Genetic strain variations in the metaphase-II phenotype of mouse oocytes matured *in vivo* or *in vitro*

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Abstract

The interplay between genetic and epigenetic factors plays a central role in mammalian embryo production strategies that superimpose *ex vivo* or *in vivo* manipulations upon strain background characteristics. In this study, we examined the relationship between genetic background and the phenotypic properties of mouse metaphase-II (M-II) oocytes that were matured under *in vivo* (IVO) or *in vitro* conditions, either in a basal (IVM) or a supplemented (IVM +) medium. Differences existed amongst inbred (C57BL/6), outbred (CF-1, Black Swiss, NU/NU) and hybrid lines (B6D2F1) induced to superovulate with regard to cytoplasmic microtubule organizing center (MTOC) number but not spindle size or shape, except for larger and asymmetrical spindles in Black Swiss oocytes. When oocytes were matured in culture, meiotic spindle and cytoplasmic phenotypic properties of M-II oocytes were affected relative to *in vivo* conditions and between strains. Specifically, measures of meiotic spindle size, shape, polar pericentrin distribution and cytoplasmic MTOC number all revealed characteristic variations. Interestingly, the overall reduction in cytoplasmic MTOC number noted upon IVM was concomitant with an overall increase in spindle and polar body size. Maturation under IVM + conditions resulted in a further decrease in cytoplasmic MTOC number, but spindle and polar body characteristics were intermediate between IVO and IVM. How these oocyte phenotypic properties of maternal origin may be linked to predictive assessments of fecundity remains to be established.

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Introduction

Despite the widespread usage of mice in the development of assisted reproductive technologies, and the availability of various genetic strains in which to assess fecundity determinants, surprisingly little is known about the genetic basis of oocyte quality. Genetic strain variations in mice with regard to *in vitro* fertilization (Kaleta 1977), cleavage rates (McLaren & Bowman 1973, Luckett & Mukherjee 1986), pH regulation (Steeves *et al.* 2001), parthenogenetic activation (Gao *et al.* 2004, Ibáñez *et al.* 2005) and preimplantation development (Suzuki *et al.* 1996) buttress the well-known relationship between fecundity and gen-

etic background amongst hybrid F1, inbred and outbred lines (Silver 1995). Additionally, variations in hormone sensitivity influence ovulation rate amongst different mouse strains (Spearow 1988, Spearow *et al.* 1999) and the rate of meiotic maturation *in vivo* or *in vitro* can vary depending on genetic background (Polanski 1986, 1997). Thus, it seems likely that some of the genetic factors that specify embryo quality find their roots in maternal pre-conceptional processes associated with oogenesis and ovulation. Of note in this regard is the potential interplay of genetic and environmental influences during oocyte maturation.

It is well established that the environment to which the oocyte is exposed during maturation influences the developmental potential of the resulting embryos and, in many mammalian species, *in vitro* matured oocytes exhibit lower rates of embryonic development than *in vivo* matured oocytes after fertilization (Eppig & O'Brien 1998, Niemann & Wrenzycki 2000, Moor & Dai 2001, Trounson *et al.* 2001). Additional properties distinguish *in vitro* from *in vivo* matured oocytes, such as mitochondria and ATP content (Combelles & Albertini 2003, Nishi *et al.* 2003), protein synthesis (Schultz *et al.* 1978), translation and transcription patterns (Eichenlaub-Ritter & Peschke 2002, Rodriguez & Farin 2004), glutathione content (de Matos *et al.* 2003) and imprinting/methylation patterns in embryos derived from *in vivo* and *in vitro* oocytes (Lucifero *et al.* 2002). Whether such potential determinants of oocyte quality result from genetic predisposition or environmental sensitivities remains unknown.

Recently we have shown that the temporal progression of cytoplasmic and nuclear remodeling events were distinctly different in *in vivo* matured oocytes compared with oocytes matured under basal *in vitro* conditions in the outbred CF-1 mouse strain (Sanfins *et al.* 2003, 2004), further supporting the notion that *in vivo* produced ova are generally of superior quality with respect to production of healthy embryos and offspring. It is well established that during *in vitro* maturation, although nuclear maturation takes place, complete cytoplasmic maturation is not assured (Eppig *et al.* 1996). Therefore, important factors must be added to the culture medium in an attempt to recapitulate the follicular environment and improve cytoplasmic maturation. With this in mind, we began to design a supplemented maturation medium (IVM+), based on several reports addressing the positive effects of certain components on improving cytoplasmic maturation and, consequently, oocyte maturation efficiency. Specifically, addition of D-glucosamine as a substrate for hyaluronic acid (HA) synthesis improves cumulus expansion (Chen *et al.* 1990, Dell'Aquila *et al.* 2004). Cysteine is a critical factor for the synthesis of glutathione (de Matos *et al.* 1996), which has been shown to be important in protecting the oocyte from oxidative damage and essential, after fertilization, for sperm chromatin decondensation, male pronuclear formation and subsequent development to the blastocyst stage (Perreault *et al.* 1988, de Matos *et al.* 1995). Ascorbic acid, another anti-oxidant, prevents follicular apoptosis in mouse follicles and increases developmental competence of *in vitro* matured oocytes (Eppig *et al.* 2000, Tatemoto *et al.* 2001). Finally, follicle-stimulating hormone (FSH) has been shown to stimulate *de novo* HA synthesis and cumulus expansion (Eppig 1979, 1980) and is routinely added to culture media to improve maturation and oocyte developmental competence (Merriman *et al.* 1998, Eppig *et al.* 2000).

The aim of the present study was to analyze the potential effect of both the genetic background and the maturation conditions on the metaphase-II (M-II) phenotype of

mouse oocytes. With this aim, M-II oocytes from five different strains of mice were obtained either after superovulation (IVO) or by culture in a basal (IVM) or in a supplemented (IVM+) maturation medium, and their phenotypic properties in terms of meiotic spindle size and organization, number of cytoplasmic microtubule organizing centers (MTOCs) and first polar body characteristics were compared. The results obtained reveal distinct phenotypic variations in the organization of the microtubule-centrosome complex based upon genetic background that are subjected to epigenetic changes during *in vitro* maturation.

Materials and Methods

Collection, culture and fixation of mouse oocytes

Mouse strains used as oocyte donors were B6D2F1 (C57BL/6 × DBA/2), C57BL/6 and CF-1, as common representatives of hybrid, inbred and outbred strains respectively. In addition, oocytes collected from Black Swiss and from immunodeficient homozygous nude (NU/NU) females, two other outbred strains that were being used at the time for other work by our group, were also included in the study. B6D2F1, C57BL/6, CF-1 and NU/NU female mice were obtained from Charles River Laboratories (Wilmington, MA, USA) and Black Swiss mice were obtained from Taconic (Germantown, NY, USA). All animals were handled according to the *Guide for Care and Use of Laboratory Animals* (National Academy of Science 1996) and to protocols approved by the Tufts University Institutional Animal Care and Use Committee. Animals were maintained on a 14 h light:10 h darkness photoperiod under constant temperature and relative humidity conditions, and food and water were provided *ad libitum*.

In vivo matured oocytes (IVO) were obtained from mice (8–12 weeks old) previously injected with 5 IU equine chorionic gonadotropin (eCG; Calbiochem, San Diego, CA, USA) to stimulate follicular development, followed 48 h later by 5 IU human chorionic gonadotropin (hCG; Calbiochem) to induce ovulation. Oocytes were collected from oviducts 16 h after hCG injection in a collection medium consisting of HEPES-buffered Eagle's minimum essential medium (MEM) with Hanks' salts (Gibco-Invitrogen Life Technologies, Grand Island, NY, USA) supplemented with 100 IU penicillin/ml, 100 µg streptomycin/ml and 0.3% (w/v) bovine serum albumin (BSA; Sigma, St Louis, MO, USA). The mean number ($\pm$ S.E.M.) of ovulated oocytes recovered per female was as follows: B6D2F1 = 20.3 ± 0.3 , C57BL/6 = 16.7 ± 2.9 , Black Swiss = 13.9 ± 1.1 , CF-1 = 19.8 ± 2.1 and NU/NU = 27.9 ± 1.3 oocytes/female (15 females per strain). Following removal of cumulus cells by a 5-min treatment with 150 U bovine testicular hyaluronidase/ml (Sigma) in collection medium at room temperature, oocytes were immediately fixed and extracted for 30 min at 37°C in a microtubule stabilizing buffer (0.1 mol

PIPES/ml, pH 6.9, 5 mmol $\text{MgCl}_2 \times 6\text{H}_2\text{O}$ /ml and 2.5 mmol EGTA/ml) containing 2% (v/v) formaldehyde, 0.1% (v/v) Triton X-100, 1 μmol taxol/ml, 10 U aprotinin/ml, 1 mmol dithiothreitol/ml and 50% (v/v) deuterium oxide (Messinger & Albertini 1991). Fixed oocytes were stored at 4°C in a blocking solution of phosphate-buffered saline (PBS) containing 2% (w/v) BSA, 2% (w/v) powdered milk, 2% (v/v) normal goat serum, 0.1 mol glycine/ml and 0.01% (v/v) Triton X-100 until further processing.

For *in vitro* maturation experiments, mice (8–12 weeks old) were injected 45–48 h earlier with 5 IU eCG and cumulus-enclosed oocytes were collected by follicular puncture in collection medium. Immature oocytes surrounded by cumulus cells were selected and were cultured for 16 h in either a basal maturation medium (IVM) consisting of Eagle's MEM supplemented with Earle's salts (Gibco-Invitrogen Life Technologies), 2 mmol glutamine/ml, 0.23 mmol pyruvate/ml, 100 IU penicillin/ml, 100 μg streptomycin/ml and 0.3% (w/v) BSA or in the same medium additionally supplemented with 0.5 mg D-glucosamine/ml (Sigma), 0.6 mmol cysteine/ml (Sigma), 0.02 μmol ascorbic acid/ml (Sigma) and 0.2 ng ovine FSH/ml (National Institute of Diabetes and Digestive and Kidney Diseases, Baltimore, MD, USA) (IVM +). All cultures were maintained in a humidified atmosphere of 5% CO_2 in air. After removing cumulus cells by gentle pipetting, oocytes were fixed and stored at 4°C as described above until further processing.

Processing of oocytes for fluorescence microscopy

For each experiment, IVO, IVM and IVM + oocytes from the five strains of mice were processed in parallel for phenotypic evaluation by fluorescence microscopy. In this way, spindle size and morphology, chromatin and cytoplasmic MTOCs could be evaluated simultaneously by digital imaging in samples processed under identical conditions. Oocytes were incubated sequentially with primary and secondary antibodies for 1 h at 37°C with shaking, followed by three 15-min washes in blocking solution. First, MTOCs were labeled using a rabbit polyclonal anti-pericentrin antibody (Doxsey *et al.* 1994) followed by affinity-purified Texas Red-conjugated donkey anti-rabbit IgG (1:150; Jackson ImmunoResearch Laboratories Inc, West Grove, PA, USA). Secondly, microtubules were labeled by using a 1:1 mixture of mouse monoclonal anti- α -tubulin and anti- β -tubulin antibodies (Sigma) at a 1:100 final dilution, followed by incubation with affinity-purified fluoresceinated donkey anti-mouse IgG (1:50, Jackson ImmunoResearch Laboratories, Inc.). Oocytes were then mounted in a 50% glycerol/PBS solution containing 0.2% (w/v) sodium azide (Sigma) and 1 μg Hoechst 33258/ml (Polysciences Inc., Warrington, PA, USA) to label chromatin. Incubation of oocytes in secondary antibodies alone failed to yield detectable staining. Labeled oocytes were analyzed on a Zeiss IM-35 inverted microscope using 50 W mercury arc lamp and a 40 $\times$ Neofluor objective. Digital images were collected with a Hamamatsu Orca ER

digital camera (model C4742-95) interfaced with a MetaMorph Imaging System 5.0 (Universal Imaging Corp., Downingtown, PA, USA). A triple band pass dichroic and automated excitation filter wheel permitted collection of spatially registered images with minimal magnification distortion. Digital images of single oocytes within each experimented group were collected under identical exposure conditions for each of the flours.

Quantification of spindle size and cytoplasmic MTOC numbers

Oocytes were first analyzed to determine meiotic status based upon previously reported criteria (Albertini 1992). Digital images were then obtained from M-II oocytes with integration times of 500–1000 ms for the fluorescein channel at subsaturation pixel intensities and thresholding, and spatial measurements were recorded with the MetaMorph Imaging System after calibration with a stage micrometer. Images with clear definition of spindle boundaries and of appropriate orientation were used to obtain measurements of spindle length (from pole to pole), spindle width at the equator and spindle pole width, and from these values the total area of the spindle was calculated for each oocyte. To account for half-spindle asymmetry, a formula was derived for determination of spindle area that would take into account differences between each half spindle (Sanfins *et al.* 2003).

For each group of oocytes, the organization of pericentrin material at the spindle poles was also analyzed, and characterized as solitary or multiple foci and c-shaped aggregates, as previously described (Carabatsos *et al.* 2000). Additionally, the number of cytoplasmic MTOCs was determined in each oocyte based on pericentrin-positive foci that were not spindle associated (Messinger & Albertini 1991).

Statistical analysis

Experiments were performed in parallel for the five different mouse strains and the three maturation conditions and were repeated twice. The percentages of IVO, IVM and IVM + oocytes at M-II in the five strains analyzed were compared by Chi-square analysis. Two-way ANOVA by strain and maturation conditions (IVO, IVM, IVM +) were used to compare spindle dimensions (length, width, pole width and area) and the number of cytoplasmic MTOCs. The Newman–Keul's multiple range test was used for post-hoc comparisons. $P < 0.05$ was considered significant.

Results

Meiotic competence differences between IVO, IVM and IVM + oocytes are strain specific

The ability of oocytes to complete the first meiotic division and arrest at the M-II stage was analyzed in oocytes

Figure 1 Maturation rates of oocytes from five strains of mice matured under *in vivo* (IVO) or *in vitro* (IVM and IVM +) conditions. Data represent the combined results of two replicate experiments for each maturation treatment, and the total number of oocytes analyzed (n) for each strain and treatment are indicated at the bottom of the columns. Different letters at the top of the columns represent significant differences ($P < 0.05$) across strains within each maturation treatment (Chi-square).

matured under *in vivo* (IVO) or *in vitro* (IVM and IVM +) conditions for each of the five strains of mice used in this study. As shown in Fig. 1, different extents of maturation were observed for each group. Under IVO conditions, all strains completed maturation except C57BL/6, which exhibited a significantly lower percentage of M-II oocytes due to a delay in meiotic progression at the

anaphase–telophase of meiosis I. Under IVM and IVM + conditions, lower maturation rates were observed for B6D2F1 and C57BL/6 oocytes respectively when compared with oocytes from the three outbred strains. Overall, for all strains, the percentage of M-II oocytes in IVM was significantly lower than in IVO ($P < 0.05$), except in the C57BL/6 group where a significantly higher

Figure 2 M-II spindles of (A–E) IVO, (a–e) IVM and (a'–e') IVM + oocytes in the five strains of mice studied. Tubulin (green), pericentriolar (red) and chromosomes (blue) are depicted. Note the pointed/tapered-shaped spindles with solitary pericentriolar foci at the poles of IVO oocytes (A–E), contrasting with the barrel-shaped spindle with fragmented pericentriolar foci at the poles of IVM oocytes (a–e). IVM + oocytes exhibit big spindles but with focused pericentriolar aggregates at the poles (a'–e').

Table 1 Spindle dimensions in M-II oocytes from five different mouse strains collected after ovulation (IVO) or matured *in vitro* in a basal (IVM) or a supplemented (IVM +) maturation medium. Values are means $\pm$ S.E.M.

Maturation conditions	Spindle dimensions	Strain of oocytes				
		B6D2F1	C57BL/6	Black Swiss	CF-1	NU/NU
IVO	Length (μm)	22.3 $\pm$ 0.4	23.1 $\pm$ 0.5	22.8 $\pm$ 0.3	22.4 $\pm$ 0.5	21.9 $\pm$ 0.3
	Width (μm)	11.5 $\pm$ 0.2 ^a	11.9 $\pm$ 0.5 ^a	14.2 $\pm$ 0.4 ^b	11.8 $\pm$ 0.3 ^a	11.7 $\pm$ 0.3 ^a
	Pole width (μm)	3.6 $\pm$ 0.1	3.9 $\pm$ 0.1	4.3 $\pm$ 0.2	3.3 $\pm$ 0.1	3.7 $\pm$ 0.1
	Area (μm^2)	168.6 $\pm$ 3.9 ^a	182.8 $\pm$ 6.8 ^{a,b}	211.8 $\pm$ 6.3 ^b	169.8 $\pm$ 5.6 ^a	170.2 $\pm$ 4.9 ^a
	<i>n</i>	29	21	30	33	31
IVM	Length (μm)	26.0 $\pm$ 0.5 ^{a,b}	25.3 $\pm$ 0.6 ^a	27.6 $\pm$ 0.7 ^b	24.6 $\pm$ 0.4 ^{a,c}	26.6 $\pm$ 0.8 ^{a,b}
	Width (μm)	13.5 $\pm$ 0.6 ^a	13.1 $\pm$ 0.4 ^a	16.9 $\pm$ 0.5 ^b	12.8 $\pm$ 0.4 ^a	12.8 $\pm$ 0.5 ^a
	Pole width (μm)	9.3 $\pm$ 0.4 ^a	6.6 $\pm$ 0.3 ^b	9.1 $\pm$ 0.4 ^{a,c}	6.7 $\pm$ 0.3 ^b	8.3 $\pm$ 0.4 ^c
	Area (μm^2)	300.2 $\pm$ 14.2 ^a	249.7 $\pm$ 9.8 ^c	360.3 $\pm$ 14.9 ^b	241.3 $\pm$ 8.5 ^c	287.4 $\pm$ 18.8 ^a
	<i>n</i>	31	31	30	34	32
IVM +	Length (μm)	24.6 $\pm$ 0.8	23.9 $\pm$ 0.7	26.2 $\pm$ 0.9	24.3 $\pm$ 0.4	24.8 $\pm$ 0.5
	Width (μm)	12.5 $\pm$ 0.3 ^a	12.5 $\pm$ 0.6 ^{a,b}	12.6 $\pm$ 0.4 ^{a,b}	14.2 $\pm$ 0.5 ^b	10.2 $\pm$ 0.4 ^c
	Pole width (μm)	6.9 $\pm$ 0.4 ^a	5.3 $\pm$ 0.5 ^{b,c}	5.8 $\pm$ 0.4 ^b	4.3 $\pm$ 0.2 ^c	5.4 $\pm$ 0.5 ^{b,c}
	Area (μm^2)	239.1 $\pm$ 11.2	214.9 $\pm$ 14.0	241.9 $\pm$ 13.1	225.9 $\pm$ 8.1	195.5 $\pm$ 12.7
	<i>n</i>	21	12	18	25	14

Pole width indicates the mean width at the two spindle poles.

^{a-c} Different superscripts denote significant differences ($P < 0.05$) across strains within the same maturation conditions for each parameter analyzed (ANOVA).

incidence of M-II oocytes was observed suggesting that the meiotic delay detected during IVO is overcome with IVM. There was no significant variation in the percentage of M-II oocytes between IVM + and IVM groups for any of the strains examined, and it is noteworthy that for all outbred lines (Black Swiss, CF-1 and NU/NU) the extent of maturation was equivalent between IVO and IVM + groups.

Variation in spindle dimensions between treatments and strains

M-II oocytes labeled for chromatin, pericentrin and α/β -tubulin were analyzed for spindle organization (Fig. 2) and from these images spindle size measurements which included spindle length, width, total area and mean spindle pole width were conducted, as summarized in Table 1.

In the IVO group, Black Swiss oocytes exhibited a significantly larger spindle area consistent with greater spindle width (Table 1 and Fig. 2C). This feature held true for IVM oocytes, since Black Swiss samples showed maximal spindle width and length in addition to broad spindle poles, and larger spindle total area as would be expected (Table 1 and Fig. 2c). At the other extreme, C57BL/6 and CF-1 oocytes exhibited smaller overall spindle areas after IVM due to their more compact spindle poles and slightly decreased overall length in comparison with the other strains (Table 1 and Fig. 2b and d). In the IVM + group, CF-1 oocytes showed wider spindles but with narrower poles and NU/NU oocytes displayed significantly narrower spindles (Table 1 and Fig. 2d' and e'). In spite of these differences, total spindle area in IVM + oocytes was equivalent across strains. Overall, oocytes matured under basal IVM conditions exhibited more heterogeneity

in spindle dimensions between strains when compared with IVO. Additionally, IVM + maturation conditions resulted in a decrease in the overall spindle size differences between strains when compared with IVM.

With reference to variations between maturation conditions within strains, a significant increase in spindle length, spindle pole width and total spindle area was evident for all strains studied as a result of IVM, when compared with IVO. However, spindle width was significantly increased only in B6D2F1 and Black Swiss oocytes. Overall, the ratio of spindle width at the equator to width at the poles decreased consistent with the transformation of spindles from a pointed/tapered shape in IVO oocytes to the more barrel-shaped IVM phenotype (Fig. 2). In contrast to the uniform increase in spindle dimensions observed for all strains in IVM with regard to IVO oocytes, oocyte maturation in IVM + conditions resulted in strain-specific variations in spindle size, except for the width of the spindle poles which was consistently intermediate between IVO and IVM in all strains. Spindle length in IVM + oocytes was similar to that in IVM oocytes, with the exception of the NU/NU strain, in which a significant decrease in length was observed. NU/NU and Black Swiss IVM + oocytes exhibited significantly narrower spindles than their IVO and IVM counterparts, while the opposite was observed in the CF-1 strain. In contrast, spindle width in B6D2F1 and C57BL/6 oocytes matured in IVM + was equivalent to both IVO and IVM oocytes. Finally, with regard to spindle total area, the effect of IVM + maturation conditions was again dependent on the strain. Specifically, spindle area was intermediate between IVM and IVO groups in B6D2F1 oocytes, similar to IVM in CF-1 oocytes, but similar to IVO in the other three strains (C57BL/6, Black Swiss and NU/NU). Thus, although some strain-specific variations in spindle size were apparent,

Figure 3 Mean number of cytoplasmic MTOCs in oocytes from five strains of mice matured under *in vivo* (IVO) or *in vitro* (IVM and IVM +) conditions. Data represent the combined results of two replicate experiments for each maturation treatment, and the total number of oocytes analyzed for each strain were 10, 30 and 20 for IVO, IVM and IVM + respectively. Different letters at the top of the columns represent significant differences ($P < 0.05$) across strains within each maturation treatment (ANOVA). No differences were detected across strains in the mean number of cytoplasmic MTOCs in IVM + oocytes.

the most consistent finding obtained from this analysis was that the M-II spindle area was increased for all strains as a result of IVM, when compared with IVO, and that maturation in a supplemented medium (IVM +) tended to recapitulate the IVO spindle dimensions in a strain-dependent manner.

Pericentrin organization and distribution at the spindle poles

For each strain, the organization of pericentrin at the spindle poles highlighted variations in spindle size and shape evidenced above for IVO, IVM and IVM + matured oocytes (Table 1). Representative images from each group of oocytes are shown in Fig. 2 to illustrate the relationship between polar constriction and overall spindle size and shape. IVO oocytes typically exhibited spindles with focused poles, solitary pericentrin foci and symmetric halves as emphasized by equatorial chromatin disposition (Fig. 2A–E). An exception to this was the Black Swiss oocytes which usually displayed asymmetric half spindles in which the shorter half was associated with one pointed pole with a pericentrin focus and the longer half being less tapered with multiple foci at the pole (Fig. 2C). In fact, C-shaped polar aggregates of pericentrin were common in Black Swiss oocyte spindles and may explain their typically larger size and more asymmetric spindles (data not shown).

In general, pericentrin organization at the spindle poles varied in all strains as a function of IVM. IVM oocytes consistently displayed flat-poled and barrel-shaped spindles regardless of the strain, re-emphasizing the effect that culture can have on spindle phenotype. It appears further that spindle phenotype was related to the organization of pericentrin at the spindle poles as, overall, IVM oocytes tended to exhibit multiple pericentrin foci at the spindle poles (Fig. 2a–e), as described originally by Doxsey *et al.* (1994). Thus, the striking effect of culture on spindle properties was apparent at both the levels of spindle size and shape as well as the organization of pericentrin at the

poles. In the case of the IVM + oocytes, an intermediate phenotype was evident with respect to spindle pole width and the presence of more compacted pericentrin at the poles relative to IVM oocytes but clearly less than that observed in IVO oocytes (Fig. 2a'–e'). Some strain variation in IVM + oocytes was evident, with B6D2F1 oocytes exhibiting the broadest spindle poles and CF-1 the most focused (Table 1). Again, the range of patterns showed that pericentrin in B6D2F1, C57BL/6 and Black Swiss oocytes organized as multiple foci (~50%) or single C-shaped aggregates (~50%), whereas in NU/NU and CF-1 oocytes solitary foci or C-shaped aggregates were most frequently observed at the spindle poles. Thus, variations in spindle size and shape were consistently associated with specific alterations in the organization of pericentrin at the spindle poles between strains and between different maturation groups.

Cytoplasmic MTOC quantification between treatments and strains

Having shown above that variations in spindle pole pericentrin distribution were associated with variable spindle pole widths and overall spindle shape, we next evaluated the distribution and number of cytoplasmic MTOCs across strains and maturation conditions. As shown in Fig. 3, IVO oocytes consistently exhibited increased numbers of cytoplasmic MTOCs across strains when compared with their IVM counterparts. Moreover, IVM + oocytes exhibited on average fewer cytoplasmic MTOCs relative to either IVM or IVO groups. Specifically, under IVO conditions, Black Swiss and CF-1 oocytes showed the greatest number of cytoplasmic MTOCs (~30/oocyte), while B6D2F1 oocytes displayed an intermediate number (~20) and C57BL/6 and NU/NU oocytes exhibited the lowest mean number (~10) of cytoplasmic MTOCs/oocyte. In the case of IVM, C57BL/6 oocytes showed the fewest cytoplasmic MTOCs (~5/oocyte) which may, in part, be related to delayed maturation *in vivo*. For IVM + oocytes, no differences were observed between strains but the overall number of

Figure 4 Cytoplasmic MTOCs and first polar body (PB) size in IVO, IVM and IVM + of NU/NU M-II oocytes. Tubulin (green), pericentrin (red) and chromosomes (blue) are depicted. Arrows indicate the position of the PB. (A–C) PB size and spindle shape and position in (A) IVO, (B) IVM and (C) IVM + oocytes. Correlative pericentrin and chromosome staining depicting a high number of cytoplasmic MTOCs in IVO oocytes, with small-sized PB, are shown in (a), IVM oocytes exhibit lower number of cytoplasmic MTOCs and bigger PBs (b) and oocytes cultured under IVM + conditions display the lowest number of cytoplasmic MTOCs and PBs of intermediate size (c). Note the presence of cytoplasmic MTOCs in the PB in IVM oocytes (b).

cytoplasmic MTOCs was significantly decreased when compared with IVM and IVO oocytes within the same strain. Interestingly, as shown above for the spindle size and shape, it appears that maturation under IVM + conditions diminished strain variance in MTOC number, with all samples exhibiting approximately five MTOCs/oocyte after IVM +. While variations in MTOC number between IVO, IVM and IVM + groups may be related to the amount of pericentrin associated with the spindle, other factors may affect the distribution of this material. For example, pericentrin-positive foci were found in the first polar body (Fig. 4). The presence of pericentrin material in the polar body was typically low in IVO oocytes (0–20%; Fig. 4A and a), intermediate in IVM + oocytes (4–45%; Fig. 4C and c) and high in IVM oocytes (40–79%; Fig. 4B and b). It is also noteworthy that, regardless of the strain, IVM oocytes consistently exhibited larger polar bodies than their IVO counterparts. In fact, IVO oocytes typically exhibited polar bodies that were either small or absent altogether. Thus, the distribution and number of pericentrin-containing MTOCs was highly variable according to genetic background and a generalized decrease in MTOC

number appeared to be associated with *in vitro* maturation. Whether increase in spindle size and appearance of MTOCs within the first polar body seen upon IVM and IVM + are interrelated consequences of culture remains to be established.

Discussion

While genetic background is well known to influence oocyte developmental competence in the mouse (Silver 1995), few studies have identified phenotypic traits in this species that might account for variations in fecundity. The present study analyzed microtubule patterning in oocytes from five strains of mice known to exhibit a wide range of fecundity. Oocytes collected following induced ovulation were remarkably similar across strains in terms of the centrosome–microtubule organization, with the exception of larger spindles in Black Swiss oocytes and significant variations in the number of cytoplasmic MTOCs, which were consistently higher in outbred mice. Given the known influence of the oocyte maturation milieu on the

developmental potential of resulting embryos (Eppig & O'Brien 1998, Moor *et al.* 1998, Trounson *et al.* 2001) we also compared IVO oocytes from each strain to oocytes matured *in vitro* under two conditions: using a basal (IVM) or a supplemented (IVM+) media. Our analysis has demonstrated a striking effect of *in vitro* maturation on the phenotype of the oocytes in terms of spindle, polar body and cytoplasmic MTOCs. While this effect has previously been reported in outbred CF-1 oocytes (Sanfins *et al.* 2003), the results of the present work in five different strains of mice and two different maturation media reinforced the idea that the environment in which the oocyte undergoes maturation is a critical factor in establishing and maintaining microtubule patterning in mouse oocytes, independent of the genetic background.

Our studies first showed that exogenous gonadotropins used to induce ovulation yielded fully mature oocytes that exhibited consistently tapered and small meiotic spindles, as previously observed by Sanfins *et al.* (2003) in CF-1 oocytes. With regard to nuclear maturation, IVM and IVM+ oocytes reached M-II at slightly varying rates across strains. The only significant strain-specific difference resided in the low numbers of M-II eggs for the inbred C57BL/6 strain after IVO in contrast to after IVM and IVM+. It is likely that variation in maturation kinetics reflects the low incidence of M-II observed *in vivo* for C57BL/6 oocytes. Furthermore, the fact that C57BL/6 oocytes reach M-II at an elevated frequency after IVM or IVM+ support the idea that the meiotic delay observed *in vivo* does not represent an intrinsic property of the oocyte, but possibly an effect of the follicular environment.

A notable feature of all *in vitro* matured oocytes was the transition in spindle phenotype from ones with tapered anastral poles (IVO) to larger barrel-shaped spindles (IVM) and an intermediate phenotype in the IVM+ groups. Thus, some aspects of the processes underlying spindle morphogenesis must be susceptible to culture conditions and some degree of caution is warranted then in attributing significance to spindle shape variations when comparing *in vivo* with *in vitro* maturation conditions. Although every effort was made to normalize experimental conditions for sample preparation and analysis, and spindle properties are known to be affected by this (see Sanfins *et al.* 2003 for a discussion), the follicular milieu during ovulation was not being recapitulated under our culture conditions and must be a primary cause for the IVO versus IVM/IVM+ differences detected. Similar to spindle shape, spindle dimensions were also modified by maturation conditions but, in this case, an effect of the oocyte genetic background was also evident. Thus, even though maturation under IVM conditions resulted in an increase in spindle dimensions in all strains when compared with IVO oocytes, it also resulted in a higher variability between strains than that observed in IVO oocytes for all the spindle parameters

analyzed (length, width, pole width and total spindle area). For instance, while the only significant difference in spindle total area in IVO oocytes was the larger spindles in Black Swiss oocytes, IVM oocytes exhibited a wider range of sizes: larger spindles in Black Swiss oocytes, smaller spindles in C57BL/6 and CF-1 oocytes and spindles of intermediate size in B6D2F1 and NU/NU oocytes. When compared with IVM, variability in spindle dimensions between strains was reduced after maturation in IVM+ conditions, as significant differences among strains were only evident in spindle width and pole width. However, strain-specific differences in all spindle parameters analyzed were evident in this case when comparing IVM+ with IVO and IVM oocytes, except for the width of the spindle poles.

Genetic variations in mouse oocytes have been described by others. Strain-specific rates of maturation were reported previously for both *in vivo* and *in vitro* matured oocytes (Polanski 1986, 1997), and whether differences in the synthesis of key cell cycle factors such as cyclins (Polanski *et al.* 1998), c-mos (Choi *et al.* 1996, Verlhac *et al.* 2000) or cdc25b (Lincoln *et al.* 2002) explain the incidence of M-II oocytes in the inbred C57BL/6 or other variations remains an interesting possibility. The fact that the overall incidence of maturation to M-II was lower with IVM oocytes when compared with IVO oocytes most likely reflects the greater developmental heterogeneity of oocytes retrieved after eCG. While this heterogeneity is in accordance with previous reports (Combelles & Albertini 2003), our results begin to uncover phenotypic variations intrinsic to the genotype that are manifest as alterations in microtubule patterning during oocyte maturation.

Our analysis of spindle dimensions revealed significant differences, with small pointed spindles in IVO oocytes when compared with larger less-tapered spindles in IVM and IVM+ oocytes. Furthermore, IVM+ spindles exhibited characteristics intermediate between IVM and IVO spindles in being both smaller and neither flat nor pointed at the poles. This finding suggested that mechanisms of spindle assembly may differ under *in vitro* and in *in vivo* conditions and further that forces shaping the spindle pole are being regulated differently *in vivo*.

Another key phenotypic variant identified in this work pertains to the disposition of the centrosomal protein pericentrin at both the spindle poles and in the oocyte cortex. Pericentrin organization has been described during *in vitro* maturation in oocytes originating from outbred mice (Doxsey *et al.* 1994, Carabatsos *et al.* 2000, Combelles & Albertini 2001). While confirming the arrangement of pericentrin at the poles of IVM spindles across five strains of mice, our work further describes the differential distribution of pericentrin at the spindle poles of IVO and IVM+ oocytes. The pointed spindles of IVO oocytes usually displayed a single focus of pericentrin while the broad, barrel-shaped spindles of IVM oocytes exhibited multiple and distinct foci at the

poles. Here again, pericentrin organization at the poles of IVM + spindles resembled more closely patterns observed after IVO than after IVM. Furthermore, in the case of the outbred Black Swiss strain, alterations in spindle shape were observed concomitantly with changes in pericentrin organization at the spindle poles. While the mechanisms underlying spindle assembly, maintenance and function remain unknown in mammalian oocytes, the present study points towards important microtubule and pericentrin differences under varying maturation conditions and genetic backgrounds. It may be speculated here that spindles in *in vivo* ovulated oocytes are under more tension when compared with spindles in *in vitro* matured ones, which display relaxed organization with respect to microtubules and pericentrin at the poles.

The distribution of centrosomal proteins involved in microtubule microtubule patterning, including pericentrin and α -tubulin, which organize meiotic spindle microtubules and microtubules associated with the oocyte cortex, is known to change according to the stage of meiotic cell cycle progression in mice (Messinger & Albertini 1991, Albertini 1992, Combelles & Albertini 2001, Sanfins *et al.* 2003). Mouse oocytes are characterized by the unique presence of cytoplasmic centrosome aggregates (Maro *et al.* 1985) that nucleate microtubules upon anaphase of both meiosis-I or meiosis-II (Combelles & Albertini 2001). It has been proposed to be a maternal store of MTOCs for later embryonic mitoses (Maro *et al.* 1985, Houliston *et al.* 1987, Schatten 1994). Although speculative, it is conceivable that differences in the number of cytoplasmic MTOCs between IVO, IVM, and IVM + oocytes and across strains illustrate the pool of centrosomal material available in each oocyte type. Our work suggests that loss to the polar body or excessive spindle integration could be related to differences in MTOC number seen in IVM and IVM + oocytes when compared with IVO. If this were the case, then the limited development of embryos seen after *in vitro* maturation, coupled with our findings of a decrease in MTOC number, might explain deficiencies in cell cycle timing (Sanfins *et al.* 2003) or cell polarity determination (Liu *et al.* 2003, Albertini & Barrett 2004) seen after *in vitro* maturation.

Numerous mouse strains are routinely being used to generate gene knockout, transgenic, nuclear transfer-cloned and new mutagenesis-induced lines. Despite the expanding utility of genetically modified mouse models, relatively little effort has been expended in defining the phenotypic properties of murine oocytes that would be of predictive value in defining the maternal origins of oocyte developmental competence. Additionally, the notion that cytoplasmic properties of the M-II oocyte influence reprogramming events after nuclear transfer (Fulka *et al.* 2001, Liu *et al.* 2001) emphasizes the need to establish markers of oocyte cytoplasmic quality. With regard to microtubule patterning as a phenotypic marker,

previous results in CF-1 oocytes (Combelles & Albertini 2003) and the present analysis on a limited number of strains establish feasibility and utility for this purpose. Moreover, most laboratories employ a single strain in their studies, thereby possibly restricting the value of their findings to a solitary genetic background. In conclusion, our studies identifying microtubule patterning variations in five strains of mice together with well-recognized epigenetic factors like culture, suggest that both genetic attributes and environmental modulators must be taken into account in studies aimed at understanding the mechanisms of meiotic spindle assembly, control of the meiotic cell cycle and determinants of oocyte quality in general.

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