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Research

Translocation of active mitochondria during pig oocyte maturation, fertilization and early embryo development *in vitro*

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The distribution of active mitochondria during pig oocyte maturation, fertilization and early embryo development *in vitro* was revealed by using MitoTracker Green staining and confocal laser scanning microscopy. The regulation of mitochondrial translocation by microfilaments and microtubules was also studied. In oocytes collected from small follicles, strong staining of active mitochondria was observed in the cell cortex. Accumulation of active mitochondria in the peripheral cytoplasm and around the germinal vesicles was characteristic of fully grown oocytes collected from large follicles. Mitochondria accumulated in the perinuclear area during meiotic progression from germinal vesicle breakdown (GVBD) to anaphase I. Larger mitochondrial foci were formed and moved to the inner cytoplasm in mature oocytes. Compared with the oocytes matured *in vivo*, in which large mitochondrial foci were distributed throughout the cytoplasm, mitochondria were not observed in the central cytoplasm in most of the oocytes matured *in vitro*. Strong staining of mitochondria was observed in the first polar bodies in metaphase II oocytes. In fertilized eggs, active mitochondria aggregated in the pronuclear region. Perinuclear clustering and a cortical ring were the most marked features of early cleavage. Active mitochondria were distributed in both inner cell mass cells and trophectoderm cells of the blastocysts. Disassembly of microtubules with nocodazole inhibited both mitochondrial aggregations to the germinal vesicle area and their inward movement to the inner cytoplasm during oocyte maturation, as well as the translocation of mitochondria to the peri-pronuclear region during fertilization, whereas disruption of microfilaments by cytochalasin B had no effects. These data indicate that: (i) oocyte maturation, fertilization and early embryo development in pigs are associated with changes in active mitochondrial distribution; (ii) mitochondrial translocation is mediated by microtubules, but not by microfilaments; and (iii) *in vitro* maturation conditions may cause incomplete movement of mitochondria to the inner cytoplasm and thus affect cytoplasmic maturation.

Introduction

Mammalian oocytes are arrested at the G2–M phase transition of the first meiotic division. *In vitro*, fully grown oocytes liberated from their follicles spontaneously reinitiate meiosis I, characterized by germinal vesicle breakdown (GVBD), chromatin condensation, spindle assembly, emission of the first polar body and progression to metaphase of the second meiotic division (MII), at which stage they undergo a second arrest until fertilization. After spermatozoa penetrate the oocyte, the second polar body extrudes, male and female pronuclei form and syngamy occurs to start early embryo development. Nuclear changes during oocyte maturation and fertilization are co-ordinated with movements of genetic material and organelles, and with biochemical changes in the cytoplasm to ensure normal embryo development. The normality of early embryogenesis is directly related to the ordered expression of these developmental programmes (Van Blerkom, 1991).

Of the numerous cytoplasmic changes that occur, the positioning of mitochondria may be involved in concentrating ATP or calcium to specific regions in oocytes or fertilized eggs to support normal developmental processes. Thus, the distribution of active mitochondria may be indicative of the energy or ion requirement of various key events during oocyte maturation, fertilization and early embryo development. In mice, the perinuclear accumulation of mitochondria between GVBD and metaphase I (MI) (Van Blerkom and Runner, 1984; Van Blerkom, 1991), and the polarized distribution of mitochondria to one half of the oocyte containing the MII spindle (Calarco, 1995) were observed and were regarded as one aspect of the developmental programme of cytoplasmic maturation. Previous observations also revealed that translocation of mitochondria is co-ordinated

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with changes in the location of microtubule-organizing centres and that the microtubule-mediated accumulation of mitochondria may be required for nuclear maturation of mouse oocytes (Van Blerkom, 1991). In hamster and human fertilized eggs, active mitochondria relocate to surround the pronuclei (Bavister and Squirrell, 2000; Van Blerkom *et al.*, 2000). Changes in the location of active mitochondria are also suggested to correlate with the ability of hamster embryos to develop *in vitro* (Barnett *et al.*, 1997; Bavister, 2000).

In pigs, the developmental ability of oocytes matured and fertilized *in vitro* is low compared with that of oocytes matured and fertilized *in vivo*, although progress with *in vitro* maturation and fertilization has been made (Prather and Day, 1998). Inadequate cytoplasmic maturation during *in vitro* maturation or abnormal *in vitro* fertilization is thought to impair embryonic development. The ultrastructure and distribution of mitochondria in pig oocytes have been observed by electron microscopy (Cran, 1985). However, unlike confocal microscopy, electron microscopy allows observations of only a limited number of samples. In pigs, the distribution of mitochondria during oocyte fertilization and early embryo development is largely unknown. In addition, the regulation of mitochondrial translocation in oocytes and fertilized eggs of this species has not been studied.

In the present study, MitoTracker Green staining was used in combination with confocal laser scanning microscopy to determine: (i) the distribution of active mitochondria during maturation of pig oocytes *in vivo* and *in vitro*; (ii) the mitochondrial distribution during fertilization and subsequent early embryo development *in vitro*; and (iii) the regulation of mitochondrial translocation by the microtubule and microfilament cytoskeleton.

Materials and Methods

In vitro *maturation and fertilization of oocytes and* in vitro *culture of early embryos*

In vitro maturation of pig oocytes was conducted in a chemically defined medium (Abeydeera *et al.*, 1998). Briefly, oocytes were aspirated from antral follicles of ovaries collected from slaughtered prepubertal gilts. Oocytes from large follicles (3–6 mm in diameter) were assumed to be meiotically competent and were used for maturation culture. Oocytes from small follicles (0.5–2.0 mm in diameter) were also collected for mitochondrial evaluation. After washing three times with Hepes-buffered Tyrodes's lactate containing 0.1% (w/v) polyvinyl alcohol, each group of 50 cumulus-enclosed oocytes was cultured for 44 h at 39°C in an atmosphere of 5% CO_2 in air in a 500 µl drop of tissue culture medium (TCM)-199 supplemented with 0.57 mmol cysteine l^{-1}, 10 ng epidermal growth factor ml^{-1} (Sigma Chemical Co., St Louis, MO), 10 iu FSH ml^{-1} (Sigma), 10 iu hCG ml^{-1} (Sigma) and 0.1% (w/v) polyvinyl alcohol.

IVF was carried out as reported by Han *et al.* (1999), with only minor modifications. Oocytes were inseminated in a 100 µl drop of modified Tris-buffered medium containing 0.2% (w/v) BSA and 2 mmol caffeine l^{-1} with frozen–thawed spermatozoa (5×10^5 cells ml^{-1}). Six hours after insemination, oocytes were removed from the fertilization medium and cultured for up to 6 days in 500 µl North Carolina State University (NCSU)-23 medium (Petters and Wells, 1993) containing 4 mg BSA ml^{-1}.

Collection of oocytes matured in vivo

Gilts were observed for spontaneous oestrus. Between 42 and 48 h after oestrus was detected, two gilts were anaesthetized for surgical recovery of *in vivo* matured oocytes. Ovulated oocytes were collected by flushing the oviduct with Hepes-buffered Tyrodes's lactate containing 0.1% (w/v) polyvinyl alcohol. Cumulus cells attached to the oocytes were removed by treatment with 0.3% (w/v) hyaluronidase in Hepes-buffered Tyrodes's lactate containing 0.1% (w/v) polyvinyl alcohol.

Treatments

Oocytes were cultured in maturation medium with or without the microtubule assembly inhibitor nocodazole (100 µmol l^{-1}) or the microfilament organization inhibitor cytochalasin B (500 nmol l^{-1}) for 44 h to evaluate the regulation of mitochondrial translocation by the cytoskeleton during oocyte maturation.

Oocytes matured *in vitro* for 44 h were fertilized *in vitro* for 6 h and transferred to the NCSU medium containing either 500 nmol cytochalasin B l^{-1}, 100 µmol nocodazole l^{-1} or 1 µmol taxol l^{-1} and subsequently cultured for up to 24 h to investigate the effects of cytoskeletal modulators on mitochondrial migration during fertilization.

Confocal microscopy of active mitochondria

A stock solution of MitoTracker Green FM at a concentration of 1 mmol l^{-1} was prepared in dimethyl sulfoxide (DMSO) and stored at –20°C. Oocytes, zygotes and early embryos were stained for active mitochondria in maturation medium or NCSU medium containing 0.5 µmol cell permeant MitoTracker Green l^{-1} (Molecular Probes, Eugene, OR) for 30 min at 39°C in 5% CO_2 in air. After washing three times, each for 20 min at 39°C in PBS, tissues were fixed in 2% (w/v) paraformaldehyde in PBS for 1 h at room temperature. Oocytes, zygotes and early embryos were washed twice for 10 min each in PBS containing 0.3% (w/v) PVA (Sigma) and 1% (v/v) Triton-X100 (Sigma) and stained with 10 µg propidium iodide ml^{-1} for 10 min to detect DNA. After washing twice in PBS, the oocytes, zygotes and embryos were mounted on glass slides and observed using laser scanning confocal microscopy (BioRad, Hercules, CA). For observation of MitoTracker staining, the 488 nM line and QH515/30 emission filter were used. The 568 nM line was used to excite propidium

iodide and emitted light was observed by using a HQ600/40 emission filter to visualize DNA. Data were saved to a disk and processed using Photoshop 5.5 software. At least 30 samples in each group were analysed in three repeated experiments except for the control group of *in vivo* matured oocytes ($n = 17$).

Results

Distribution of active mitochondria during pig oocyte maturation

Aggregation of active mitochondria in the cortex was a common feature of oocytes at the GV stage. In GV stage oocytes derived from small follicles of 0.5–2.0 mm in diameter, strong staining of a peripheral active mitochondrial band was observed in approximately half (19/40) of the oocytes (Fig. 1a). In the other oocytes (21/40), in addition to the mitochondrial aggregation in the cortex, some mitochondria were distributed in the sub-cortical region, but few were localized around the germinal vesicles or in the centre of the cytoplasm (Fig. 1b). The mitochondria were distributed homogeneously in the cell cortex (Fig. 1c). Sub-plasma membrane distribution of mitochondria was also a characteristic of GV stage oocytes derived from larger follicles of 3–6 mm in diameter; however, mitochondria were dispersed to the inner cytoplasm to different degrees in different oocytes (Fig. 1d–f), and in particular, mitochondria were mostly accumulated in the perinuclear region in many oocytes (15/31) (Fig. 1e,f). In all 37 oocytes that reached metaphase I or anaphase I after 24 or 30 h of culture, mitochondria were aggregated in the perinuclear area (Fig. 1g). The major characteristics of mitochondrial distribution in MII oocytes matured *in vitro* were as follows: (i) in most of the matured oocytes (61/68), mitochondria were located more centrally, although few, if any, translocated to the very centre of the oocytes (Fig. 1h); (ii) relatively few but larger mitochondrial foci were distributed evenly in the cytoplasm except for the central region in all mature oocytes observed (Fig. 1h,i); (iii) strong staining in the sub-plasma membrane region was no longer apparent (Fig. 1h,i); and (iv) strong active mitochondrial staining was common in the first polar bodies (51/68) (Fig. 1h,i). In all seven oocytes derived from large follicles that failed to undergo GVBD after 44 h of culture, mitochondria were located both around the GV and close to the plasma membrane, and no large mitochondrial foci were observed (data not shown).

Compared with the oocytes matured *in vitro*, the most marked feature of oocytes matured *in vivo* was that mitochondria were distributed throughout the cytoplasm, although mitochondria were more abundant in the peripheral cytoplasm than in the inner cytoplasm in most of the oocytes. Of the 17 samples observed, mitochondrial foci filled the entire ooplasm in 12 oocytes (Fig. 1j), but were absent in the central cytoplasm in three oocytes (Fig. 1k) and were distributed in the central cytoplasm in two oocytes (Fig. 1l). Strong staining was also observed in the first polar body (Fig. 1l).

Distribution of active mitochondria in fertilized eggs

As expected, the tails, particularly the principal segments, of spermatozoa attached to the surface of the zonae pellucidae of fertilized eggs were stained by MitoTracker Green (Fig. 2a). After fertilization, mitochondrial aggregations were associated with the pronucleus (Fig. 2b), close to the plasma membrane (Fig. 2b,c), or between the pronucleus and the plasma membrane (Fig. 2c) in all 37 fertilized eggs examined, although some mitochondrial foci were observed in the inner cytoplasm of some fertilized eggs (Fig. 2b,d). Mitochondria were associated with both pronuclei (Fig. 2e). There was typically strong mitochondrial staining in the second polar bodies (21/37) 24 h after insemination (data not shown).

Distribution of active mitochondria during early embryo development in vitro

During early cleavage, mitochondria aggregated in the region in which the blastomeres were still in contact (Fig. 2f). The consistency of perinuclear clustering and of the cortical ring of mitochondria in the blastomeres was another characteristic feature (Fig. 2g). In morula, mitochondria were distributed mainly in the outer region of the blastomeres (data not shown). Active mitochondria were distributed homogeneously in both inner cell mass cells and trophectoderm cells in all pig blastocysts (Fig. 2h,i).

Regulation of mitochondrial translocation by microtubule and microfilament modulators during oocyte maturation and fertilization

When oocytes at the GV stage were cultured for 44 h in medium containing 500 nmol cytochalasin B l^{-1}, meiotic progression was arrested at MI. Mitochondria migrated to the inner cytoplasm in the control MII oocytes (29/36) (Fig. 3a), whereas mitochondria aggregated in the region near to the MI chromosomes in most cytochalasin B-treated oocytes (34/40) (Fig. 3b,c), although a few mitochondrial foci were observed in the inner cytoplasm (Fig. 3c). In all five cytochalasin B-treated oocytes that failed to undergo GVBD, there were numerous mitochondria aggregated around the GV (Fig. 3d). In oocytes treated for 44 h with 100 µmol nocodazole l^{-1}, MI chromosomes formed, but further progression of meiosis was inhibited. Inward and perinuclear accumulation of mitochondria were blocked in all oocytes ($n = 36$). Mitochondria were aggregated mainly in the peripheral cytoplasm (Fig. 3e), although in some cases (15/36) a few mitochondria were distributed around the MI chromosomes (Fig. 3f).

Microfilament disruption did not influence the aggregation of mitochondria around the pronuclear region in all 36 fertilized eggs examined at 24 h after insemination (Fig. 3g). When the two pronuclei were closely apposed, mitochondria were aggregated either around or between the two pronuclei. In contrast, the microtubule disruptor nocodazole inhibited aggregation of mitochondria in the

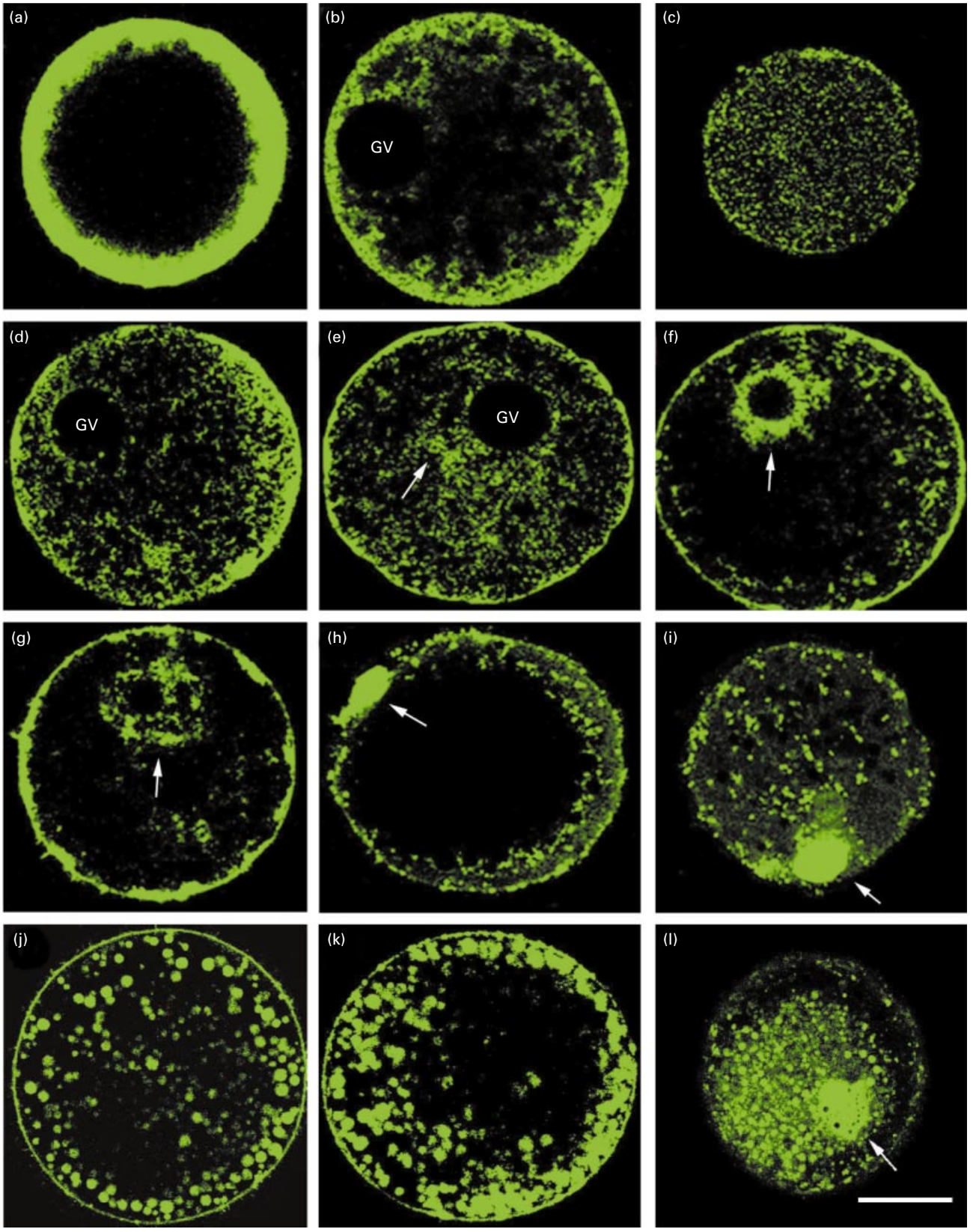

Fig. 1. Distribution of mitochondria during maturation of pig oocytes. (a) An example of a peripheral band of mitochondria in a germinal vesicle (GV) oocyte derived from a small follicle. (b) A GV oocyte from a small follicle, indicating the distribution of mitochondria in the sub-cortical region, but few distributed in the vicinity of GV. (c) Mitochondria were distributed homogeneously in

peri-pronuclear region in 30 of 41 eggs examined. Mitochondrial foci were no longer observed and mitochondria were distributed homogeneously throughout the cytoplasm (Fig. 3h). Although taxol inhibited the centration of pronuclei, it did not affect aggregation of mitochondria in the vicinity of the pronuclei (28/35) at 24 h after insemination (Fig. 3i).

Discussion

Mitochondrial ultrastructure and changes in distribution during maturation of pig oocytes have been studied by electron microscopy. These studies revealed that mitochondria are clustered at the periphery of the cell before injection of human chorionic gonadotrophin (hCG), but disperse during maturation (Cran, 1985). In the present study, MitoTracker staining revealed a similar pattern of mitochondrial distribution. The restricted peripheral distribution of mitochondria was also reported in sheep and bovine oocytes (Cran *et al.*, 1980; Sun and Qin, 1989). This pattern of distribution may be related to the high energy requirement in the cortex, as oocytes require cumulus cell support at this stage, and intimate association between the oocyte and the cumulus cells is maintained by gap junctions from cumulus cell process endings on the surface of the oolemma (Fleming and Saacke, 1972; Eppig, 1982). In addition to the sub-membrane aggregation, mitochondria accumulated progressively to the GV area before culture in immature oocytes collected from large follicles. Van Blerkom (1991) also observed the gradual perinuclear accumulation of mitochondria before GVBD in mouse oocytes. The accumulation of active mitochondria in the perinuclear region may not be related to the synthetic activity of the nucleus, as developing oocytes characteristically have high RNA synthesis, but fully grown oocytes cease rRNA synthesis, and no RNA synthesis is observed after initiation of meiosis (Prather, 1993; Sun and Qin, 1990; Sun *et al.*, 1996). It is possible that perinuclear accumulation of mitochondria plays a role in GVBD. One possibility is that the specific patterns of perinuclear mitochondrial aggregation provide ATP for GVBD. Studies on mitochondrial distribution and ATP content in human pronuclear embryos show that a decrease in the number of mitochondria is correlated with a diminished capacity for ATP generation (Van Blerkom *et al.*, 2000). However, other studies indicate that meiotic maturation occurs in both mouse and human oocytes over a wide range of ATP concentrations (Van Blerkom *et al.*, 1995) and that mitochondrial contribution to ATP concentrations may be small in early development (Trimarchi *et al.*, 2000). Furthermore, ATP production was reported not to be the mechanism whereby glucose mediates a stimulatory action on meiosis in mouse oocytes (Downs and Mastropolo, 1994). Another possibility is that perinuclear mitochondrial clustering plays a role in GVBD by modulating local free calcium concentrations, as increases in endogenously derived intracellular calcium are a prerequisite for GVBD in pig oocytes (Kaufman and Homa, 1993) and mitochondrial distribution is related to the ability to regulate intracellular homeostasis, as revealed in hamster embryos (Lane and Bavister, 1998). Mitochondrial aggregation around the GV was also observed in oocytes arrested at the GV stage after 44 h of culture, indicating that aggregation of active mitochondria in the GV area is not sufficient to induce GVBD, although this may be one of the factors involved in this event under physiological conditions.

After GVBD, mitochondria continue to aggregate around the perinuclear area at MI and anaphase I, which is probably related to the high energy requirement of meiotic events, such as spindle assembly, chromatin condensation and movement, and polar body emission. In laboratory mice, perinuclear accumulation of mitochondria is one aspect of the developmental programme of cytoplasmic maturation observed between GVBD and MI (Van Blerkom and Runner, 1984; Van Blerkom, 1991; Calarco, 1995). The strong staining of mitochondria in the perinuclear area may be caused by either mitochondrial translocation or by activation of mitochondria *in situ*. Although examination of bovine oocytes demonstrates that the subcellular organization of the oocyte cytoplasm remains unchanged during maturation to MII (Van Blerkom *et al.*, 1990), it has been reported that mitochondria actively relocate during oocyte maturation and fertilization in several species (Bavister and Squirrell, 2000). The present study revealed no changes in active mitochondrial distribution of pig oocytes treated with 100 µmol nocodazole l^{-1}, a concentration that inhibits microtubule assembly (Sun *et al.*, 2001), but aggregation of mitochondria was found in oocytes treated with 500 nmol cytochalasin B l^{-1}, a concentration that blocks microfilament assembly (Sun *et al.*, 2001). In mouse oocytes, mitochondrial movement toward the GV area is mediated by microtubules but not by microfilaments (Van Blerkom and Bell, 1986). There is a

the cortex of GV oocytes derived from small follicles. (d,e,f). Examples of progressive inward translocation and aggregation of mitochondria around the GV in oocytes derived from large follicles. Arrows indicate the accumulation of mitochondria in the vicinity of the GVs. (g) Mitochondria accumulated in the perinuclear region (arrow) after 30 h of culture. (h) Mitochondria were translocated to the inner cytoplasm, but only a few were found in the centre of the cytoplasm in mature oocytes after 44 h of culture. Note strong mitochondrial staining in the first polar body (arrow). (i) An observation plane through the cortex of a mature oocyte, showing homogeneous distribution of larger mitochondrial foci. Arrow indicates the first polar body. (j) An *in vivo* matured oocyte, showing the distribution of mitochondria throughout the cytoplasm. (k) An example showing the absence of mitochondria in the central cytoplasm of an *in vivo* matured oocyte. (l) An *in vivo* matured oocyte, showing the absence of mitochondria in the peripheral cytoplasm. Note the strong staining of the polar body (arrow). Scale bar represents 40 µm.

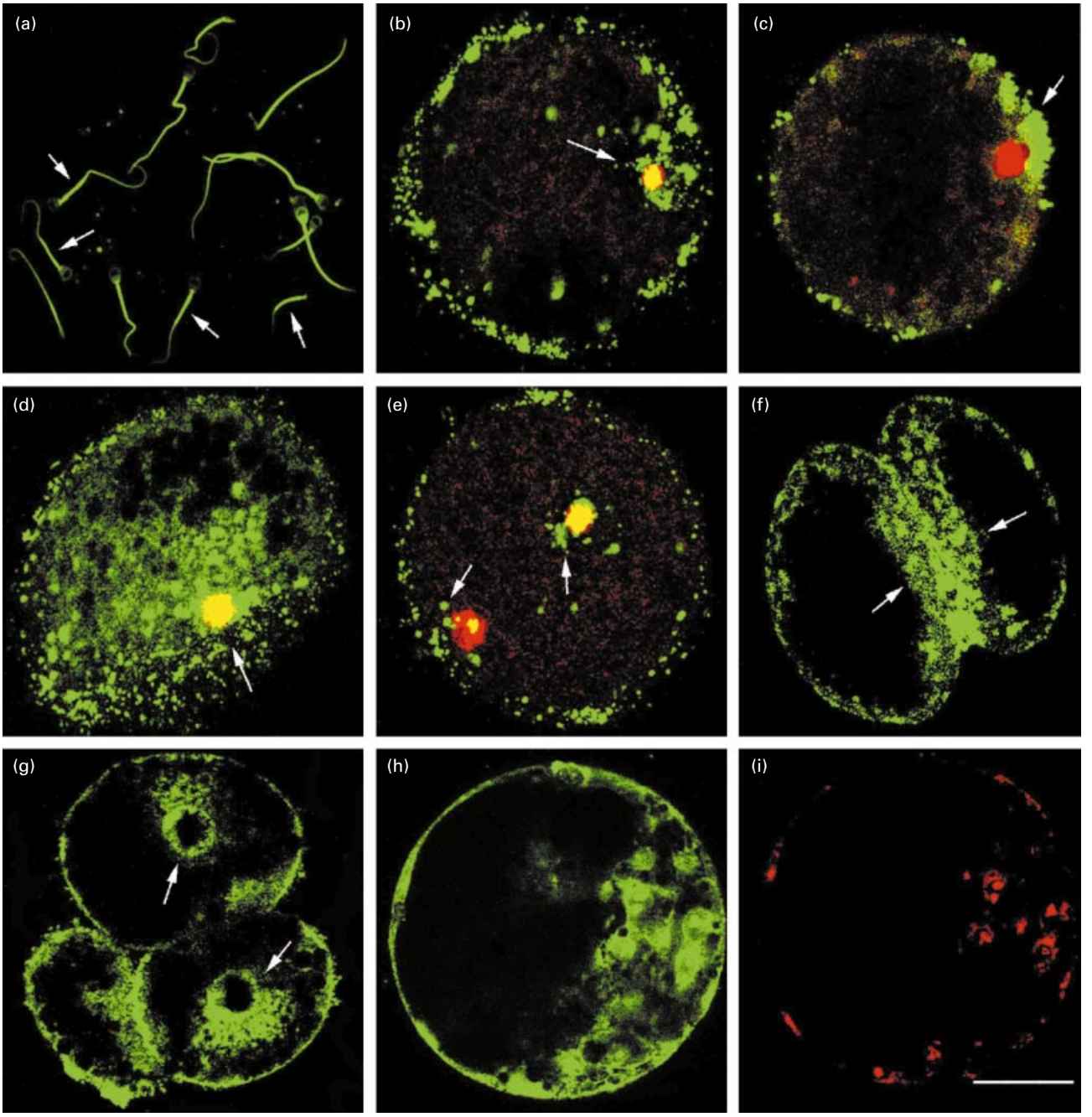

Fig. 2. Mitochondrial distribution in fertilized eggs and early embryos in pigs. (a) The tails, especially the principal segments (arrows), of spermatozoa that attached to the zona surface of fertilized eggs were stained green. (b) A fertilized egg, indicating the mitochondria distributed close to the plasma membrane and in the vicinity of a pronucleus (arrow). (c) Mitochondrial aggregation between the pronucleus and the plasma membrane (arrow). (d) In addition to the perinuclear distribution (arrow), some mitochondrial foci were observed in the inner cytoplasm of a few fertilized eggs. (e) Mitochondrial aggregation around both pronuclei (arrows). (f) Mitochondrial aggregation in the region in which the blastomeres are still in contact (arrows) during early cleavage. (g) Mitochondrial clustering around the blastomere nuclei (arrows) and in the cortex of the cell. (h,i) Mitochondria present in both inner cell mass cells and trophectoderm cells of blastocysts. Green, mitochondria; red, chromatin; yellow, overlapping of green and red. Scale bar represents 40 μm.

temporal, spatial and developmental relationship between the location of microtubule-organizing centres and the progressive translocation of mitochondria to the nuclear region (Van Blerkom, 1991). This finding indicates that strong staining of mitochondria in the perinuclear area in pig oocytes is caused by active mitochondrial translocation and that, as in mouse oocytes, mitochondrial translocation is mediated by microtubules but not by microfilaments.

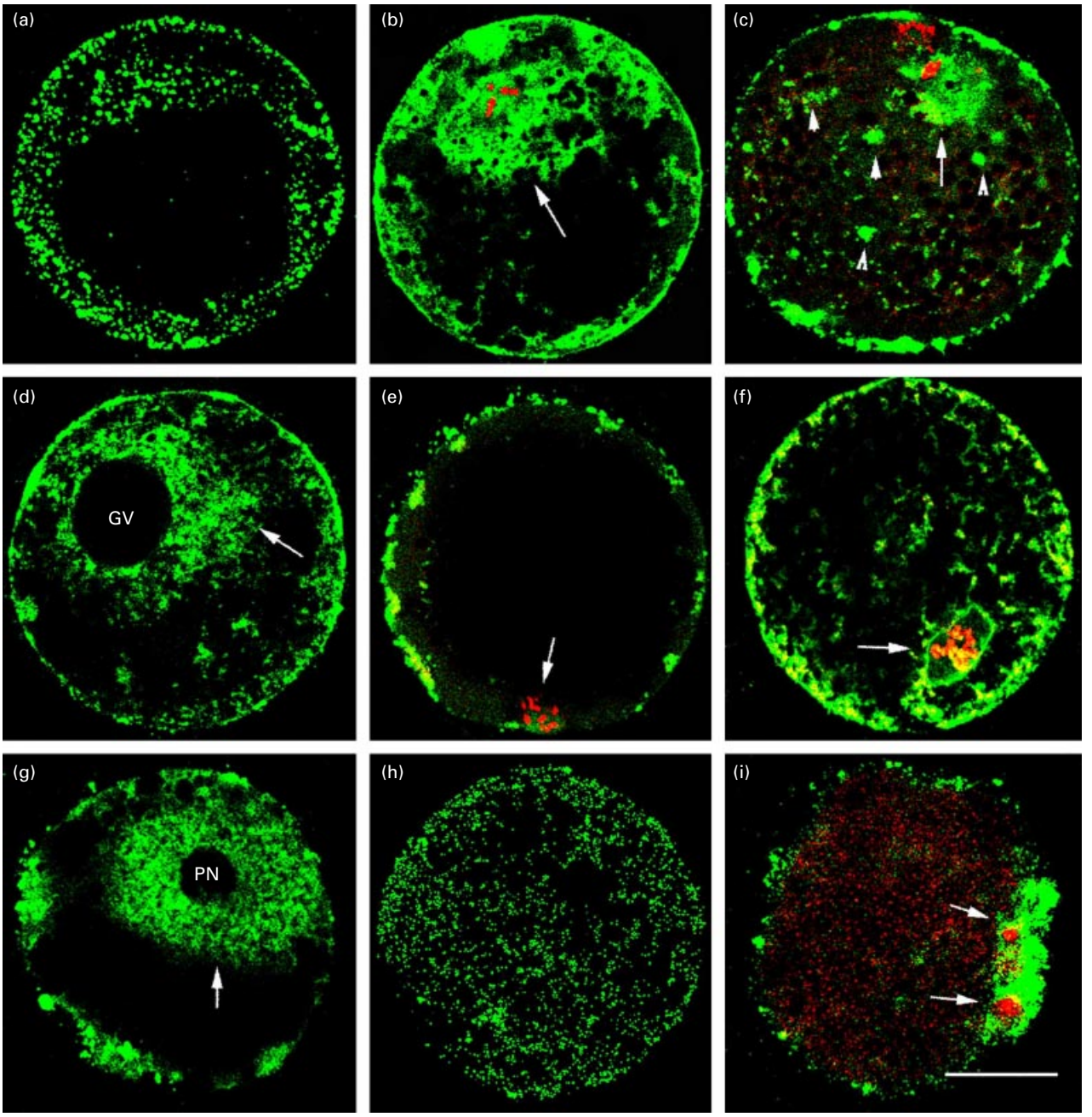

Fig. 3. Regulation of mitochondrial translocation by microtubule and microfilament modulators during maturation and fertilization of pig oocytes. (a) Mitochondrial distribution in a control oocyte after 44 h of culture. (b) Mitochondrial accumulation in the vicinity (arrow) of the metaphase I (MI) chromosomes in an oocyte arrested at MI by cytochalasin B after 44 h of culture. (c) Mitochondrial foci (arrowheads) distributed in the cytoplasm, in addition to the accumulation of mitochondria around the MI chromosomes (arrow). (d) Mitochondrial distribution around the GV (arrow) in an oocyte arrested at the GV stage treated with cytochalasin B after 44 h of culture. (e) Inhibition of inward movement of mitochondria toward the nuclear area (arrow) in an oocyte treated with nocodazole for 44 h. (f) A few mitochondria are distributed in the vicinity of MI chromosomes (arrow) in some oocytes treated with nocodazole for 44 h. (g) Aggregation of mitochondria around the pronucleus (arrow) in a fertilized egg treated with cytochalasin B. (h) Distribution of mitochondria in a fertilized egg after microtubule disruption with nocodazole. (i) Inhibition of centration of pronuclei and accumulation of mitochondria around the pronuclei (arrows) in a fertilized egg treated with taxol. Green, mitochondria; red, chromatin; yellow, overlapping of green and red. Scale bar represents 40 μm.

Observations in the present study revealed that larger but scarcely distributed mitochondrial foci formed after *in vitro* maturation of pig oocytes. This observation is consistent with electron microscopy. Cran (1985) reported a decrease in the number of mitochondria such that at 50 h after hCG the number of mitochondria was only one-third of that at 0 h. Concomitantly, there was > 300% increase in individual mitochondrial volume. In addition, small groups of mitochondria became enveloped in cisternae of endoplasmic reticulum. Thus, the larger mitochondrial foci in the mature oocytes in the present study, as revealed by MitoTracker staining, may represent the increase in mitochondrial volume and the aggregation of mitochondria.

Peripheral mitochondria showed an even spatial distribution during *in vitro* maturation of bovine oocytes (Hyttel *et al.*, 1986) and *in vivo* maturation of mouse and hamster oocytes (Tokura *et al.*, 1993; Barnett *et al.*, 1996). However, Calarco (1995) reported that fully mature mouse oocytes showed obvious polarity of mitochondrial distribution. The present study revealed that accumulation of mitochondria around the MII spindle did not occur, but instead they were distributed homogeneously in the ooplasm except for in the inner cytoplasm during *in vitro* maturation of pig oocytes. The absence of mitochondria in the inner cytoplasm may be caused by *in vitro* culture conditions and represents poor cytoplasmic maturation of *in vitro* matured oocytes, as both electron microscopy (Cran, 1985) and MitoTracker staining (present study) show that mitochondria are dispersed to the inner cytoplasm in most oocytes matured *in vivo*.

Staining of active mitochondria was strong in the first polar bodies of both *in vivo* and *in vitro* matured oocytes. Intensive mitochondrial staining was also observed in the second polar body of fertilized hamster (Barnett *et al.*, 1996) and pig eggs (present study). Mitochondria are the most apparent cytoplasmic organelle in the first polar body of rabbit oocytes matured *in vivo* as revealed by electron microscopy (Zamboni and Mastroianni, 1966).

There is little information regarding mitochondrial changes during oocyte fertilization and early embryo development in pigs. The present study showed that, in fertilized eggs, the homogeneous distribution of mitochondria in mature oocytes was no longer apparent and that mitochondria aggregated to the peri-pronuclear region. This finding is consistent with results obtained in mouse, hamster and human zygotes, in which mitochondria showed perinuclear accumulation (Tokura *et al.*, 1993; Barnett *et al.*, 1996; Sun *et al.*, 1996; Van Blerkom *et al.*, 2000). The accumulation of mitochondria in the perinuclear area indicates that pronuclear apposition and syngamy may require high concentrations of ATP or free calcium.

The present study also revealed that aggregation of mitochondria in the pronuclear area was mediated by microtubules: whereas exposure of sperm-penetrated pig oocytes to taxol, which promotes the assembly of microtubules, did not affect mitochondrial aggregation in the vicinity of pronuclei, the centration of pronuclei was inhibited. When mouse oocytes at the GV or GVBD stages were treated with taxol, bundles of microtubules formed and large aggregates of mitochondria were arranged along the microtubule bundles at the periphery of the nucleus (Rime, 1987).

As reported in hamsters (Barnett *et al.*, 1996), the consistency of perinuclear clustering and cortical ring of mitochondria in the blastomeres was the most marked feature of cleavage of pig early embryos. In addition, more mitochondria were located in the region between the blastomeres. The translocation of active mitochondria was correlated with the ability of mouse embryos to develop *in vitro*. In mouse embryos with a developmental block, the translocation of mitochondria was inhibited, whereas translocation of mitochondria was restored when the developmental block was released by adding superoxide dismutase or thioredoxin to the culture medium (Tokura *et al.*, 1993). In contrast to hamster blastocysts, in which mitochondria were detected most readily in trophectoderm cells (Barnett *et al.*, 1996), mitochondria were distributed homogeneously in both inner cell mass cells and trophoctoderm cells in pig blastocysts.

In conclusion, the present study revealed several new characteristics of mitochondrial distribution during pig oocyte maturation: (i) mitochondria accumulate in the perinuclear area from the GV stage to anaphase I; (ii) fewer but larger mitochondrial foci form and relocate to the inner cytoplasm in mature oocytes, and mitochondrial translocation in oocytes matured *in vitro* is not as complete as in oocytes matured *in vivo*; and (iii) strong staining of active mitochondria is observed in the first polar body. Mitochondria aggregate to the pronuclear area in fertilized eggs, and perinuclear clustering of mitochondria is also a marked feature in early cleavage. Mitochondria are distributed homogeneously in both inner cell mass cells and trophectoderm cells of blastocysts. Finally, the apparent translocation of mitochondria is mediated by microtubules but not by microfilaments in pig oocytes.

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