu et al., 2001). We used colchicine as a model aneugen to demonstrate the suitability of this follicle culture method for the detection of chemically induced aneuploidy.

The main signs of follicle growth preceding oocyte maturation and ovulation in vitro are a dramatic increase in oocyte size, the efficient proliferation and differentiation of granulosa cells and the recruitment of the endocrine, steroidogenic theca cells from the interstitial tissue. At the age of 12–14 days, the ovary of the mouse strain used in this study possesses the first wave of growing preantral follicles, with two layers of cuboidal granulosa cells (type 3b) (Pedersen and Peters, 1968). Mechanical isolation of homogeneous populations of follicles is therefore fairly easy since the ovaries contain large numbers of preantral follicles but only a few more advanced stages (Cortvrindt et al., 1996). Preantral follicles cultured for 6–8 days consist already of a mean of 1350 and 4500 granulosa cells, respectively (Cortvrindt and Smitz, 1997), sufficient to form an antral follicle in vivo (Gosden et al., 1993). Concurrently, the granulosa cells differentiate into two subpopulations with distinct endocrine characters, the mural and cumulus granulosa cells. An antrum is formed in most follicles by 12 days of culture. The mural granulosa cells are close to the basement membrane and connected to each other by gap junctions (see for example Ackert et al., 2001). The oocyte is surrounded by the cumulus granulosa cells, which are also connected to each other and the germ cell by gap junctions (for a review see Eppig, 2001). The present data show that under appropriate culture conditions, follicle development in vitro follows the same differentiation pattern as seen in vivo.

An intrinsic, neuronally governed gonadotrophin surge initiates the maturation of the cumulus–oocyte complex in vivo, and induces the muficication and detachment of cumulus from the oocyte accompanied by resumption of meiosis. We showed that >80% of the follicles cultured in vitro developed to the pre-ovulatory stage with an antrum-like cavity on day 12 (Table I). Comparable with the in vivo situation, the majority of follicles exhibited expansion and mucification of the cumulus 16 h after the in vitro ovulatory stimulus by recombinant hormones.

Unlike the cyclical patterns of gonadotrophin surges characteristic of in vivo folliculogenesis, preantral follicles were continuously exposed to supra-physiological concentrations of FSH, leading to the survival of the majority of follicles recruited for culture. This presumably influences the yield of oocytes capable of fully maturing to metaphase II and explains the differences in numbers of oocytes per female emitting a PB between the three experimental groups. In vivo only a few growing follicles will be selected to release their oocytes for fertilization, although a large number of primordial follicles can start to grow initially (Faddy et al., 1976). Most follicles are destined to undergo atresia and die because they cannot respond to the low concentrations of hormones and survival factors, e.g. fibroblast growth factor, kit ligand, etc. (Reynaud and Driancourt, 2000), available in vivo at the appropriate developmental stage (Pedersen, 1970; Baker et al., 2001). In the present study >90% of the follicles survived to the pre-ovulatory stage (Table I). The handling and refreshment steps were easy and provided conditions for mass culture required for quantitative assays.

**Hormone secretion profiles in culture**

Hormonal homeostasis modulates the kinetics of in vivo maturation of mouse oocytes (Polanski, 1986). During folliculogenesis, the theca cells begin to respond to LH and synthesize androgens (Erickson et al., 1985). Granulosa cells are incapable of producing oestradiol until the theca differentiates into androgen-producing cells. The secretion of oestra diol progressively increases in vivo up to the late pre-ovulatory stage of folliculogenesis. In our study, we isolated follicles which expressed steroidogenic activity. On day 4 of culture oestradiol was already detectable in spent medium and the release of oestradiol increased progressively in the medium from surviving follicles until day 12 of culture, although there was some variation in the absolute levels of steroids (for a discussion see Cortvrindt et al., 1998).

Granulosa cells transform in vivo from an oestrogen-secreting tissue into a progesterone-secreting one in response to the final LH surge (McNatty and Sawers, 1975; McNatty et al., 1979; McNatty, 1981). We also found a marked increase in the rate of progesterone production in the preantral follicle cultures after the addition of rHCG/rEGF, indicating that the granulosa cells were competent to express LH receptors and to respond normally to ligand binding. Therefore, preantral follicle culture allows the examination of the influences of potentially endocrine disrupting chemicals on folliculogenesis and oocyte quality in a well-defined system, especially with a view of disturbances reducing the capacity of oocytes to form a functional spindle and segregate chromosomes with high fidelity.

**Oocyte growth and maturation to meiosis II during follicle culture**

During oocyte growth most molecules necessary for the resumption of meiosis, fertilization and early development until full zygotic gene activation are already synthesized (for a review see Eichenlaub-Ritter and Peschke, 2002). In the present study, mature oocytes of the IVG group had a mean
diameter of 69.7 μm, comparable with that of the IVM group but significantly smaller than in the IVC (Table II). More importantly, our cytogenetic results show that nearly 90% of the oocytes obtained from follicles could resume meiosis in response to the ovulatory stimulus and 75% of all oocytes from surviving follicles were capable of extruding a first PB (Table II). Oocytes obtained by the same culture method were also capable of normal embryonic development to the blastocyst stage following in vitro fertilization (Cortvrindt et al., 1996; Smitz et al., 1996) and developed to normal, live young after transfer to foster mothers (Cortvrindt and Smitz, 2002). Therefore, the preantral follicle culture method appears suitable to analyse first and second meiotic divisions as well as parameters important for early embryogenesis. Nearly all oocytes (94%) obtained by superovulation (IVC) and by in vivo growth (IVM) were capable of maturation to metaphase II, while the rate was significantly lower (80.2%) in the IVG group (Table II). In contrast, the yield of PB oocytes or oocytes at meiosis II per individual animal was higher in the IVG group (44.7 oocytes per female) as compared with the two other groups (24.3 and 34.7 per female, respectively, in the IVM and IVC groups). It is remarkable that, despite the reduced selection, none of the metaphase II oocytes from follicles entirely grown in vitro suffered from non-disjunction to produce hyperploid oocytes. Thus, preantral follicle culture appears compatible with high fidelity chromosome segregation, which is a prerequisite for assessments of aneuploidy induction by possible or suspected aneugens. Therefore, it is possible that the method can help to reduce in vivo testing with administration of chemicals to experimental animals and may restrict the numbers of animals needed for aneuploidy research.

Under the applied conditions, in vitro growth and maturation resulted in uncoupling of oocyte nuclear and cytoplasmic maturation in some oocytes from preantral follicle culture (Hu et al., 2001). Cytoplasmic maturation can be improved by slightly modifying culture conditions in the future, for example by reducing the concentrations of gonadotrophins, since asynchrony between nuclear and cytoplasmic maturation has been documented in some strains of mice which respond to exogenous gonadotrophins by ovulating metaphase I as well as diploid oocytes (Maudlin and Fraser, 1977; Hansmann and El-Nahass, 1979; Hansmann et al., 1983; Polanski, 1986; Mailhes et al., 1995). Diploidy levels are fairly constant (5–7%) in the preantral follicle culture method and comparisons between controls and treated groups of follicles can disclose whether or not a test chemical has polyploidy-inducing activity (see below).

An increased risk of errors in chromosome segregation at anaphase II appears to be correlated with prolonged metaphase II arrest and displacement of chromosomes (Eichenlaub-Ritter et al., 1986), spindle pericentral migration (Eichenlaub-Ritter et al., 1986; Carabatsos et al., 2000) and precocious segregation of chromatids (Mailhes et al., 1998). Mouse oocyte spindles shown by anti-tubulin immunofluorescence at 16 h after hCG/rEGF administration fully confirmed previous reports (Hu et al., 2001). All oocytes possessed a normal barrel-shaped anastral spindle and well-aligned chromosomes as seen in vivo (Hu et al., 2001), thereby fulfilling a prerequisite for normal chromosome segregation at meiosis II.

High fidelity of chromosome segregation in IVG oocytes

We focused this study on the cytogenetic analysis of metaphase II oocytes, since metaphase II presents the end-point of oocyte development within a follicle before ovulation. Human trisomy data indicate that the first meiosis of oogenesis is particularly susceptible to disturbances of chromosome segregation (Eichenlaub-Ritter, 2000; Hassold and Hunt, 2001). We here showed for the first time that the vast majority of PB oocytes from preantral follicle culture with defined sizes of follicles isolated initially and grown and matured under strictly controlled conditions to meiosis II in vitro were euploid with 20 metaphase II chromosomes (>95%; Table III). There were no significant differences in the frequencies of euploid or hyperploid oocytes between the IVG group and the IVM or IVC group. Although one of the IVG oocytes (0.4%) possessed two chromatids, this probably presented a balanced chromosomal constitution, and low levels of presegregation may also be observed in in vivo matured oocytes from our hybrids or other strains of mice (Mailhes et al., 1998; Yin et al., 1999b).

Other numerical chromosomal aberrations, such as single chromatids and non-disjunction, were not observed. Also, there was no evidence for structural chromosomal aberrations. In conclusion, when follicles were cultured as a whole, oocyte growth and maturation in vitro displayed high fidelity chromosome segregation.

Preantral follicle culture to assess the aneugenic potential of chemicals in mouse oocytes

Faithful chromosome segregation during anaphase requires the assembly and proper function of a microtubular spindle apparatus. A so-called ‘spindle checkpoint’ can detect a multitude of spindle defects, ranging from massive spindle disruption to the presence of a single unattached kinetochoore or chromosome displacement in mitotic and male meiotic cells (Rudner and Murray, 1996) and minute alterations in microtubule polymerization kinetics (Hauf et al., 2003). A spindle checkpoint also exists during oogenesis (Fulka et al., 1995; Soewarto et al., 1995; Brunet et al., 1999). However, evidence from human oocytes of reproductively aged woman (Battaglia et al., 1996; Volarcik et al., 1998) and from mutant or chemical-exposed mouse oocytes (LeMaire-Adkins et al., 1997; Yin et al., 1999b; Hodges et al., 2002) indicate that it is rather permissive under certain conditions. This leaky checkpoint may be the major cause of the high rates of non-disjunction in female gametogenesis. It is known that aneugens may directly affect spindle formation during resumption of oocyte maturation. However, aneugens may also indirectly interfere with spindle formation by disturbing the orchestrated interactions between the oocyte and the somatic compartment during folliculogenesis and thereby contribute to compromised checkpoint control (Hodges et al., 2002). Therefore, we exposed oocytes maturing within a follicle in this pilot study to the classical spindle poison colchicine.

Initially we used a higher concentration of colchicine in the in vitro oocyte assay (20 nM; data not shown), which was the lowest concentration capable of increasing aneuploidy in human lymphocyte cultures (Elhajouji et al., 1997). However, this concentration completely arrested meiotic progression of the oocytes. Thus, it appears that the naked oocytes as well as the follicle-enclosed in vitro grown oocytes, many of which became arrested in first M phase already with 2.5 nM colchicine, are much more susceptible to this aneugen than mitotically dividing somatic cells. Thus an almost 10-fold lower concentration of the chemical (2.5 nM colchicine) inducing mitotic non-disjunction already influenced the rate of meiotic progression to metaphase II in oocytes from follicle
culture and a 4-fold lower concentration increased the frequency of hyperploidy significantly. These observations suggest that colchicine-like aneugens may have two targets, which could contribute to the high susceptibility for disturbances in chromosome segregation in a synergistic fashion: the oocyte and its companion follicle cells. An influence of colchicine on cumulus cells can be deduced from the reduced rate of mitoculization. At the initiation of meiotic resumption, oocytes are still in direct contact with the surrounding follicle cells. It is therefore conceivable that altered signalling between the two compartments may contribute directly and/or indirectly to disturbances of chromosome segregation.

Conclusions

The present studies suggest that the preantral follicle culture may present a suitable method to assess the significance of alterations in the microenvironment of the follicle and oocyte before and during the nuclear maturation process. It may be useful to explore the different responses of oocytes and somatic cells in the follicle to exposures with environmental chemicals. Furthermore, it may provide a method to study the hazards from acute as compared with chronic exposures of oocytes and follicles to environmental chemicals, endocrine-disrupting agents and pharmaceuticals, as well as their active metabolites for chromosomal constitution and developmental potential of the ovum and the embryo.

The IVG method presented here, together with the demonstration of the chromosomal integrity of oocytes and of the colchicine-induced aneuploidy effects are a promising step towards establishing a novel in vitro assay for aneugen screening in female meiosis. It certainly requires further verification and validation with aneugens with different mechanisms of action. The ease of obtaining large numbers of high quality oocytes in a narrow time frame from a physiological follicle culture system suggests that it can be employed effectively for the detection of adverse influences of environmental chemicals on female germ cells at earlier, intermediate and late developmental stages. More importantly, this assay allows exploration of the significance of germ cell–somatic cell interactions, which are not only vital for full fertility but are probably vulnerable processes involved in the genesis of trisomy and genetic diseases in humans.

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References


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