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Chromatin and microtubule organisation in maturing and pre-activated porcine oocytes following intracytoplasmic sperm injection

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Summary

Chromatin and microtubule organisation was determined in maturing and activated porcine oocytes following intracytoplasmic sperm injection in order to obtain insights into the nature of sperm chromatin decondensation and microtubule nucleation activity. Sperm chromatin was slightly decondensed at 8 h following injection into germinal vesicle stage oocytes. Sperm-derived microtubules were not seen in these oocytes. Following injection into metaphase I (MI)-stage oocytes, sperm chromatin went to metaphase in most cases. A meiotic-like spindle was seen in the sperm metaphase chromatin. In a few MI-stage oocytes, sperm chromatin decondensed at 8 h after injection, and a small sperm aster was seen. Sperm injection into oocytes at 5 h following activation failed to yield pronuclear formation. Maternally derived microtubules were organised near the female chromatin in these oocytes, and seemed to move condensed male chromatin closer to the female pronucleus. At 18 h after sperm injection into pre-activated oocytes, a condensed sperm nucleus was located in close proximity to the female pronucleus. These results suggest that the sperm nuclear decondensing activity and microtubule nucleation abilities of the male centrosome are cell cycle dependent. In the absence of a functional male centrosome, microtubules of female origin take over the role of microtubule nucleation for nuclear movement.

Keywords: Centrosome, ICSI, Oocyte maturation, Microtubules, Pig eggs, Pronucleus

Introduction

During fertilisation, penetrating sperm rapidly decondense to become a male pronucleus. The sperm nucleus-decondensing activity of the oocytes appears to be cell cycle dependent. In mice, immature oocytes do not promote sperm nuclear decondensation prior to germinal vesicle breakdown (GVBD); the oocyte cytoplasm only acquires this ability after GVBD and loses it a few hours after fertilisation (Usui & Yanagimachi, 1976; Borsuk & Tarkowski, 1989; Szollosi *et al*., 1990).

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Using conventional *in vitro* fertilisation techniques, Wang & Niwa (1997) observed in the pig that spermatozoa could penetrate into immature and maturing oocytes with a high incidence of polyspermy. The spermatozoa in immature oocytes were partially decondensed soon after penetration, and could be transformed into metaphase chromatin when the oocytes were cultured to the metaphase stage. Although sperm penetration in pre-activated oocytes in pigs has been reported (Funahashi *et al*., 1993), little information is available on the fertilisation processes, such as pronuclear formation and movement, in pre-activated pig oocytes.

As in most other animals, in the pig the paternal centrosome is typically introduced into oocytes during fertilisation (for review see Schatten, 1994; Kim *et al*., 1996, 1997). The paternal centrosome seems to nucleate microtubules for positioning the male and female pronuclei. Interestingly, in the absence of functional

male-derived microtubules, the maternally derived microtubules take over the role of pronuclear movement (Kim *et al.*, 1998, 1999). Similarly, in the absence of sperm-derived microtubules in rabbit, such as in parthenogenetically activated oocytes, microtubules of female origin are organised near the female nucleus, which functions to centre the nucleus (Terada *et al.*, 2000). Many comparative studies have been done on microtubule and chromatin assembly during fertilisation and parthenogenesis in mammals (Breed *et al.*, 1994; Navara *et al.*, 1994; Kim *et al.*, 1996). However, little information is available on the microtubule nucleation ability of centrosomes of either male or female origin in maturing or pre-activated porcine oocytes.

Intracytoplasmic sperm injection (ICSI) is a widely used technology for clinically assisted fertilisation as well as a good tool for studying basic fertilisation processes in mammals. In this study, we determined chromatin morphology and microtubule assembly in maturing and activated porcine oocytes following ICSI in order to gain insights into the nature of the sperm decondensation activity and the microtubule nucleation ability of centrosomes in pig oocytes.

Materials and methods

In vitro maturation

Prepubertal porcine ovaries were collected from a local slaughterhouse and transported to the laboratory at 25 °C in Dulbecco's phosphate-buffered saline (PBS) supplemented with 5.54 mM D-glucose, 0.33 mM sodium pyruvate, 75 mg/ml potassium penicillin G and 50 mg/ml streptomycin sulfate. Cumulus–oocyte complexes (COC) were aspirated with an 18 gauge needle into a disposable 10 ml syringe from follicles 3–6 mm in diameter. The COC were washed three times with TCM-HEPES medium. Groups of 50 COC were matured in 500 µl of bovine serum albumin (BSA)-free NCSU23 medium (Petters & Wells, 1993) supplemented with 10% follicular fluid, 0.6 mM cysteine, 10 IU/ml human chorionic gonadotropin (hCG; Sigma, St Louis, MO) and 10 IU/ml pregnant mare's serum gonadotropin (PMSG; Sigma) under mineral oil at 39 °C for 46–48 h.

Oocyte activation

The procedures for electrical stimulation of porcine oocytes were as described by Kim *et al.* (1996). Electrical stimulation to induce activation was delivered with an Electro Cell Manipulator (BTX) to a chamber with two parallel platinum wires. At 3 or 5 h before sperm cell injection, cumulus-cell-denuded oocytes were stimulated by a 30 µs pulse at 1.36 kV/cm DC at room temperature (25 °C). The oocytes were then transferred to 500 µl of NCSU23 medium and cultured at 39 °C in an atmosphere of 5% CO_2 in air until sperm cell injection.

Preparation of spermatozoa and sperm injection

The porcine sperm-rich fraction (15 ml) was collected from a boar by the gloved hand method and, after adding antibiotic–antimycotic solution (Sigma), the semen sample was kept at 20 °C for 16 h. The semen was washed three times by centrifugation with 0.9% (w/v) NaCl supplemented with 10 mg/ml BSA (fraction V; Sigma). Spermatozoa were suspended in 1.5 ml of TL-HEPES (Prather *et al.*, 1995) for 30 min to induce capacitation. Spermatozoa were washed in TL-HEPES and then suspended for 1 h in 1.5 ml of TL-HEPES. The sperm were resuspended to 1 ml with heparin-containing (10 µg/ml) TL-HEPES in an Eppendorf tube and kept at 39 °C for 30–60 min to induce capacitation. In order to see the sperm tail in porcine oocytes following ICSI, spermatozoa were stained with the MitoTracker fluorochrome (Molecular Probes, Eugene, OR) for 5 min at a final concentration of 10 µM, and then washed in TL-HEPES medium before injection.

A microdrop (5 µl) of sperm injection was placed on a slide, and the slide placed on a Nikon Differential Interference Contrast inverted microscope equipped with Narishige micromanipulators. The oocytes were denuded of cumulus cells by repeated pipetting. Oocytes with visible polar bodies and of excellent morphology were used for this experiment. Oocytes were centrifuged for 10 min at 12 000 *g* in 1.2 ml Eppendorf centrifuge tubes. The injection of a spermatozoon into the oocyte cytoplasm was performed using the method of Lee *et al.* (1998). Briefly, the injection needle used was of 6–7 µm inner and 8–9 µm outer diameter. The polar body was at 6 or 12 o'clock and the point of injection at 3 o'clock. An oocyte was penetrated by the injecting micropipette, a small amount of cytoplasm was drawn into the micropipette, and then the cytoplasm together with the spermatozoon and a small amount of medium was expelled into the oocyte. Immediately after ooplasmic injection, the injecting micropipette was withdrawn quickly, and the oocyte released from the holding pipette to reduce the intracytoplasmic pressure being exerted on it. After injection, all the oocytes were transferred to NCSU23 medium and cultured at 39 °C under 5% CO_2 in air.

Immunofluorescence microscopy

Microtubules and DNA were detected by the indirect immunocytochemical techniques described by Kim *et*

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al. (1996). Briefly, the oocytes were permeabilised in a modified Buffer (Simerly & Schatten, 1993; 25% glycerol, 50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid, 1 mM β-mercaptoethanol, 50 mM imidazole pH 6.7, 3% Triton X-100 and 25 mM phenylmethylsulfonyl fluoride) for 20 min, fixed in methanol at –20 °C for 10 min and stored in PBS containing 0.02% sodium azide and 0.1% BSA for 2–7 days at 4 °C. Microtubule localisation was performed using an anti-α-tubulin monoclonal antibody (Sigma). Fixed oocytes were incubated for 90 min at 39 °C with the antibody diluted 1:300 in PBS. After several washes with PBS containing 0.5% Triton X-100 and 0.5% BSA, oocytes were incubated in a blocking solution (0.1 M glycine, 1% goat serum, 0.01% Triton X-100, 1% powdered milk, 0.5% BSA and 0.02% sodium azide) at 39 °C for 1 h. The blocking was followed by incubation in fluorescein isothiocyanate (FITC)-labelled goat anti-mouse antibody (Sigma). DNA was fluorescently detected by exposure to 5 mg/μl propidium iodide (Sigma) for 1 h. Stained oocytes were then mounted under a coverslip with an antifade mounting medium (Universal Mount, Fisher Scientific, Huntsville, AL) to retard photobleaching. Slides were examined using laser-scanning confocal microscopy, performed using a Bio-Rad MRC 1024 equipped with a krypton–argon ion laser for the simultaneous excitation of fluorescein for microtubules and propidium iodide for DNA. The images were recorded digitally and archived on an erasable magnetic optical disk.

Statistical analysis

The data were pooled from at least four replications. Differences in the percentages of oocytes developing to a particular stage were determined by chi-square procedures.

Results

Sperm injection into oocytes maturing *in vitro*

Thirty porcine oocytes at 0, 22 or 44 h of maturation were each injected with a spermatozoon and cultured for 8 h in maturation medium. Following sperm injection into 0 h oocytes, all oocytes remained at the germinal vesicle (GV) stage (Table 1). A large proportion (60%) of these oocytes had slightly decondensed sperm chromatin (Table 2, Fig. 1*A*) and microtubules were not seen in the oocyte cytoplasm or near the male chromatin (Fig. 1*A*). At 8 h following injection into oocytes at 22 h of maturation, female chromatin formed in the metaphase I (MI) stage in 24 of the 30 oocytes (Table 1). In these oocytes, sperm chromatin went to metaphase in most cases (21/24; Table 2, Fig. 1*B*), and in these cases a meiotic-like spindle was seen in the sperm metaphase chromatin ($n = 21$; Fig. 1*B*). Some spermatozoa injected into 22 h matured oocytes underwent decondensation, in which case a small sperm aster was likely to be seen (4/6). However, fully developed microtubule asters were not observed. Following

Table 1 Meiotic maturation of porcine oocytes maturing *in vitro* at 8 h following intracytoplasmic sperm injection

Time of maturation (h)	No. of oocytes examined (r)	No. of oocytes (%)				
		GV	GVBD	MI	MII	fPN
0	30 (3)	30 (100)	0	0	0	0
22	30 (3)	3 (10)	3 (10)	24 (80)	0	0
44	30 (3)	0	0	0	06 (20)	24 (80)

r, replication; GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II; fPN, female pronucleus.

Table 2 Transformation of sperm nuclei in porcine oocytes maturing *in vitro* at 8 h following intracytoplasmic sperm injection

Time of maturation (h)	No. of oocytes examined (r)	No. of oocytes with sperm chromatin (%)			
		Condensed	Decondensed	M phase	mPN
0	30 (3)	12 (40)	18 (60)	0	0
22	30 (3)	3 (10)	6 (20)	21 (70)	0
44	30 (3)	0	6 (20)	6 (20)	18 (60)

r, replication; mPN, male pronucleus.

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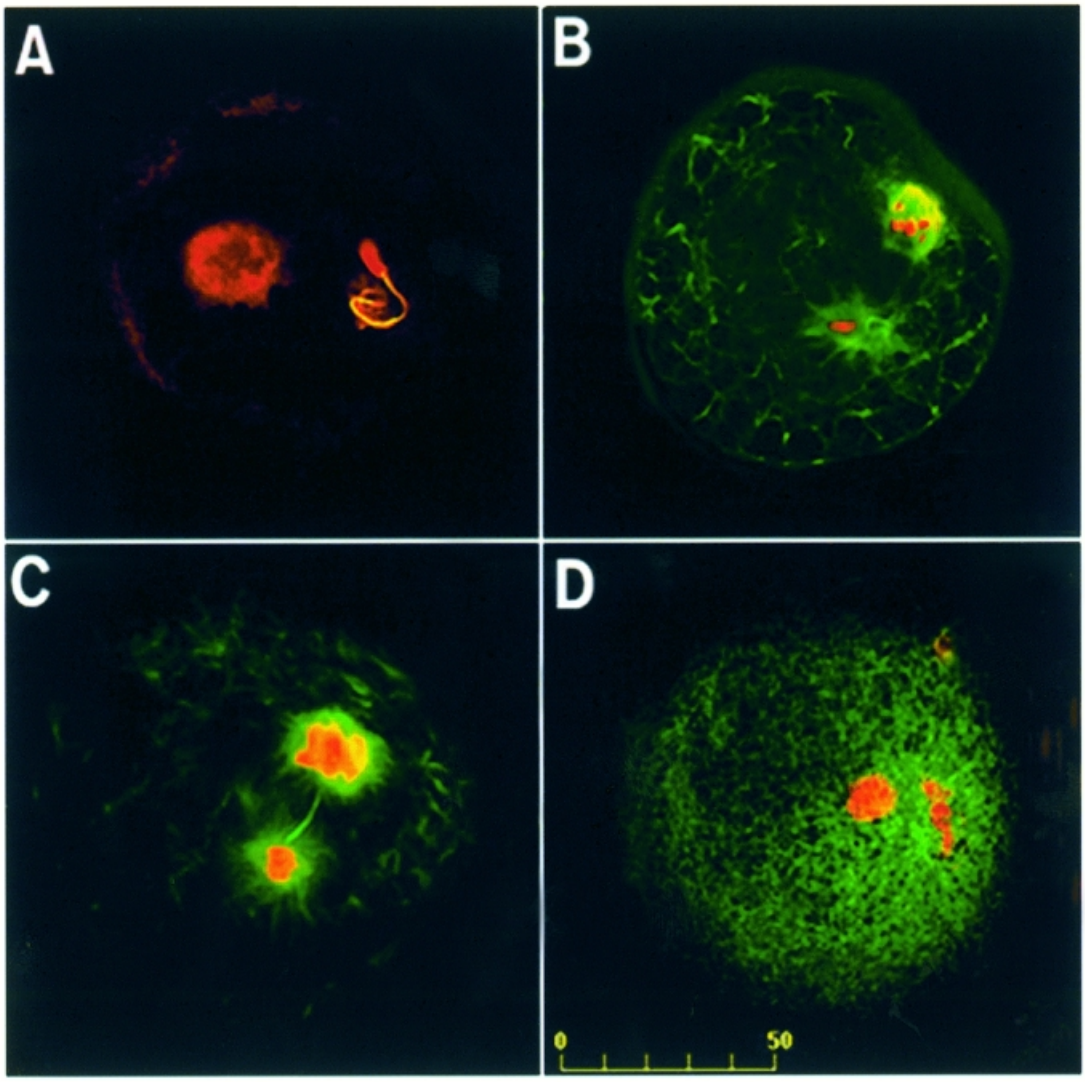

Figure 1 Microtubule and chromatin assembly in porcine maturing oocytes following intracytoplasmic sperm injection (ICSI). Red image, DNA; green image, microtubules. (*A*) Germinal vesicle (GV) and slightly decondensed sperm were seen in GV-stage oocytes following ICSI. (*B*), (*C*) ICSI into 22 h matured oocytes. Decondensed sperm chromatin and a small sperm aster are seen at the base of the sperm head (*B*). In the other case, sperm chromatin developed to metaphase chromatin. (*D*) Microtubule aster was well developed during pronuclear movement in matured porcine oocytes following ICSI.

sperm injection into mature oocytes, well-developed microtubule asters were seen, which seemed to move male and female chromatin close together for the formation of syngamy (see Kim *et al*., 1998; Fig. 1*D*).

Injection into preactivated oocytes

While female chromatin developed into a pronucleus at 6 h following sperm injection into activated oocytes (Fig. 2*A*), in only a few cases did sperm chromatin decondense and form a male pronucleus (Table 3, Fig. 2*B*). Sperm-derived microtubules were not observed in or near the condensed sperm chromatin. Instead the maternally derived microtubules were organised near the female chromatin in most cases, which often seemed to move the condensed male chromatin nearer to the female pronucleus (12/19; Fig. 2*B*). At 18 h after sperm injection into pre-activated oocytes, a condensed sperm nucleus was located in close proximity to the female pronucleus in most cases (39/52; Fig. 2*C*). The condensed sperm chromatin remained in all parthenogenetically developed embryos, suggesting that male chromatin did not participate in mitosis (Fig. 2*D*).

Discussion

The cytoplasm of maturing oocytes has an activity that can transform sperm nuclei into metaphase

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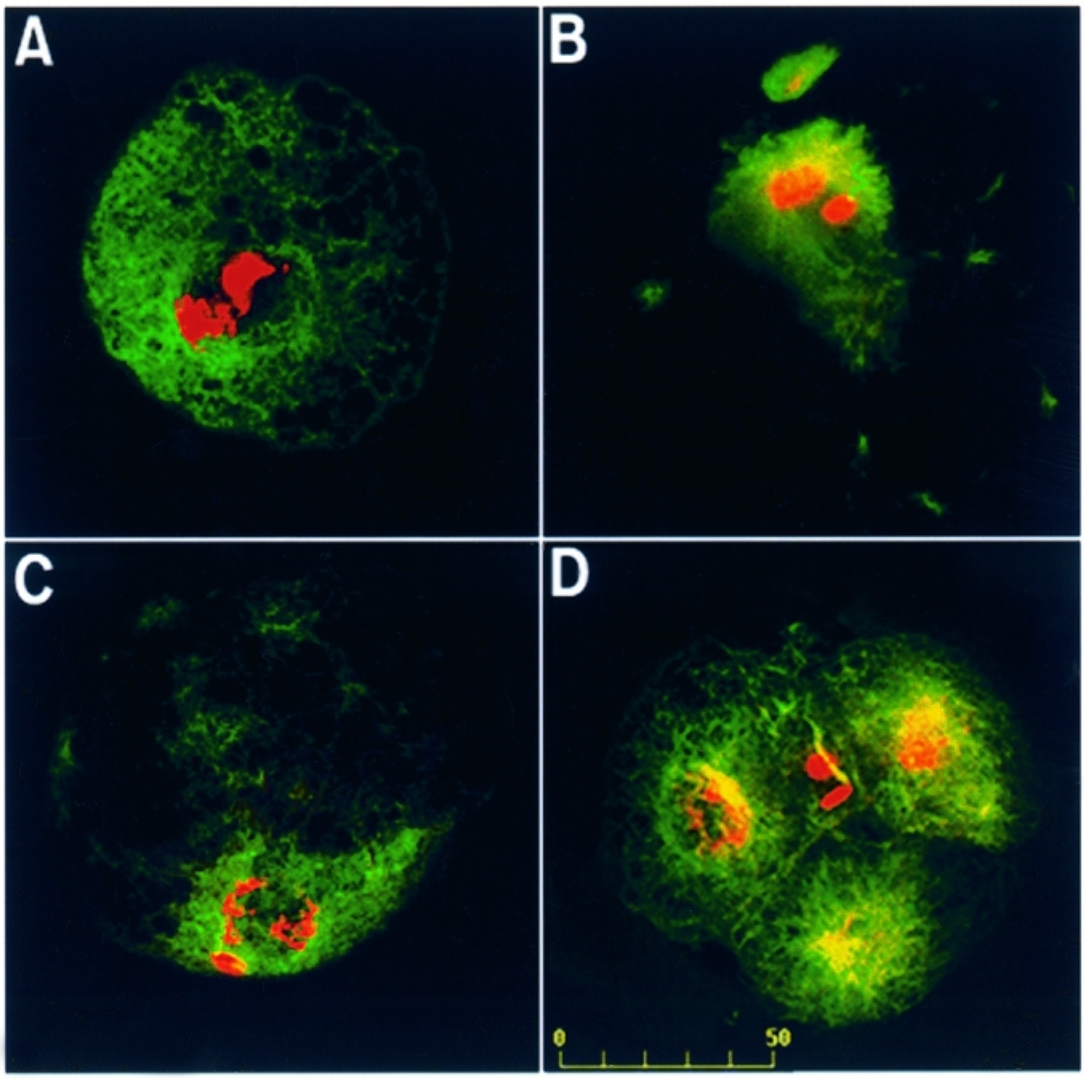

Figure 2 Microtubule and chromatin assembly in maturing porcine oocytes following intracytoplasmic sperm injection (ICSI). Red image, DNA; green image, microtubules. (*A*) Extensive microtubules around male and female pronuclei during pronuclear apposition. (*B*) Microtubules were seen to be associated with the female pronucleus in pre-activated oocytes following ICSI. (*C*) Condensed sperm head was located close to the female pronculeus. (*D*) Intact sperm head remained in a parthenogenetically developed 4-cell embryo.

Table 3 Chromatin morphology of pre-activated porcine oocytes at 6 and 18 h following intracytoplasmic sperm injection

Time of			No. of oocytes with sperm chromatin (%)			
Activation (h)	Observation (h)	Examined (r)	Condensed	Decondensed	mPN	Others
0	6	32 (3)	3 (9)	13 (41)	14 (44)[a]	2 (6)
	18	25 (3)	2 (8)	5 (20)	15 (60)[a]	3 (12)
3	6	31 (4)	17 (55)	6 (20)	5 (15)[b]	3 (10)
	18	44 (4)	12 (27)	20 (45)	5 (12)[b]	7 (16)
5	6	26 (3)	13 (50)	9 (35)	0[c]	4 (15)
	18	25 (3)	10 (40)	10 (40)	0[c]	5 (20)

r, replication
[a,b,c]p <0.05.

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chromosomes in mice (Clarke & Masui, 1986), cattle (Abeydeera *et al*., 1992) and pigs (Wang & Niwa, 1997). Similarly, we observed transformation of male chromatin into metaphase chromatin following ICSI into MI-stage oocytes. The possible participation of a maturation promoting factor (MPF) in maturing oocytes for transformation of sperm nuclei into metaphase chromosomes has been suggested in bovine and porcine oocytes (Abeydeera *et al*., 1992; Wang *et al*., 1994). The MPF activity appears that GVBD and is very high at MI and MII in pig oocytes (Mattioli *et al*., 1991; Naito & Toyoda, 1991). Similar to what was found in another study (Wang & Niwa, 1997), in the present study almost all sperm metaphase chromosomes were observed in oocytes with metaphase female chromatin, and not in GV-stage or GVBD-stage oocytes, suggesting that MPF is critical for the transformation of sperm nuclei to metaphase chromosomes in maturing pig oocytes.

During the transit of spermatozoa through the epididymis, sperm nuclei are made very stable by an extensive cross-linking of protamines, which are sperm-specific basic proteins (Bedford & Calvin, 1974). Following sperm penetration into the oocyte cytoplasm, the protamines are removed and replaced by histones, and the sperm nucleus is remodelled into a pronucleus with assembly of the nuclear envelope. The process of pronuclear formation is not fully understood at present. Reduction of the sulfate bond by glutathione (Sutovski *et al*., 1996) and nucleoplasmin from the germinal vesicle (Philpott *et al*., 1991; Maeda *et al*., 1998) seem to play key roles in the decondensation of sperm and the formation of the male pronucleus. In the present study, slight sperm decondensation was seen in GV-stage oocytes following sperm injection. Previously, sperm penetration into the zona pellucida of GV-stage oocytes and sperm decondensation in these oocytes have been reported in dog, cow and pig (Mahi & Yanagimachi, 1976; Niwa *et al*., 1991; Wang *et al*., 1994). However, the degree of sperm nuclear decondensation appears different among these species; in the dog and cow, sperm nuclei are fully decondensed, but in the pig they are only partially decondensed. It seems that the amount or activity of the factor, such as reduced glutathione (Wiesel & Schultz, 1981), that is contained in the cytoplasm of GV oocytes may vary in different species. Much lower amounts of glutathione are contained in GV-stage porcine oocytes compared with fully matured oocytes (Yoshida *et al*., 1993), which may induce incomplete progress of the decondensation of male chromatin following sperm injection into GV-stage oocytes.

In the mouse, spermatozoa penetrating the oocyte 3 h or more after activation form abnormal chromatin and fail to form a normal pronucleus (Maleszewski *et al*., 1999). Similarly, we observed that much less sperm chromatin developed into male pronuclei in 3 h early-activated oocytes. Further, all sperm chromatin injected into oocytes at 5 h following activation failed to form pronuclei. This suggests that the cytoplasmic factors for sperm decondensation and pronuclear formation, such as glutathione or nucleoplasmin, may be gradually inactivated during the interphase following activation, and/or may be depleted by the female pronucleus.

Following ICSI, similar to conventional fertilisation, microtubules that are organised by the sperm centrosome appear to play an important role in bringing the male and female pronuclei into close apposition and in forming syngamy in rhesus monkey (Hewitson *et al*., 1996; Sutovsky *et al*., 1996) and pig oocytes (Kim *et al*., 1998). In the present study, a sperm aster was not seen in GV-stage oocytes following sperm injection, but was seen in some MI-stage oocytes, suggesting that the microtubule nucleation ability of the sperm centrosome is cell cycle dependent.

In the present study, we observed that sperm-derived microtubules following ICSI into activated oocytes were not organised. Instead, maternal microtubules were organised in association with the fully grown female nucleus, which seems to bring condensed sperm chromatin close to the female pronucleus. This supports the hypothesis that in the absence of a functional sperm centrosome, the maternally derived microtubules take over the role of nuclear positioning of oocytes (Kim *et al*., 1998). The mechanism whereby the maternally derived microtubules organise and move pronuclei to the centre of oocytes is unclear. As shown in *Xenopus* eggs (Heald *et al*., 1996) and rabbit oocytes (Terada *et al*., 2000), in unfertilised, activated porcine oocytes the chromatin (or DNA particles) may have ability to induce assembly of microtubules to transport the nucleus in the absence of a sperm-derived centrosome.

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