Effect of genetic background and activating stimulus on the timing of meiotic cell cycle progression in parthenogenetically activated mouse oocytes

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Abstract

With the aim of investigating the effects of oocyte genotype and activating stimulus on the timing of nuclear events after activation, oocytes collected from hybrid B6D2F1, inbred C57BL/6 and outbred CF-1 and immunodeficient nude (NU/+) females were activated using ethanol or strontium and fixed at various time-points. Meiotic status, spindle rotation and second polar body (PB2) extrusion were monitored by fluorescence microscopy using DNA-, microtubule- and microfilament-selective probes. Although activation efficiency was similar in all groups of oocytes, a significant percentage of CF-1 and NU/+ oocytes treated with ethanol and of C57BL/6 oocytes treated either with ethanol or strontium failed to complete activation and became arrested at a new metaphase stage (MIII) after PB2 extrusion. C57BL/6 oocytes also showed slower release from MII arrest but faster progression to telophase (TII) after ethanol exposure, and they exhibited the most rapid exit from TII under both activation treatments. Strontium caused delayed meiotic resumption, spindle rotation and PB2 extrusion, but rapid TII exit, in B6D2F1, CF-1 and NU/+ oocytes when compared with ethanol. Compared with all other strains, NU/+ oocytes were significantly slower in completing spindle rotation and PB2 extrusion, irrespective of the activating stimulus, and a significant decrease in activation rates and pace of meiotic progression was observed after strontium exposure. Thus, our findings demonstrated that the kinetics of meiosis resumption and completion, spindle rotation and PB2 extrusion following parthenogenetic activation depends on both genotype-specific factors and on the activation treatment applied.

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Introduction

In most mammalian species, ovulated oocytes are arrested at metaphase of the second meiotic division (MII). This arrest is characterized by a high maturation-promoting factor (MPF) activity (Nurse 1990), which is maintained by a cytosolic factor (CSF) (Masui 1991). Under natural conditions, exit from the MII arrest is accomplished by fertilization, which triggers a series of transient rises in intracellular Ca^{2+} concentration that last for several hours (Cuthbertson & Cobbold 1985, Swann 1990). These calcium transients cause inactivation of CSF, possibly through activation of Ca^{2+} -calmodulin-dependent protein kinase II (CaMKII), and subsequent inactivation of MPF (Collas *et al.* 1995, Zernicka-Goetz *et al.* 1995). Intracellular Ca^{2+}

oscillations at fertilization also initiate a series of subcellular and biochemical changes in oocytes, including cortical granule exocytosis, modifications of zona pellucida glycoproteins, recruitment of maternal mRNAs, resumption of meiosis, second polar body (PB2) extrusion, pronuclear formation, DNA synthesis and first mitotic cleavage (Schultz & Kopf 1995). Collectively, these events are referred to as oocyte activation. Meiotic resumption after activation is characterized by chromatid segregation and spindle elongation during the transition from MII to anaphase (AII) and telophase II (TII). At the same time, in mouse oocytes, the spindle rotates from a parallel to a perpendicular orientation relative to the plasma membrane, and the PB2 is extruded (Maro *et al.* 1984, Liu *et al.* 2000, Ibáñez *et al.* 2003). Finally, chromatin is decondensed,

pronuclear envelopes form, spindle microtubules depolymerize and the interphase microtubular network is assembled. MPF inactivation is essential for meiotic resumption and progression to interphase, whereas the inactivation of MAP kinase (MAPK), a component of CSF, is associated with chromatin decondensation, pronuclear formation, initiation of DNA synthesis and changes in microtubule dynamics (Moos *et al.* 1995, Carroll *et al.* 2000).

Although sperm supply the natural stimulus responsible for oocyte activation, oocytes may also be activated parthenogenetically, without the contribution of sperm, by a variety of physical and chemical stimuli (reviewed by Macháty & Prather 1998, Alberio *et al.* 2001). Most artificial stimuli activate oocytes by a mechanism similar to that used by the sperm, i.e. inducing an increase in the intracellular Ca^{2+} levels. In mouse oocytes, ethanol has been frequently employed as an activating agent (Cuthbertson *et al.* 1981, Cuthbertson 1983, Winston & Maro 1995). Ethanol activates oocytes by promoting the formation of inositol 1,4,5-trisphosphate (IP_3) at the plasma membrane and the influx of extracellular Ca^{2+} (Ilyin & Parker 1992), causing a large, single rise in intracellular Ca^{2+} concentration (Cuthbertson *et al.* 1981). Strontium is another popular activating agent, currently used in mouse nuclear transfer protocols (Wakayama *et al.* 1998). It induces repetitive Ca^{2+} transients, which last for several hours, probably by displacing bound Ca^{2+} in the oocyte, but also by inducing Ca^{2+} release from intracellular stores (Kline & Kline 1992, Bos-Mikich *et al.* 1995).

Parthenogenetic activation can be used as a model to study biochemical and morphological changes occurring in the oocyte during fertilization and early embryonic development, and it is a critical component of the cloning procedure. Because of this, several studies have been performed to date on the activation of mouse oocytes using various artificial stimuli. The majority of these studies have focused on the comparison of activation rates, usually measured by the presence of a pronucleus several hours after activation, or on the comparison of parthenogenetic development up to the blastocyst stage, of oocytes from the same strain activated with different treatments (Cuthbertson 1983, O'Neill *et al.* 1991, Bos-Mikich *et al.* 1995, 1997, Kishikawa *et al.* 1999). According to these criteria, differences in oocyte activation efficiencies have been observed depending on the activating stimulus used. Nevertheless, only a few studies have been reported on the activation efficiencies induced by artificial stimuli on oocytes from different strains of mice, and the results of these studies indicate that parthenogenetic activation and development is clearly dependent upon oocyte genetic background (Marcus 1990, Rybouchkin *et al.* 1996, Gao *et al.* 2004).

In the present work, oocytes collected from several strains of mice were subjected to parthenogenetic activation, using either ethanol or strontium, with the aim of investigating whether oocyte genetic background and

activating stimulus influence nuclear events following artificial activation. In particular, the effects of these two factors on the kinetics of meiotic cell cycle resumption and completion, spindle rotation and PB2 extrusion were evaluated. Mouse strains chosen as oocyte donors were B6D2F1, C57BL/6 and CF-1, as common representatives of hybrid, inbred and outbred strains respectively. Oocytes collected from immunodeficient heterozygous nude females (NU/+), of an outbred background, were also included in the study, as delayed PB2 extrusion after activation had been observed in these oocytes in previous studies by our group (E Ibáñez, DF Albertini and EW Overström, unpublished results).

Materials and Methods

Collection of MII oocytes

Hybrid B6D2F1 (C57BL/6 × DBA/2), inbred C57BL/6 and outbred CF-1 and immunodeficient nude (NU/+) female mice (Charles River Laboratories, Wilmington, MA, USA) were used as oocyte donors. Animal care and procedures were conducted according to protocols approved by the Tufts University Institutional Animal Care and Use Committee. Females (8–10 weeks old) were induced to superovulate by intraperitoneal injection of 5 IU equine chorionic gonadotropin (eCG; Calbiochem, San Diego, CA, USA) followed 48 h later by 5 IU human chorionic gonadotropin (hCG) (Calbiochem). *In vivo* ovulated MII oocytes were recovered from oviducts 16 h post-hCG injection in Hepes-buffered potassium simplex optimized medium (H-KSOM; Speciality Media, Phillipsburg, NJ, USA) and cumulus cells were dispersed by incubation in 150 U bovine testicular hyaluronidase/ml (Sigma, St Louis, MO, USA) in H-KSOM at 37 °C for 5 min. Cumulus-free oocytes were then washed three times in fresh H-KSOM and immediately activated.

Oocyte activation and culture

Oocytes were parthenogenetically activated using two different chemical treatments. For ethanol activation, oocytes were treated for 5 min at 37 °C in H-KSOM containing 7% (v/v) ethanol. Oocytes were then washed twice in H-KSOM and cultured for up to 6 h at 37 °C under 5% CO_2 in air in KSOM medium containing both non-essential and essential amino acids (Specialty Media) and supplemented with 1 mg bovine serum albumin (BSA)/ml. When strontium was used as the activating agent, oocytes were cultured for up to 6 h in Ca^{2+} -free KSOM (Specialty Media) containing 10 mmol SrCl_2 /ml (Sigma). Initial exposure of MII oocytes to either ethanol or strontium was considered as time zero post-activation (p.a.).

To monitor meiotic cell cycle resumption and progression after activation, ethanol- and strontium-treated oocytes were fixed at 5-min intervals, from 5 min to 20 min p.a., and then at 30-min intervals until 5 h 50 min p.a.

Fixation of oocytes and processing for immunofluorescence analysis

At the defined time-points after activation, oocytes were fixed and extracted for 30 min at 37°C in a microtubule stabilizing buffer (0.1 mol PIPES/ml, pH 6.9, 5 mmol MgCl₂/ml and 2.5 mmol EGTA/ml) containing 3.7% (v/v) formaldehyde, 0.1% (v/v) Triton X-100, 1 µmol taxol/ml, 0.01% (w/v) aprotinin, 1 mmol dithiothreitol/ml and 50% (v/v) deuterium oxide (Messinger & Albertini 1991). Fixed oocytes were stored until processing at 4°C in a phosphate-buffered saline (PBS) blocking solution containing 1% (w/v) BSA, 0.2% (w/v) powdered milk, 2% (v/v) normal goat serum, 0.1 mol glycine/ml, 0.2% (w/v) sodium azide and 0.01% (v/v) Triton X-100 (Wickramasinghe & Albertini 1992).

A triple-labeling protocol was used for the detection of microtubules, microfilaments and chromatin by fluorescence microscopy (Herman *et al.* 1983). Oocytes were first incubated for 1 h at 37°C in a mixture of mouse monoclonal anti α -tubulin and anti β -tubulin antibodies (Sigma) at a 1:1000 final dilution in PBS blocking solution. After two washes in 0.1% (w/v) polyvinylpyrrolidone (PVP)/PBS at room temperature, oocytes were incubated at 37°C in PBS blocking solution alone for 30 min, and then in a 1:150 dilution of a donkey anti-mouse fluorescein-conjugated IgG (Jackson ImmunoResearch, West Grove, PA, USA) for 45 min at 37°C. Oocytes were washed again twice in 0.1% PVP/PBS, and incubated at 37°C for 30 min with 10 U Texas Red-conjugated phalloidin/ml (Molecular Probes, Eugene, OR, USA) to stain actin filaments. Finally, after extensive washing in 0.1% PVP/PBS, oocytes were incubated at room temperature for 15 min in 10 µg Hoechst 33258/ml (Molecular Probes) and mounted in 50% (v/v) glycerol/PBS containing 25 mg sodium azide/ml.

Microscopic analysis and scoring criteria

Labeled oocytes were examined using a Zeiss IM-35 inverted epi-fluorescence microscope (Zeiss, Thornwood, NY, USA) fitted with filters selective for Hoechst, fluorescein and Texas Red and a 50W mercury lamp. Digital images were acquired using a Photometrics Cool Snap CCD camera (Roper Scientific Inc., Trenton, NJ, USA) running on Metamorph software (version 5.0; Universal Imaging Corp., Downingtown, PA, USA).

For each oocyte, meiotic status, meiotic spindle orientation relative to the plasma membrane and presence of PB2 were recorded. Activation was considered to have occurred if the oocytes had exited MII arrest and resumed meiosis.

Statistical analysis

Each activation treatment was repeated at least three times on separate days in each strain of oocytes. Approximately 50 oocytes were examined per strain and treatment

at each defined time-point, with the exception of the C57BL/6 strain (approximately 30 oocytes analyzed per time-point for each activation treatment). The results obtained in the replicate experiments were pooled and analyzed by chi-square test or Fisher's exact test. A probability value of $P < 0.05$ was considered to be statistically significant.

Results

Effect of oocyte genotype on meiotic cell cycle progression following ethanol exposure

The majority of ethanol-treated oocytes, regardless of the strain, exited MII arrest during the first 20 min p.a. and reached the TII stage between 50 min and 1 h 20 min p.a. However, significant differences were detected in the kinetics of meiotic resumption and of AII–TII transition depending on the genotype of the oocytes. Whereas a similar percentage of oocytes from the four strains were at early AII stage by 5 min p.a. (46.4–64%), a significantly higher number of C57BL/6 oocytes were still at MII at 10 min (58.1%) and 20 min p.a. (32.3%) when compared with CF-1 (26 and 10% respectively) and NU/+ oocytes (33.3 and 6% respectively), indicating a slower release from MII arrest (Fig. 1). By 50 min after ethanol exposure, most B6D2F1 (98%) and CF-1 (92.5%) oocytes and 100% of NU/+ oocytes had resumed meiosis, while 25.8% of C57BL/6 oocytes were still arrested at MII. The majority of CF-1 oocytes that had exited MII by 50 min p.a. were already at the TII stage (87.1%), a percentage significantly higher than that of B6D2F1 (60%) and NU/+ oocytes (68.8%) but similar to that of C57BL/6 oocytes (78.3%). By 1 h 20 min p.a. and onward, no more differences were observed among genotypes in the rates of activation or in the percentage of activated oocytes that had progressed further than AII. Therefore, in spite of the fact that C57BL/6 oocytes were slow to resume meiosis after ethanol activation treatment, they were quick to catch up. In fact, exit from TII after PB2 extrusion commenced earlier in C57BL/6 oocytes (1 h 20 min p.a.) than in CF-1 and NU/+ oocytes (1 h 50 min p.a.) or in B6D2F1 oocytes (2 h 20 min p.a.). In addition, by 2 h 20 min after ethanol exposure, 30.8% of activated C57BL/6 oocytes had exited the TII stage, a percentage similar to that of CF-1 (10.4%) and NU/+ oocytes (14%) but significantly higher than that of B6D2F1 oocytes (1.9%; Fig. 1).

Activated oocytes showed a different behavior in their meiotic progression after TII exit and PB2 extrusion depending on the strain. By 5 h 50 min p.a. 94% of activated B6D2F1 oocytes had progressed from TII into interphase and formed a pronucleus. However, in a significant and similar percentage of activated C57BL/6 (20%), CF-1 (49%) and NU/+ oocytes (37%) a spindle formed around the condensed chromosomes after PB2 extrusion, and the oocytes became arrested at a new metaphase stage (MIII) (Figs 1 and 2h).

Figure 1 Meiotic cell cycle resumption and progression in mouse oocytes from hybrid B6D2F1, inbred C57BL/6, outbred CF-1 and immuno-deficient mutant nude (NU/+) females after parthenogenetic activation with ethanol. Data at each time-point represent the combined results of at least three replicate experiments. Numbers at the top of the columns indicate the total number of oocytes analyzed at each time-point.

Effect of oocyte genotype on meiotic cell cycle progression following strontium exposure

When strontium was used as the activating agent, no differences were observed between genotypes in the speed of meiotic resumption during the first 20 min after treatment (Fig. 3). However, from 50 min p.a. and onward, NU/+ oocytes displayed the lowest activation rates, except for some groups of C57BL/6 oocytes (50 min, 2 h 20 min and 2 h 50 min). Progression from All to TII between 50 min and 2 h 50 min p.a. was also delayed in NU/+ oocytes when compared with B6D2F1 and CF-1 oocytes, which also showed higher percentages of activated oocytes in TII by 50 min p.a. (93.1 and

94.2% respectively) than in the C57BL/6 strain (62.5%; Fig. 3).

As previously observed with ethanol, strontium-treated C57BL/6 oocytes were the earliest in exiting TII (starting at 1 h 20 min p.a.) and also the fastest until 2 h 50 min p.a. Whereas the first NU/+ oocytes to exit TII were detected at 2 h 20 min p.a. (2.4%), as in B6D2F1 (10%) and in CF-1 (1.9%) oocytes, the timing of the TII–interphase transition between 2 h 50 min and 3 h 50 min was significantly delayed in this strain. At 5 h 50 min p.a., however, 100% of the oocytes that were activated had progressed further than the TII stage, regardless of the genotype. All activated B6D2F1 and CF-1 oocytes and 97.8% of activated NU/+

Figure 2 Sequential images of meiotic cell cycle progression, spindle rotation and PB2 extrusion in parthenogenetically activated mouse oocytes. MII arrested oocytes (a) were treated with either ethanol or strontium and fixed at several time-points after activation. Microtubules (green), microfilaments (red) and chromatin (blue) were stained with anti-tubulin antibodies, phalloidin and Hoechst 33258 respectively. Although differences in timing were detected depending on the strain of the oocyte and the activating stimulus (see text for details), a significant percentage of oocytes had resumed meiosis and were found at early All stage by 5 min p.a. (b). As the oocytes progressed from All to TII, two cortical protrusions formed on top of each pole of the spindle, which was initially parallel to plasma membrane (c). As early as 15–20 min p.a. the spindle started to rotate towards one of the protrusions, which enlarged as the other one regressed (d). By 50 min p.a. only a single cortical protrusion was evident in some of the activated oocytes, and the telophase spindle showed an orientation perpendicular to the plasma membrane (e). Between 1 h 20 min and 1 h 50 min p.a. PB2 extrusion had occurred in the majority of the activated oocytes (f). Depending on the strain and the activating stimulus applied, activated oocytes progressed from TII to interphase after PB2 extrusion and formed a pronucleus (g), or became arrested at a new metaphase stage (MIII) and a new metaphase spindle formed (h). Arrowheads and arrows indicate the presence of the first and the second PB respectively. Original magnification 400x.

Figure 3 Meiotic cell cycle resumption and progression in mouse oocytes from hybrid B6D2F1, inbred C57BL/6, outbred CF-1 and outbred immunodeficient nude (NU/+) females after parthenogenetic activation with strontium. Data at each time-point represent the combined results of at least three replicate experiments. Numbers at the top of the columns indicate the total number of oocytes analyzed at each time-point.

oocytes were in interphase at this point, while a significant number of activated C57BL/6 oocytes (13.3%) remained arrested at MIII and only 86.7% were able to form a pronucleus (Fig. 3).

Effect of the activating stimulus on meiotic cell cycle progression following parthenogenetic activation

In comparison with ethanol, meiotic resumption during the first 20 min after strontium exposure was significantly delayed in B6D2F1, CF-1 and NU/+ oocytes, but not in C57BL/6 oocytes. In addition, in the NU/+ strain, the percentage of activated oocytes between 50 min (78%) and 2 h 20 min p.a. (83.7%) was significantly lower than when

ethanol was used as the activating agent (100%), and the AII–TII transition between 50 min and 1 h 50 min p.a. was also delayed. By contrast, in activated B6D2F1 oocytes, progression from AII to TII at 20 min and 50 min p.a. was faster when strontium rather than ethanol was used as the activating stimulus (Figs 1 and 3).

Differences between activation protocols were also observed in the timing and the speed of TII exit, but only for B6D2F1, CF-1 and NU/+ oocytes. In the B6D2F1 strain, the first activated oocytes that exited TII after strontium exposure were detected at 2 h 20 min p.a. (10%), as when ethanol activation treatment was applied (1.9%). However, a significantly higher percentage of strontium- than of

ethanol-treated oocytes had formed a pronucleus by 2 h 50 min and 3 h 20 min p.a. (50 vs 24% and 95.9 vs 68% respectively). Therefore, the TII–interphase transition was accelerated in strontium-treated B6D2F1 oocytes when compared with the ethanol-treated group. A similar pattern was observed in the CF-1 strain. Although the first strontium-treated CF-1 oocytes to exit TII were detected 30 min later than when ethanol was used as the activating stimulus (2 h 20 min vs 1 h 50 min), the difference was not significant, and the percentage of activated oocytes that had exited TII by 3 h 20 min, 4 h 20 min and 4 h 50 min was significantly higher after strontium than after ethanol exposure (85.4 vs 64.6%, 96.1 vs 80% and 100 vs 87.5% respectively). Likewise, TII exit was delayed for 30 min in strontium-treated NU/+ oocytes in comparison with the ethanol-treated group (2 h 20 min vs 1 h 50 min). However, no significant differences were detected in this strain in the speed of the TII–interphase transition between the two activation protocols, except for a slight delay at 2 h 50 min p.a. and a slight acceleration at 4 h 50 min p.a. in the strontium-treated oocytes.

For all genotypes, strontium treatment resulted in an increase in the rate of pronuclear formation in comparison with ethanol, although this increase was only significant for the CF-1 and NU/+ strains. Thus, by 5 h 50 min after strontium exposure, 100% of CF-1 and 97.8% of NU/+ activated oocytes had formed a pronucleus, a percentage significantly higher than that observed when ethanol was applied (51 and 63% respectively). In contrast, 13.3% of activated C57BL/6 oocytes were arrested at MIII by 5 h 50 min after strontium exposure, a percentage equivalent to that obtained in oocytes from the same strain treated with ethanol (20%).

Effect of oocyte genotype and activating stimulus on meiotic spindle rotation following parthenogenetic activation

In MII-arrested oocytes, the spindle was parallel to the plasma membrane. Shortly after activation, as the oocyte exits MII and progresses to AII and subsequently to TII, the

sister chromatids migrated to opposite poles of the spindle and two cortical protrusions formed adjacent to each spindle pole. The meiotic spindle then started rotating towards one of the protrusions, as the other one regressed, and achieved an orientation perpendicular to the plasma membrane. Eventually, this protrusion was constricted at the oolemma and the PB2 containing half of the chromatin of the oocyte was extruded (Fig. 2).

Initiation of spindle rotation after ethanol exposure followed a similar timing in the four strains of oocytes analyzed, but significant differences were detected in the rate of completion of the rotation depending on the genotype of the oocyte. Specifically, completion of spindle rotation was significantly slower in NU/+ oocytes than in the other three strains (Table 1). In addition, even though CF-1 oocytes were faster than B6D2F1 oocytes to progress from AII to TII (Fig. 1), they were significantly slower to complete spindle rotation (Table 1). By 2 h 50 min p.a. almost all activated oocytes (92–100%) showed a perpendicularly oriented spindle and no more significant differences in the kinetics of spindle rotation were detected among genotypes from this time-point and onward (data not shown).

When compared with ethanol, strontium induced an acceleration of partial spindle rotation in B6D2F1 oocytes at 50 min p.a., but by 1 h 20 min p.a. a significantly lower number of strontium- than of ethanol-treated oocytes had completed spindle rotation (Tables 1 and 2). Completion of spindle rotation was also delayed in strontium-treated C57BL/6 oocytes at 1 h 20 min p.a. and in CF-1 oocytes at 50 min p.a., relative to ethanol exposure. In addition, in the NU/+ strain, both the rate of partial spindle rotation at 50 min p.a. and the rate of complete spindle rotation at 1 h 20 min and 1 h 50 min p.a. was decreased in strontium-treated oocytes in comparison with ethanol-treated oocytes of the same strain. In fact, NU/+ oocytes were the slowest again of all groups of strontium-treated oocytes both to initiate and to complete spindle rotation (Table 2).

Table 1 Number (% in parentheses) of activated mouse oocytes showing a partially or completely rotated meiotic spindle at various times after ethanol exposure.

Category	Strain	Time post-activation						
		15 min	20 min	50 min	1 h 20 min	1 h 50 min	2 h 20 min	2 h 50 min
Oocytes with partially rotated spindle	B6D2F1	0/34 (0)	1/44 (2)	12/50 (24)	1/51 (2) ^a	0/58 (0)	0/53 (0)	0/50 (0)
	C57BL/6	1/19 (5)	1/21 (5)	8/23 (35)	2/26 (8) ^{a,b}	0/24 (0)	0/26 (0)	0/26 (0)
	CF-1	0/39 (0)	2/45 (4)	19/62 (31)	3/65 (5) ^a	1/72 (1)	0/48 (0)	0/50 (0)
	NU/+	0/41 (0)	0/47 (0)	21/48 (44)	13/48 (27) ^b	2/51 (4)	1/50 (2)	0/48 (0)
Oocytes with completely rotated spindle	B6D2F1	0/34 (0)	0/44 (0)	20/50 (40) ^a	50/51 (98) ^a	58/58 (100) ^a	53/53 (100) ^a	49/50 (98)
	C57BL/6	0/19 (0)	0/21 (0)	10/23 (43) ^a	24/26 (92) ^{a,b}	24/24 (100) ^{a,b}	25/26 (96) ^{a,b}	26/26 (100)
	CF-1	0/39 (0)	0/45 (0)	22/62 (35) ^a	52/65 (80) ^b	63/72 (87) ^b	40/48 (83) ^b	46/50 (92)
	NU/+	0/41 (0)	0/47 (0)	0/48 (0) ^b	29/48 (60) ^c	45/51 (88) ^b	47/50 (94) ^{a,b}	45/48 (94)
Total oocytes with rotated spindle (partially + completely)	B6D2F1	0/34 (0)	1/44 (2)	32/50 (64) ^{a,b}	51/51 (100) ^a	58/58 (100) ^a	53/53 (100) ^a	49/50 (98)
	C57BL/6	1/19 (5)	1/21 (5)	18/23 (78) ^a	26/26 (100) ^{a,b}	24/24 (100) ^{a,b}	25/26 (96) ^{a,b}	26/26 (100)
	CF-1	0/39 (0)	2/45 (4)	41/62 (66) ^a	55/65 (85) ^b	64/72 (89) ^b	40/48 (83) ^b	46/50 (92)
	NU/+	0/41 (0)	0/47 (0)	21/48 (44) ^b	42/48 (87) ^b	47/51 (92) ^{a,b}	48/50 (96) ^a	45/48 (94)

^{a–c}Values with different superscripts within the same column and category differ significantly between strains ($P < 0.05$).

Table 2 Number (% in parentheses) of activated mouse oocytes showing a partially or completely rotated meiotic spindle at various times after strontium exposure.

Category	Strain	Time post-activation						
		15 min	20 min	50 min	1 h 20 min	1 h 50 min	2 h 20 min	2 h 50 min
Oocytes with partially rotated spindle	B6D2F1	0/25 (0)	1/35 (3)	23/49 (47) ^a	10/50 (20)	1/49 (2) ^a	0/50 (0) ^a	0/48 (0)
	C57BL/6	2/13 (15)	2/14 (14)	7/24 (29) ^{a,b}	7/27 (26)	0/25 (0) ^a	0/23 (0) ^a	0/27 (0)
	CF-1	0/27 (0)	0/36 (0)	19/52 (36) ^a	8/52 (16)	2/53 (4) ^a	6/53 (11) ^b	0/53 (0)
	NU/+	0/22 (0)	0/26 (0)	4/39 (10) ^b	14/41 (34)	10/42 (24) ^b	1/41 (2) ^{a,b}	0/43 (0)
Oocytes with completely rotated spindle	B6D2F1	0/25 (0)	0/35 (0)	11/49 (22) ^{a,c}	39/50 (78) ^a	47/49 (96) ^a	50/50 (100) ^a	48/48 (100) ^a
	C57BL/6	0/13 (0)	0/14 (0)	7/24 (29) ^c	18/27 (67) ^a	24/25 (96) ^a	23/23 (100) ^{a,b}	26/27 (96) ^{a,b}
	CF-1	0/27 (0)	0/36 (0)	4/52 (8) ^a	40/52 (77) ^a	49/53 (92) ^a	45/53 (85) ^b	53/53 (100) ^a
	NU/+	0/22 (0)	0/26 (0)	0/39 (0) ^b	14/41 (34) ^b	27/42 (64) ^b	36/41 (88) ^b	37/43 (86) ^b
Total oocytes with rotated spindle (partially + completely)	B6D2F1	0/25 (0)	1/35 (3)	34/49 (69) ^a	49/50 (98) ^a	48/49 (98)	50/50 (100) ^a	48/48 (100) ^a
	C57BL/6	2/13 (15)	2/14 (14)	14/24 (58) ^{a,b}	25/27 (93) ^b	24/25 (96)	23/23 (100) ^{a,b}	26/27 (96) ^{a,b}
	CF-1	0/27 (0)	0/36 (0)	23/52 (44) ^b	48/52 (92) ^a	51/53 (96)	51/53 (96) ^{a,b}	53/53 (100) ^a
	NU/+	0/22 (0)	0/26 (0)	4/39 (10) ^c	28/41 (68) ^b	37/42 (88)	37/41 (90) ^b	37/43 (86) ^b

^{a–c}Values with different superscripts within the same column and category differ significantly between strains ($P < 0.05$).

Effect of oocyte genotype and activating stimulus on the timing of PB2 extrusion following parthenogenetic activation

The presence of an extruded PB2 was first detected by 50 min after ethanol exposure (16% B6D2F1, 13% C57BL/6, 9.7% CF-1), except in the NU/+ strain (Fig. 4a). Extrusion of PB2 was in fact delayed in NU/+ oocytes, relative to the other oocyte genotypes, until 2 h 20 min p.a. No significant differences were detected in the timing of PB2 extrusion among the other three genotypes, except for a lower percentage of CF-1 activated oocytes showing an extruded PB2 by 1 h 20 min (72% in CF-1 vs 96% in B6D2F1 and 88% in C57BL/6 oocytes; Fig. 4a).

Strontium caused a delay in the rates of PB2 extrusion by 1 h 20 min and 1 h 50 min in B6D2F1 and CF-1 oocytes when compared with ethanol. However, no significant differences in the timing of PB2 extrusion were detected among B6D2F1, C57BL/6 or CF-1 oocytes treated with strontium (Fig. 4b). Strontium also resulted in a delayed extrusion of the PB2 in NU/+ oocytes when compared with ethanol, although it was only significant by 1 h 50 min and 3 h 20 min p.a. In comparison with the other three genotypes, strontium-treated NU/+ oocytes showed the lowest rates of PB2 extrusion between 1 h 20 min and 3 h 20 min p.a. (Fig. 4b).

By 3 h 50 min p.a. extrusion of PB2 had occurred in the majority (96–100%) of both ethanol- and strontium-treated oocytes and no more differences were observed either among genotypes or between activation treatments in the rates of PB2 extrusion from this time-point and onward (data not shown).

Discussion

In this work, experiments were undertaken to investigate the effects of oocyte genotype and activating stimulus on the kinetics of key nuclear events of oocyte activation, such as meiotic cell cycle resumption and progression,

Figure 4 Rates of PB2 extrusion at various times p.a. in (a) ethanol- and (b) strontium-treated mouse oocytes from four different strains. Data at each time-point represent the combined results of at least three replicate experiments. Asterisks at the top of the columns denote significant differences ($P < 0.05$) among strains at each time-point.

spindle rotation and PB2 extrusion. With this aim, oocytes collected from hybrid B6D2F1, inbred C57BL/6 and outbred CF-1 and immunodeficient NU/+ mice were parthenogenetically activated with either ethanol or strontium and analyzed at several time-points after activation. These two artificial activating agents were chosen because of the differential Ca^{2+} signal dynamics they induce in the treated oocytes (Cuthbertson *et al.* 1981, Kline & Kline 1992, Bos-Mikich *et al.* 1995).

No differences were detected among strains or between activation treatments in the final activation efficiency, measured as the percentage of oocytes that exited MII arrest by 6 h p.a., except for the group of strontium-treated NU/+ oocytes. However, significant differences were evident in the timing of meiotic cell cycle progression and in the rate of pronuclear formation after activation depending on both the oocyte genotype and the activating stimulus. In fact, comparison of the kinetics of oocyte activation in oocytes from four different strains of mice subjected to two different activation treatments allowed us to identify some strain-specific characteristics that appear to be independent of the activating stimulus and other characteristics that vary according to the activation treatment applied. For instance, fast exit from the TII stage after PB2 extrusion and arrest at MIII appear as inherent characteristics of C57BL/6 oocytes, at least under the artificial activation conditions examined in the present study, as they are not modified by the type of activating stimulus applied. In contrast, delayed meiotic resumption and progression in NU/+ oocytes was only related to strontium exposure. In addition, the low percentage of CF-1 and NU/+ oocytes that were able to progress to interphase and form a pronucleus after PB2 extrusion following ethanol exposure could be significantly improved by the use of strontium as the activating stimulus. Nevertheless, in spite of the clear influence of the activating stimulus on the timing of meiotic progression in NU/+ oocytes and in the rate of pronuclear formation in both NU/+ and CF-1 oocytes, the genetic background of the oocyte must also play a role, as these effects are not observed in oocytes from hybrid B6D2F1 and inbred C57BL/6 strains.

Because the mechanism of action of various artificial activating stimuli is well known, some of the effects of the activation treatment on the kinetics and the end-point of meiotic progression observed in this study are easily explained. Thus, while ethanol induces a single, large Ca^{2+} rise for the period of its addition (Cuthbertson *et al.* 1981), strontium triggers repetitive Ca^{2+} rises which last for several hours (Kline & Kline 1992, Bos-Mikich *et al.* 1995). Even though a single Ca^{2+} increase is sufficient to induce early activation events, such as cortical granule exocytosis and the resumption of meiosis, repetitive Ca^{2+} oscillations are required for late events such as mRNA recruitment, pronuclear formation and DNA synthesis to occur (Schultz & Kopf 1995, Soloy *et al.* 1997). In fact, our results in CF-1, NU/+ and C57BL/6 oocytes agreed with previous findings that oocytes treated with ethanol or

other activating agents inducing a single Ca^{2+} rise often arrest at MIII instead of forming a pronucleus after PB2 extrusion, due to insufficient stimulation (Kubiak 1989, Vincent *et al.* 1992). Similarly, the significant increase in the rates of pronuclear formation in CF-1 and NU/+ oocytes as seen after strontium exposure was consistent with the idea that repetitive Ca^{2+} oscillations are instrumental in ensuring the completion of meiosis (Swann & Ozil 1994, Ducibella *et al.* 2002). However, the high number of B6D2F1 oocytes that formed a pronucleus and of C57BL/6 oocytes that arrested at MIII after PB2 extrusion, irrespective of the activating stimulus applied, suggest that these rules are not absolute. In light of our results, the genetic background of the oocyte may also dictate the dynamics of the Ca^{2+} signal that is needed for a complete activation of the oocyte under artificial conditions. In particular, for the strains analyzed in this study, pronuclear formation in B6D2F1 oocytes appeared to require the lower Ca^{2+} stimulation and C57BL/6 oocytes the higher.

On the other hand, because ethanol induces a large Ca^{2+} rise for the period of the oocyte exposure, it presumably results in a higher intensity of Ca^{2+} stimulation than strontium during the first minutes p.a. However, in the long term, the continued Ca^{2+} transients induced by strontium over a longer time-period probably contribute to a higher overall Ca^{2+} stimulation in the oocytes. This differential Ca^{2+} signal dynamics triggered by the two activating agents may account for the faster meiotic resumption observed in ethanol-treated B6D2F1, CF-1 and NU/+ oocytes during the first 20 min p.a., when compared with strontium exposure, and the faster TII exit in strontium-treated B6D2F1 and CF-1 oocytes in comparison with ethanol-treated oocytes of the same genotype. The latter is consistent with the observations by Ozil (1990, 1998) that an increase in the intensity of the Ca^{2+} stimulation decreases the time to pronuclear formation after oocyte activation. Nevertheless, this effect was not observed for all the strains analyzed (e.g. C57BL/6 oocytes), thus indicating again an influence of the oocyte genetic background.

Our study also revealed strain- and treatment-dependent differences in the timing of spindle rotation and of PB2 extrusion after parthenogenetic activation. Strontium exposure resulted in a delay in the completion of spindle rotation and in PB2 extrusion in B6D2F1, CF-1 and NU/+ oocytes when compared with ethanol exposure. Also, NU/+ oocytes were significantly slower than oocytes from the other three genotypes in completing meiotic spindle rotation and in extruding the PB2, both after ethanol and strontium treatment. In general, a positive correlation was observed between the apparent rate of spindle rotation and the rate of PB2 extrusion. This finding is not surprising as the spindle needs to achieve the proper orientation, perpendicular to the plasma membrane, before extrusion of the PB2 can occur.

Earlier studies on the development of androgenetic mouse embryos have revealed strain-specific differences in how the oocytes modify paternal genomes after fertilization (Latham & Solter 1991, Latham 1994, Latham & Sapienza 1998). Similarly, maternal pronuclear exchanges between oocytes from different strains have also demonstrated a genotype effect on the modification of maternal chromosomes during oogenesis (Roemer *et al.* 1997). On the other hand, strain-specific differences in the timing of germinal vesicle breakdown and of first PB extrusion have been reported among oocytes derived from recombinant strains and progenitor inbred strains (Polanski 1997a,b). Finally, with regard to parthenogenesis, differences in the rate of cleavage after parthenogenetic activation using several treatments have been described in oocytes derived from two different outbred mouse strains (Marcus 1990). In addition, in a more recent study, oocytes of different strains (B6D2F1, C57BL/6 and DBA/2) have shown a different ability to support both parthenogenetic and cloned embryo development in different culture media (Gao *et al.* 2004). The origin of the oocyte strain-specific differences detected in these previous studies, as well as in the present study, is unclear, but it may reflect differences in ooplasm composition. The finding of at least 17 proteins showing significant quantitative differences in the rate of synthesis between inbred DBA/2 and C57BL/6 oocytes (Latham 1994) would support this idea.

Considering this egg composition effect, several possibilities could account for the strain-specific differences detected here on the timing and end-point of meiotic progression after parthenogenetic activation. Among them, it is possible that oocytes from different genotypes show differences in the size, density and properties of the Ca^{2+} channels located in the endoplasmic reticulum resulting in quantitative or temporal differences in the Ca^{2+} transients induced by the activating stimulus. As the kinetics of cell cycle progression after activation and the choice between MIII arrest or progression to interphase is dependent on the profile of internal Ca^{2+} release in the oocyte after activation (Ozil 1990, 1998, Vincent *et al.* 1992), strain-dependent variations in the quantity of free Ca^{2+} liberated upon the same activation treatment could explain some of the genotype-specific differences detected in the present study. These variations could also be related to different oocyte maturation kinetics in the four mouse strains analyzed, as it has been reported that the amount of IP_3 receptor (IP_3R), a key channel in regulating intracellular Ca^{2+} oscillations during activation (Miyazaki *et al.* 1993), increases during meiotic maturation (Mehlmann *et al.* 1996, He *et al.* 1997). In fact, it is well documented in several mammalian species that complete activation and pronuclear formation is dependent upon the age of the oocyte with regard to ovulation. In general, while aged oocytes are easily activated even with artificial stimuli inducing a single Ca^{2+} rise, recently ovulated oocytes often arrest at MIII and repetitive Ca^{2+} transients or combined treatments with protein synthesis or phosphorylation

inhibitors are required for the completion of meiosis and entry into interphase in these young oocytes (Collas *et al.* 1989, Kubiak *et al.* 1993, Presicce & Yang 1994, Swann & Ozil 1994, Krivokharchenko *et al.* 2003). Although it is beyond the scope of the present study, further analysis of oocyte maturation kinetics after hCG injection, quantification of IP_3R levels and measurement of the Ca^{2+} transients induced by ethanol and strontium in oocytes from the four strains of mice could be performed to assess this possibility.

Alternatively, the proteins responsible for exit from MII arrest and pronuclear formation (i.e. MPF, MAPK, CaMKII) may possess different activation/inactivation thresholds in response to variations in the level or the dynamics of the intracellular Ca^{2+} concentration in oocytes from different strains. Likewise, oocytes from different genotypes might also differ in the time required for chromatin decondensation or for the assembly of the pronuclear envelope or of the contractile ring, due to quantitative or qualitative variations among strains in the proteins involved in these processes. This again could result in differences in the timing of TII exit and pronuclear formation or in the timing of PB2 extrusion depending on the genetic background of the oocyte.

Finally, it is interesting to note that hybrid B6D2F1 oocytes exhibited the best rates of pronuclear formation regardless of the activation treatment applied, indicating a better capacity for full activation than inbred C57BL/6 and outbred CF-1 and NU/+ oocytes. In a recent study, Gao *et al.* (2004) showed that hybrid B6D2F1 oocytes were able to support better *in vitro* development to the blastocyst stage after parthenogenetic activation with strontium than inbred C57BL/6 and DBA/2 oocytes. On the other hand, it is well documented that the rates of preimplantation embryo development both *in vivo* and *in vitro* are also strain dependent, and that embryos derived from hybrid strains develop more efficiently and faster than those derived from inbred and outbred strains (Goldbard & Warner 1982, Warner *et al.* 1987, Du & Wales 1993, Scott & Whittingham 1996). Therefore, in light of these previous results by others and our results presented here, it is possible that strain-specific differences in the dynamics of oocyte activation could be related to later differences in the rate of early embryo development.

In conclusion, our results have demonstrated that the timing of nuclear events following oocyte activation, such as meiotic cell cycle progression, spindle rotation and PB2 extrusion, as well as the ability of the oocyte to complete activation and progress into interphase after PB2 extrusion depend not only on the parthenogenetic treatment applied but also on the genotype of the oocyte. Because of this, activation treatments that are efficient in oocytes from one strain may not necessarily be efficient for the full activation of oocytes from other strains. In particular, while the majority of B6D2F1 oocytes exit MII and progress to interphase after ethanol exposure, a relatively

high proportion of C57BL/6, CF-1 and NU/+ oocytes become arrested at MIII. In a recent study, Kishikawa *et al.* (1999) reported similar parthenogenetic development up to the morula/blastocyst stage of B6D2F1 oocytes, irrespective of the activating stimulus applied (strontium, ethanol, electric pulse or sperm). However, the results might have been different if oocytes from a different strain (e.g. C57BL/6) had been used in the study. Identification of the specific ooplasmic factors that contribute to the genotype-specific differences in meiotic progression after activation and in parthenogenetic development should lead to a better understanding of the process of oocyte activation and may improve the efficiency of both artificial activation and cloning procedures.

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