Domains of high-polarized and low-polarized mitochondria may occur in mouse and human oocytes and early embryos

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BACKGROUND: The magnitude of the inner mitochondrial membrane potential (ΔΨm) appears to influence the level of certain mitochondrial activities including regulation of ionic fluxes and ATP liberation, activities that are often compartmentalized or location dependent in cells. Recent evidence suggests that within cells, mitochondria can be heterogeneous with respect to ΔΨm, and that high-polarized mitochondria (high ΔΨm) may occur in the subplasmalemmal cytoplasm where intercellular contact is absent. Here, we investigated whether ΔΨm in oocytes and preimplantation embryos was heterogeneous and cell contact-associated. METHODS: Mouse and human oocytes and preimplantation stage embryos stained with mitochondria-specific probes rhodamine 123, MitoTracker Orange, and the ΔΨm-sensitive probe JC-1, (5,5′,6,6′-tetrachloro-1,1,3,3′-tetraethylbenzimidazolocarbocyanine iodide), were examined by epifluorescence, scanning laser confocal, and transmission electron microscopy. The possibility that intercellular contact and ΔΨm are associated was examined for oocytes, where transzonal coronal cell contacts were terminated naturally or experimentally, and for intact, disaggregated, and reconstructed cleavage stage mouse embryos. RESULTS: For both oocytes and embryos, clusters of apparently high-polarized mitochondria occur in the pericortical cytoplasm in regions free from intercellular contact. CONCLUSIONS: The findings suggest that mitochondria in oocytes and preimplantation embryos may be heterogeneous with respect to ΔΨm. We propose that high-polarized pericortical mitochondria may have a role in the acquisition of oocyte competence and the regulation of early developmental processes that may be associated with elevated metabolism or intracellular signalling through calcium-induced calcium release pathways.

Key words: JC-1 and J-aggregate/mitochondrial polarization/mitochondrial heterogeneity/oocytes/preimplantation embryos

Introduction

It is generally thought that mitochondria have a central role in early mammalian embryogenesis, and, for human oocytes and early embryos in particular, pre-existing mitochondrial (mtDNA) defects (DiMauro, 1998; Chinnery and Turnbull, 1999; Christodoulou, 2000), or heteroplasmy which may develop with advanced reproductive age (Keefe et al., 1995), have been suggested as causes of early embryonic failure (Brenner et al., 1998; Barritt et al., 1999). Mitochondrial dysfunction has also been implicated in certain instances of infertility where restoration of competence after ooplasmic transfer in IVF procedures may involve the coincident introduction of normal mitochondria derived from competent oocytes (Barritt et al., 2000). Asymmetric mitochondrial distributions at the pronuclear stage have been associated with disproportionate mitochondrial segregation between blastomeres following cell division, and, for cleavage stage blastomeres with significantly diminished mitochondrial inheritance, adverse developmental consequences may include cell division arrest and death resulting from inadequate ATP production (Van Blerkom et al., 2000).

Mitochondrial activity can differ within cells owing in part to adaptive cytoskeletal-mediated mechanisms which redistribute mitochondria to different cytoplasmic locations (Agutter and Wheatley, 2000; Aw, 2000). For example, the generation of ATP by highly condensed and comparatively undifferentiated mitochondria in maturing oocytes and early preimplantation embryos is thought to occur at relatively low levels (Ginsberg and Hillman, 1973; Biggers and Borland, 1976; Magnusson et al., 1986; Gott et al., 1990; Houghton et al., 1996; Trimarchi et al., 2000), and microtubule-mediated, stage-specific changes in mitochondrial distribution in maturing oocytes and newly fertilized oocytes may be indicative of adaptive, spatial remodelling that redistributes mitochondria to areas requiring elevated levels of ATP (Van Blerkom and Runner, 1984; Van Blerkom, 1991; Tokura et al., 1993; Barnett et al., 1996, 1997). During early development, cytoplasmic remodelling that concentrates mitochondria to perinuclear regions may also be involved in cell cycle regulation, perhaps by means of calcium (Ca²⁺) mobilization (Sousa et al., 1997; Van Blerkom et al., 2000). In exceptionally large cells such as oocytes and early blastomeres, levels of mitochondrial...
metabolism could be influenced by local differences in intracellular oxygen tension resulting from oxygen diffusion gradients extending inward from the plasma membrane (Aw, 2000). However, some current views of mitochondrial function in early mammalian embryogenesis may require re-evaluation owing to the demonstration that activities driven by the inner mitochondrial membrane potential (ΔΨm), such as regulation of Ca2+ fluxes and ATP liberation, may be compartmentalized within cells (Diaz et al., 1999) and within individual mitochondria as well (Smiley et al., 1991).

Here, we used the mitochondria-specific fluorescent probes rhodamine 123 (R123, Johnson et al., 1980) and MitoTracker Orange (MO) (Van Blerkom et al., 1998) to identify all living mitochondria (Chen, 1988), and the detection of putative JC-1 (5,5',6,6'-tetrachloro-1,1,3,3'-tetraethylbenzimidazoylcarbocyanine iodide), J-aggregates (Reers et al., 1991, 1995; Smiley et al., 1991; Salvioli et al., 1997), to determine whether mitochondria with different inner membrane potentials (ΔΨm) exist within individual mouse and human oocytes and preimplantation embryos. The results suggest that relatively high-polarized (high ΔΨm) mitochondria are localized in small clusters within subplasmalemmal/pericortical cortical cytoplasm of maturing oocytes and early embryos in areas free of intercellular contact. The findings are discussed with respect to current notions of ΔΨm that may relate to mitochondrial activity and function during oocyte maturation and preimplantation embryogenesis.

Materials and methods

**Mouse and human oocytes and embryos**

Fully grown germinal vesicle (GV) stage mouse oocytes were harvested from the ovaries of unstimulated 6–8 week old ICR animals, denuded of cumulus and corona cells by passage through a glass micropipette, and matured in vitro in human tubal fluid medium supplemented with 5% bovine serum albumin. Metaphase II (MII) stage oocytes were recovered from the oviducts of mice which ovulated at 12 h after the administration of HCG. Preimplantation stage embryos from the pronuclear and hatched blastocyst stages were obtained on days 1–5 (day 1 = day of vaginal plug detection) from mice having undergone ovulation induction with 5 IU pregnant mare serum gonadotrophin and 5 IU HCG. Normal-appearing MII human oocytes were obtained under the following circumstances: (i) patients who instructed that only a specific number of oocytes be exposed to sperm, (ii) male factor, conventional IVF procedures where ICSI was not elected, and (iii) normospermic inseminations where oocytes showed no sign of activation (second polar body or pronuclei) at 20–24 h and reinsemination by ICSI was not an option. Early human embryos resulting from dispersmer penetration and monospermic embryos donated to research were used in this study.

**Staining with mitochondria-specific fluorescent probes**

In preliminary studies, the specificity and conditions of JC-1 staining were determined with human cumulus cell cultures, and with in-vitro matured mouse oocytes at the GV, circular bivalent (CBV) and MII stage. Specimens were stained with the mitochondria-specific fluorescent probes (Molecular Probes, Eugene, OR, USA) prepared from stock solutions immediately preceding staining and used at the following concentrations: (i) R123 (1–10 µg/ml; 2.6–26 µmol/l), (ii) MO (0.08–0.8 µg/ml; 19.5–195 nmol/l), and JC-1 (0.5–2 µg/ml; 0.75–3.0 µmol/l). Cumulus cells were obtained in ICSI procedures by exposure of intact oocytes to hyaluronidase (100 IU/ml), centrifugation of enzymatically dispersed cells, vortex mixing of the pellet in normal medium, followed by seeding glass coverslips and culture for 24 h. Cumulus cells were stained with the above probes for up to 30 min and examined at 10 min intervals. Mouse GV, CBV and MII oocytes, and embryos from the pronuclear to hatched blastocyst stage were stained for 1, 5, 10, 30, 60 and 90 min, and representative oocytes and embryos were examined at 5–15 min intervals by epifluorescence microscopy with appropriate narrow band filter sets. The same intensity and pattern of R123, MO, JC-1 and J-aggregate fluorescence was observed in intact and zona-free oocytes and embryos. From these studies, a standard concentration (1.5 µmol/l) and duration (25 min) of JC-1 staining was found to be optimal for the documentation of putative J-aggregate fluorescence and consistent with oocyte and embryo viability, continued maturation and preimplantation development, respectively. To determine whether J-aggregate formation was inhibited when mitochondria were depolarized, cumulus cells and representative mouse oocytes and preimplantation stage embryos were exposed to the proton ionophore FCCP [carbonyl cyanide p-(trifluoromethoxy)phenyl-hydrazone, Sigma Chemical Co., St Louis, MO, USA] at 10 µg/ml for 5–10 min prior to staining with JC-1.

**Experimental manipulations**

**Oocyte–cumulus complexes**

Intact (cumulus-enclosed) GV stage mouse oocytes, obtained by gentle disaggregation of the ovary, were exposed to JC-1 for 20–30 min. Other complexes were manipulated such that 20–80% of the cellular mass was carefully removed by applying repeated suction to selected regions with a small-bore glass micropipette (inner diameter, 20–50 µm), or by a single passage through a standard micropipette (inner diameter, 85–100 µm). Partially denuded complexes were cultured as follows: (i) immediate exposure to JC-1 for up to 30 min, with fluorescent microscopic examination at 5 min intervals or (ii) preloaded with JC-1 for 30 min followed by culture for 30–60 min in normal medium, with fluorescent microscopic examination at 10 min intervals after removal of some or all of the residual corona and cumulus cells.

**Mitochondrial compartmentalization and blastomere disaggregation**

Mitochondria-enriched cytoplasts and mitochondria-depleted karyoplasts were produced by centrifugation of cytochalasin D-treated MII mouse oocytes preloaded with R123, MO, or JC-1 (Van Blerkom et al., 1998). Specimens were examined by conventional and scanning laser confocal fluorescence microscopy (SLCM) prior to and after separation into cytoplasts and karyoplasts. Intact 2- and 4-cell stage mouse embryos were preloaded with JC-1, cultured in normal medium (M16 or KSOM) for 1 h, disaggregated into individual blastomeres by passage through a glass micropipette, and examined by fluorescence microscopy as described below. Individual blastomeres from 2- and 4-cell embryos preloaded with JC-1 were cultured and examined after subsequent divisions. Where areas of previous intercellular cell contact were evident after separation, blastomeres were repositioned such that these regions were either juxtaposed, or rotated 180° so that regions of the plasma membrane that were formerly free were in opposition. The stability of this association during culture was documented by time-lapse video microscopy as described previously (Van Blerkom et al., 1995).

**Microscopic analysis**

Oocytes and embryos stained with R123, MO and JC-1 were analysed by conventional epifluorescence microscopy in the fluorescein
isothiocyanate (FITC) and rhodamine isothiocyanate (RITC) channels with narrow band filter sets. SLCM was used as previously described (Van Blerkom et al., 2000) to obtain high-resolution images of mitochondrial fluorescence. Most of the images shown here were obtained by conventional epifluorescence microscopy because our experience indicated that only a limited number (one to three) SLCM sections could be captured before putative J-aggregate fluorescence was quenched. During microscopic analysis, oocytes, embryos and disaggregated blastomeres were maintained in 30 µl microdroplets under oil at 37°C using the ΔT culture system (Biopics Inc., Butler, PA, USA) (Van Blerkom et al., 1995). SLCM and most of the epifluorescent images are presented as observed. However, for clarity of presentation, some epifluorescent images of putative J-aggregate fluorescence involved digital subtraction of JC-1 fluorescence that extended into the RITC channel (where J-aggregate fluorescence was visualized) from the FITC channel. A similar manipulation was performed when light microscopic and J-aggregate fluorescent images were superimposed. The accuracy of putative J-aggregate fluorescence shown in these images was demonstrated by SLCM, and by digital analysis of the FITC fluorescence spectrum that extended into the RITC channel in oocytes and embryos exposed to FCCP, which completely inhibits J-aggregate formation (see below). Because of the quenching of J-aggregate fluorescence, SLCM ratiometric determinations of fluorescence at FITC and RITC emission wavelengths were not determined in this study. Representative mouse oocytes at each stage of maturation, and uninseminated human MII oocytes were partitioned by centrifugation into cytoplasts (Figure 1J1) and karyoplasts (Figure 1J6) that have been shown previously to be enriched with, or virtually devoid of, mitochondria (Van Blerkom et al., 1998). The epifluorescent images shown in Figure 1I and 1I1 demonstrate R123 and MO fluorescence, respectively, in an oocyte in the process of compartmentalization, with mitochondrial fluorescence contained in one compartment. Figure 1J2 shows R123 fluorescence in a separated cytoplasm, and Figure 1J3 shows JC-1 and punctate J-aggregate fluorescence in another cytoplasm from the same preparation. No R123 or J-aggregate fluorescence was observed in karyoplasts (Figure 1J6, J7). The same results were obtained after analysis by SLCM. For example, Figure 1J4 and J5 shows regions of regions of high intensity JC-1 FITC emission (asterisk, Figure 1J4) that are not completely coincident with RITC J-aggregate fluorescence (asterisk, Figure 1J5) detected in the same mitochondria-enriched cytoplasm. This finding suggests that domains of potentially high-polarized, J-aggregate-forming pericortical mitochondria present in the intact oocyte (e.g. Figure 2B1) may differentially segregate within forming cytoplasm during centrifugation.

As described below, putative J-aggregate fluorescence is largely localized to the pericortical cytoplasm of maturing oocytes and developing embryos. To determine whether this staining pattern is uptake or concentration dependent, such that peripherally located mitochondria are the first to show fluorescence, oocytes at each stage of maturation were examined at timed intervals after exposure to mitochondria-specific probes at increasing concentrations. Similar to earlier findings (Van Blerkom and Runner, 1984), staining of GV mouse stage oocytes with R123, MO and JC-1 showed uniform cytoplasmic fluorescence from the earliest time point (1 min). With increasing concentrations or exposure, the relative intensity of fluorescence increased, but the distribution of mitochondrial fluorescence was unaffected. For example, Figure 1F1 and F2 shows the distribution of mitochondrial fluorescence in GV-stage mouse oocytes cultured in the presence of R123 at 5 and 10 µg/ml respectively, for 40 min. The areas of high intensity mitochondrial fluorescence indicated by an asterisk are typical of mitochondrial distributions at this stage of maturation. Figure 1G shows mitochondrial fluorescence in GV stage mouse oocyte after 30 min of culture followed by a 15 min exposure to MO at the lowest concentration used in this study (0.08 µg/ml). The asterisk in Figure 1G indicates a region of high mitochondrial density. A similar dose and time course for mouse oocytes at the CBV stage showed intense perinuclear fluorescence typical of in-vitro matured mouse oocytes were undetectable in all oocytes, embryos and isolated blastomeres exposed to FCCP.

The presence of tens of thousands of mitochondria with diameters <0.5 µm make it virtually impossible to detect individual stained mitochondria in living oocytes and early embryos by conventional microscopy or SLCM. Consequently, here the term putative J-aggregate fluorescence is used (or understood) to refer to the FCCP-sensitive, punctate fluorescence detectable in the RITC channel of JC-1-stained oocytes and embryos. To determine whether putative J-aggregate fluorescence was associated with mitochondria, MII oocytes were partitioned by centrifugation into cytoplasm (Figure 1J1) and karyoplasts (Figure 1J6) that have been shown previously to be enriched with, or virtually devoid of, mitochondria (Van Blerkom et al., 1998). The epifluorescent images shown in Figure 1I and 1I1 demonstrate R123 and MO fluorescence, respectively, in an oocyte in the process of compartmentalization, with mitochondrial fluorescence contained in one compartment. Figure 1J2 shows R123 fluorescence in a separated cytoplasm, and Figure 1J3 shows JC-1 and punctate J-aggregate fluorescence in another cytoplasm from the same preparation. No R123 or J-aggregate fluorescence was observed in karyoplasts (Figure 1J6, J7). The same results were obtained after analysis by SLCM. For example, Figure 1J4 and J5 shows regions of regions of high intensity JC-1 FITC emission (asterisk, Figure 1J4) that are not completely coincident with RITC J-aggregate fluorescence (asterisk, Figure 1J5) detected in the same mitochondria-enriched cytoplasm. This finding suggests that domains of potentially high-polarized, J-aggregate-forming pericortical mitochondria present in the intact oocyte (e.g. Figure 2B1) may differentially segregate within forming cytoplasm during centrifugation.

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Fluorescent microscopic images of human cumulus cells (A–E2) and immature and maturing mouse oocytes (F1–O) stained with the mitochondria-specific dyes R123 (A, F1, F2, H2, H3, I), MitoTracker Orange (B, G, I1), and JC-1 (C, D1, D2, E1, E2, J2–O). With the exception of J4 and J5, which are scanning laser confocal pseudocolour images, all other images were obtained by conventional epifluorescence microscopy. (J2–7) Fluorescent images of mitochondria-enriched mouse oocyte cytoplasts (J2–5) and mitochondria-depleted karyoplasts (J6, J7) stained with JC-1. The arrows in C–D2 and K4, K5, L2, M2 and O indicate regions of putative high-polarized mitochondria indicated by apparent JC-1, J-aggregate fluorescence. An asymmetrical distribution of putative J-aggregate fluorescence in maturing mouse oocytes, indicated by regions of the pericortical cytoplasm with lower density fluorescence, is noted by an asterisk in L2 and M2. Asterisks in F1–G and arrowheads in H2 and H3 indicate regions of high mitochondrial density. (See text for details.)
Mitochondrial heterogeneity in early development

Figure 2. Fluorescent microscopic images of mouse (A–B3) and human (C–E2) oocytes and early embryos (human, F–F4; mouse, G–G5), stained with R123 (insert A, F, F1) or JC-1 (all others) and examined by scanning laser confocal (A1–3, F–F4, G3–5) or conventional epifluorescence microscopy (B–E2, G1, G2). The colour bar in A1 shows relative fluorescent intensity (0, blue, lowest; 255, white, highest) in pseudocolour images. The pronounced perinuclear localization of R123-stained mitochondria (M, A) characteristic of the circular bivalent-to-metaphase I stage of mouse meiotic maturation in vitro observed by epifluorescence microscopy (insert, A) is also observed in JC-1-stained oocytes by scanning laser confocal microscopy at the FITC wavelength (arrows, A1, A2), but not at the RITC wavelength (A3), which detected putative J-aggregate fluorescence (arrows, A3) in the pericortical cytoplasm. A similar situation occurs in human pronuclear embryos where perinuclear mitochondrial (M) aggregation detected with R123 (F, F1) is also detected with JC-1 at the FITC emission wavelength (F2), but not at the RITC wavelength, where putative J-aggregate fluorescence is localized in the pericortical cytoplasm (arrows, F3). (F4) Composite scanning laser confocal image of F2 and F3 in which actual fluorescence at the two wavelengths was superimposed. Arrows in B1, B3, C2, D2, F4 G2, G4, G5 denote putative pericortical J-aggregate fluorescence detected at the RITC emission wavelength. Asterisk in F2, F4 indicates high intensity JC-1 fluorescence in the peri-pronuclear region. (See text for details.)
oocytes at this stage. For example, CBV stage oocytes from the same cohort (e.g. Figure 1H1) were stained with R123 at 5 µg/ml and examined by conventional epifluorescence microscopy after 5 and 15 min exposures. As shown in Figures 1H2 and H3, the relative intensity of perinuclear mitochondrial fluorescence was somewhat more intense at 15 min (Figure 1H3), but the pattern of perinuclear staining was unchanged. As described below, similar results were obtained with JC-1, indicating that stage-specific patterns of mitochondrial fluorescence are not influenced by either the concentration or duration of staining used in this study.

Putative J-aggregate fluorescence during in-vitro maturation of mouse oocytes

Mouse oocytes were stained with JC-1 at timed intervals (5–60 min) at the GV stage after retrieval from the ovary and during maturation in vitro at the germinal vesicle breakdown (GVB: 75 and 100 min), CBV (2, 3, 4 h), metaphase I (MI, 6 and 8 h) and MII (10 and 12 h) stages. A minimum of 150 mouse oocytes was examined by conventional epifluorescence and representative oocytes were studied by SLCM at each stage and at each time after the initiation of culture. Two hundred and twenty normal-appearing in-vivo matured MII oocytes were examined shortly after ovulation. As described above, putative J-aggregate fluorescence was detected in the RITC channel as punctate elements localized to subplasmalemmal/pericortical cytoplasm. At the standard concentration of JC-1 used in this study, virtually no apparent J-aggregate fluorescence was detectable in 93% (163/175) of fully grown and completely denuded GV stage oocytes (Figure 1K1) during the first 15 min of culture, although by epifluorescence microscopy, JC-1 fluorescence in the FITC channel was uniform (Figure 1K2) and extended into the RITC channel (Figure 1K3). For 7% (12/175) of newly isolated GV oocytes, an occasional putative J-aggregate cluster was observed in the pericortical cytoplasm at the first time point. For all maturing mouse oocytes, putative J-aggregate fluorescence was clearly detectable in subplasmalemmal/pericortical cytoplasm of all denuded GV stage oocytes at ~20 min of culture (arrowheads, Figure 1K4), with only marginal increases in the intensity and no change in distribution occurring with additional exposure times up to 60 min (arrowhead, Figure 1K5, 40 min).

From the late GV through MII stages, the distribution of putative J-aggregate fluorescence was largely asymmetrical (arrowheads, Figure 1K4, K5; arrows, Figures 1L2, M2 and 2B1, B3) with portions of the pericortical cytoplasm showing scant fluorescence. Beginning at ~45 min of culture (late GV stage), one putative J-aggregate fluorescence was observed at all stages of maturation within the first 5 min of staining, and with additional exposure times, no apparent changes in pericortical distribution were evident with staining times up to 45 min. For example, Figure 1L1, L2, M1 and M2 shows representative JC-1 fluorescence in the FITC (1L1, M1) and RITC channels (after subtraction of the FITC background in Figure 1L2, M2) after 20 min exposure to JC-1 at the late GV and GVB stages respectively. Approximately 5% (9/186) of cultured GV stage oocytes examined in this study failed to undergo GVB. In all instances, punctate J-aggregate fluorescence was detected in the perinuclear region (arrow, Figure 1O, FITC background subtracted) but was largely absent from the pericortical cytoplasm (white square, Figure 1O) after an initial 1.5–2 h of culture before JC-1 staining. The position of the oolemma in a representative GV-arrested oocyte is delineated by a solid white square in Figure 1O. Because relatively clear distinctions can be made between structures that differentially fluoresce at FITC and RITC emission wavelengths, SLCM of selected JC-1-stained oocyte and embryos was used to obtain a single, high resolution scan through the middle of the specimen. The relative intensity fluorescence in pseudocolour SLCM images is indicated numerically (from 0 to 255) and by a colour scale bar in Figure 2A1, where blue (relative intensity 0) and white (relative intensity 255) represent the lowest and highest intensity of fluorescence respectively. A pronounced perinuclear accumulation of mitochondria occurs in the CBV stage mouse oocyte (Van Blerkom and Runner, 1984), and as a result, this stage was particularly useful to examine specificity and spatial distribution of JC-1 and putative J-aggregate fluorescence. With differential interference contrast microscopy, the perinuclear accumulation of mitochondria appears as a ring-like structure (M, Figure 2A) surrounding the developing bivalent chromosomes (C, Figure 2A). Figure 2A1 (rapid scan, 1 µm section), 2A2 (20 µm section) and 2A3 (20 µm section) shows representative SLCM images of a CBV stage mouse oocyte stained with JC-1. The intensity of JC-1 fluorescence (arrows, Figure 2A1, A2) paralleled the distribution observed with R123 (arrow, insert, Figure 2A), namely, it was highest in the perinuclear region and decreased progressively towards the oolemma. In contrast, putative J-aggregate fluorescence was detected only in the pericortical cytoplasm, as indicated in Figure 2A3.

Epifluorescent images of putative J-aggregate fluorescence in representative newly ovulated MII mouse oocytes (12–14 h post HCG, n = 220) are shown in Figure 2B1 and B3 (RITC channel, FITC background subtracted, with putative J-aggregate fluorescence superimposed upon the light microscopic image of the oocyte). Figure 2B2 is an unmanipulated image of the oocyte shown in 2B3 as observed in the RITC channel, while Figure 2B is a representative epifluorescent image of a newly ovulated mouse oocyte stained with R123. The following distinct patterns of putative J-aggregate fluorescence were observed in in-vivo and in-vitro matured mouse oocytes: (i) no detectable fluorescence in the first polar body (PB1, Figure 2B1) with signal detected in the cortical cytoplasm subjacent to the first polar body, and (ii), putative J-aggregate fluorescence in the first polar body (PB1, Figure 2B3) but not in the subjacent ooplasm. For these MII oocytes, JC-1 fluorescence in the FITC channel was present throughout the cytoplasm at an intensity comparable to earlier stages of maturation (e.g. Figure 1L1, M1). At MII, the typical pattern of J-aggregate fluorescence was one that extended laterally from the pericortical cytoplasm subjacent to the first polar body (e.g. Figure 2B1). For 87% (196/220) of newly ovulated MII oocytes, the pericortical cytoplasm opposite the first polar body was virtually devoid of detectable J-aggregate fluorescence (e.g. Figure 2B3).
Mitochondrial heterogeneity in early development

**Figure 3.** Transmission electron microscope images of mouse oocytes at the germinal vesicle (GV) (A), germinal vesicle breakdown (B) and circular bivalent (C) stages. Transzonal (ZP) corona cell (CC) processes are indicated by arrows in A and E. (D) Pericortical mitochondrial cluster (M) surrounding a mass of smooth endoplasmic reticulum (SER) cisternae. We suggest that pericortical mitochondrial aggregates may be the source of J-aggregate fluorescence (see text for details). nu = nucleolus; M = mitochondria. Original magnifications: (A) ×1300; (B) ×5000; (C) ×30 000; (D) ×10 000; (E) ×1800; (F) ×6000; (G) ×650.

**JC-1 staining of human oocytes**

Within 8 h after aspiration from fully grown follicles, normally appearing MII human oocytes (n = 12; e.g. Figure 2C1) were denuded of cumulus and corona cells, stained with JC-1 and examined by conventional fluorescence microscopy. Similar to the situation in the mouse, JC-1 fluorescence was detected in the FITC channel throughout the ooplasm (Figure 2C1) and punctate J-aggregate fluorescence detected in the RITC channel was asymmetrically localized in the subplasmalemmal cytoplasm (J, Figure 2C2, background fluorescence subtracted). This pattern of putative J-aggregate fluorescence was also observed in MII oocytes (Figure 2D) stained after 24–48 h of culture (e.g. Figure 2D1, FITC; Figure 2D2, RITC channel). All oocytes exposed to FCCP (n = 7) exhibited JC-1 fluorescence in the FITC channel (Figure 2E1) but no J-aggregate fluorescence (Figure 2E2).

**Transmission electron microscopy of mouse and human oocytes**

TEM analysis of representative stages of mouse oocyte maturation were undertaken to correlate stage-specific spatial remodelling of mitochondria with J-aggregate distributions observed in living oocytes. Figure 3A is a representative image of a fully grown GV stage oocyte (n = 8) with intact coronal cells (CC) fixed immediately after isolation from the ovary. These oocytes are characterized by numerous transzonal (arrows, ZP) corona cell processes (M) present throughout the cytoplasm. As reported previously (Van Blerkom and Runner, 1984, Tokura et al., 1993), a clustering of mitochondria in the maturing GV stage oocyte is detectable at ~30–45 min of culture, and, as shown here, the clusters are clearly evident in the pericortical (asterisk, Figure 3B) and perinuclear cytoplasm at the GVB stage (black arrow, Figure 3B). In serial sections, linear arrays of mitochondria located in the pericortical cytoplasm are also evident (white arrows, Figure 3B). As shown in Figure 3C, the CBV stage is characterized by a pronounced perinuclear aggregation of mitochondria (black arrows) and distinct pericortical mitochondrial clusters (white arrows). Although a specific association between punctate, J-aggregate fluorescence observed in living oocytes and mitochondrial clusters identified by TEM could not be made, the findings provide both a temporal and spatial basis for pericortical mitochondrial clusters as a possible source of putative J-aggregate fluorescence in the maturing mouse oocyte. In the same respect, preliminary TEM analysis of putative J-aggregate fluorescent MII human oocytes (n = 6)
showed clusters of mitochondria in the subplasmalemmal/pericortical cytoplasm (M, Figure 3D) surrounding massed cisternae of the smooth endoplasmic reticulum (SER).

**JC-1 staining of mouse and human embryos**

Figure 2F and F1 shows a fully compiled image and 1 µm SLCM sections respectively, of a tripronuclear human oocyte (18 h post insemination, conventional IVF) stained with R123 in which peri-pronuclear (PN, Figure 2F1) mitochondrial aggregation (M) is evident. Figure 2F2 and F3 shows representative SLCM images of a JC-1-stained tripronuclear oocyte (n = 12) examined at FITC (Figure 2F2) and RITC (Figure 2F3) emission wavelengths. Similar to CBV stage mouse oocytes (see Figure 2A2, A3), significant JC-1 fluorescence occurred in the perinuclear region (asterisk, Figure 2F2) while putative J-aggregate fluorescence was specifically localized to the subplasmalemmal/pericortical cytoplasm (arrows, Figure 2F3). The same finding was obtained with monospermic pronuclear embryos (n = 4), and to date, 5 of 17 fertilized oocytes have exhibited an asymmetric distribution of putative J-aggregate fluorescence. For example, JC-1 (green fluorescence) and putative J-aggregate fluorescence (arrows, red fluorescence) have been superimposed in Figure 2F4, which is an SLCM image of a tripronucleate human embryo stained with JC-1 at 18 h after insemination. The highest intensity of JC-1 fluorescence was detected in the peri-pronuclear region (asterisk, Figure 2F4). For pronuclear stage mouse embryos (n = 24), a very similar pattern of pericortical J-aggregate fluorescence was observed (data not shown).

JC-1 and putative J-aggregate fluorescence were examined for cleavage (n = 90), morula (n = 50) and blastocyst stage mouse embryos (n = 75). Nine normal-appearing, early cleavage stage human embryos from monospermic (n = 2) and dispermic (n = 7) fertilizations were also available for analysis. In all cleavage stage embryos examined by conventional microscopy and SLCM, putative J-aggregate fluorescence was localized to the subplasmalemmal/cortical cytoplasm, and were virtually undetectable where blastomeres were in contact (asterisks, Figure 2G2, G4, G5). For example, Figure 2G1–4 shows representative epifluorescent (Figure 2G1, G2) and pseudocolour SLCM (Figure 2G3–5) images of a 2-cell mouse embryo (Figure 2G) observed at FITC (Figure 2G1, G3) and RITC (Figure 2G2, G4) emission wavelengths. Figure 2G2 is presented after subtraction of JC-1 background fluorescence, and Figure 2G5 was obtained by combining images taken at the green and red emission wavelengths. Typically, the distribution and apparent density of punctate J-aggregate fluorescence were comparable in each blastomere of 2–4-cell embryos. However, pronounced differences in punctate J-aggregate fluorescence between blastomeres were detected in some human embryos. For example, the arrows in Figure 4A2 indicate blastomere-specific differences in the occurrence of pericortical J-aggregate fluorescence in a normally fertilized 2-cell human embryo, and this same pattern was detected in four 2-cell embryos and one 4-cell human embryo. The asterisks in Figure 4A2 denote areas of cell contact where no putative J-aggregate fluorescence was detected. Figure 4A1 shows JC-1 fluorescence at the FITC wavelength. The higher intensity observed in the perinuclear region (N) is probably associated with significant perinuclear mitochondrial aggregation that has been previously reported for the human 2-cell embryo (Van Blerkom et al., 2000).

For the human 4-cell embryo (Figure 4B1–3) and all cleavage stage mouse embryos examined through the morula stage (Figure 4C1–3, D and E), putative J-aggregate fluorescence detected by epifluorescence (Figure 4B3, D) or SLCM (4C1–3, E) was localized in the apical pericortical cytoplasm. In contrast, intense JC-1 fluorescence was present throughout the cytoplasm of all blastomeres in cleavage stage embryos (Figure 4B2, C1, C3). For the mouse, J-aggregate fluorescence occurred at the expanded (Figure 4F1) and hatched blastocyst stages (Figure 4G1, 2), but was localized to the trophoderm (arrow, Figure 4F3). The occurrence of JC-1 fluorescence observed at the FITC emission wavelength in both trophoderm and the ICM (arrows, Figure 4F1, F2, G1) of these embryos is shown in representative epifluorescent (Figure 4F2) and SLCM images (Figure 4G1). Figure 4F4 is a high magnification image of a trophodermal cell from the region indicated by an arrow on Figure 4F3. The rod-shaped structures surrounding the nucleus (N) appear to be fully developed and elongated mitochondria characteristic of the blastocyst stage, and, owing to the intensity of JC-1 staining detected in individual mitochondria in the RITC channel, these organelles are presumed to be high-polarized and capable of forming J-aggregates. For some collapsed blastocysts stained with JC-1 during re-expansion, putative J-aggregate fluorescence was detected in cells free within the blastocoelic cavity (data not shown). This finding indicates that the absence of detectable J-aggregate fluorescence in the ICM is unlikely to be related to the availability of JC-1 within the blastocyst.

**Cell contact and putative J-aggregate fluorescence**

Whether contact between the oocyte and coronal cells was associated with the occurrence of putative J-aggregate fluorescence was examined in preliminary studies with cumulus-enclosed GV stage mouse oocytes that were exposed to JC-1 at low and high concentration for 30 min, followed by culture in normal medium for 20 min. Oocytes were either retained in culture intact (n = 120) or were manipulated such that between 20 and 80% of the corona and cumulus was removed from the zona pellucida (n = 120). Intact oocytes were denuded at 5, 10, 20, 30 and 90 min, and examined immediately by conventional fluorescence microscopy. Whereas all intact oocytes showed intense JC-1 fluorescence in the FITC channel (similar to Figure 1K2) which carried over into the RITC channel (similar to Figure 1K3), none showed putative J-aggregate fluorescence typical of oocytes stained with JC-1 after denudation. However, preliminary findings indicate that putative J-aggregate fluorescence appears in the pericortical cytoplasm of intact oocytes preloaded with JC-1 ~20 min after denudation. For manipulated oocytes, punctate J-aggregate fluorescence was detected in the pericortical cytoplasm subjacent to the denuded portion of the zona pellucida at approximately the same time (15–20 min) as observed in fully denuded oocytes. For example, the cumulus–coronal complex was undisturbed in the region
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**Figure 4.** Scanning laser confocal (A1, A2; C1–3; G1, G2) and conventional epifluorescence images of JC-1-stained preimplantation stage mouse and human embryos (A1–G2), manipulated GV stage mouse oocytes (H1–4), and individual blastomeres (I1–3) and embryos (J–O) that developed from disaggregated blastomeres. Arrows in A2, B3, C2 and C3 indicate an apparent pericortical localization of putative J-aggregate fluorescence in cleavage stage embryos. During the mouse blastocyst stage (F1), J-aggregate fluorescence was detected in the trophectoderm (arrows, E, F3, G2), but not the inner cell mass (ICM, F1), which exhibited only JC-1 fluorescence at the FITC wavelength (F2, G1). The rod-shaped structures surrounding the nucleus (N) in F4 are consistent with mitochondria, and their fluorescence in the RITC channel is indicative of J-aggregate formation associated with high polarization. (H3, 4) Representative images showing putative pericortical J-aggregate fluorescence in JC-1-stained germinal vesicle stage mouse oocytes in which portions of the cumulus–corona complex (CC) were removed during culture. Putative JC-1, J-aggregate fluorescence occurred in the same oocyte (H1) in (i) regions of the pericortical cytoplasm subjacent to portions of the plasma membrane denuded of coronal cells at the start of culture (region below arrows in F3) or (ii) during culture, after additional portions of the complex were mechanically removed (region below arrows in H4). JC-1 fluorescence in the FITC channel was detected throughout the ooplasm (H2). (I1–O) Fluorescent images of individual blastomeres from early cleavage stage mouse embryos preloaded with JC-1 prior to disaggregation and culture. Immediately after disaggregation, regions of intercellular contact, which in the intact embryo were largely devoid of putative J-aggregate fluorescence, were still detectable (I1), but within minutes, punctate pericortical J-aggregate fluorescence was observed in these areas (I2, I3). Within the continued cell division of individual blastomeres, putative J-aggregate fluorescence was largely absent in the pericortical cytoplasm at regions of intercellular contact (J, L, M2). The asterisks in A2 denote areas of cell contact where no putative J-aggregate fluorescence was detected. (See text for details).
above the white arrows in the representative oocyte shown in Figure 4H1. Small clusters of cumulus cells (e.g. CC, 4H1) were retained in some of these oocytes as positional markers. Figure 4H3 shows no putative J-aggregate fluorescence (after subtraction of JC-1 FITC fluorescence, Figure 4H2) in the newly exposed portion of the oocyte (above white arrows) but some fluorescence in the residual coronal cells (CC, Figure 4H3). When the same oocytes were examined 20 min later (Figure 4H4), putative J-aggregate fluorescence was detected in pericortical regions that were previously intact, but were absent in regions subjacent to the residual cumulus complex (arrows, Figure 4H4). TEM images showed the persistence of contact between the oolemma and transzonal cell processes in this undisturbed region (Figure 3E).

In a similar series of experiments, early cleavage stage embryos were preloaded with JC-1 followed by disaggregation into individual blastomeres. As described above for 2- and 4-cell embryos, putative J-aggregate fluorescence was undetectable in the region of intercellular contact. The epifluorescent images shown in Figure 4I1–O are presented as observed in the RITC channel, i.e. without manipulation or background subtraction. Figure 4I1, I2 and I3 are representative images of single blastomeres from a 2-cell embryo (n = 30) that were examined at precisely 1, 3 and 5 min after disaggregation, respectively. The arrow in 4I1 and I2 indicates where cell contact occurred in the intact embryo. The area of former cell contact was consistently detectable in the pericortical cytoplasm by the absence of J-aggregate fluorescence at 1 min, but at 3 min, some J-aggregate fluorescence was observed. However, by 5 min, putative J-aggregate fluorescence was observed around the entire circumference of isolated blastomeres. Blastomeres from intact embryos preloaded with JC-1 were juxtaposed for re-aggregation either to retain their former positions (n = 50) or by approximating a 180° rotation such that previously free margins were now in opposition (n = 40). After cell contact was clearly evident at the light microscopic level (between 30 and 60 min), reconstructed 2-cell embryos were examined by fluorescence microscopy. As indicated by arrows in Figure 4J, punctate J-aggregate fluorescence was virtually undetectable in the newly established regions of intercellular contact. In contrast, for those blastomeres that did not re-establish contact, putative J-aggregate fluorescence was detected throughout the pericortical cytoplasm, including regions where cells were in very close proximity (arrows, Figure 4K). Microscopic examination of couplets (n = 15) and quartets (n = 17, e.g. Figure 4L) which developed from individual blastomeres preloaded with JC-1 at the 2-cell stage, showed that J-aggregate fluorescence was largely absent in zones of intercellular contact (arrows, Figure 4L), as was observed in intact embryos at the same stage. With continued culture, compaction occurred in the quartets (n = 25; Figure 4M1) and most formed compacted morulae (n = 20; Figure 4O). The distribution of putative J-aggregate fluorescence in compacting quartets (arrows, Figure 4M2) and compacted morulae (arrows, Figure 4O) was comparable with the situation observed in intact embryos. However, for individual blastomeres from 2- and 4-cell embryos that remained unassociated and undivided during culture, clusters of putative J-aggregate fluorescence were observed throughout the cytoplasm (arrows, Figure 4N), including the perinuclear region (N). No J-aggregate fluorescence was detected in individual blastomeres or resulting embryos exposed to FCCP (data not shown). The above findings suggest that the occurrence of putative J-aggregate fluorescence does not require the persistence of JC-1 in the medium and is therefore unlikely to be related to such factors as stain uptake or regional concentration differences with the cytoplasm.

Discussion
Outward proton pumping across the inner mitochondrial membrane creates a proton gradient that has two components, a $\Delta \Psi \text{m}$ and a pH gradient, and the energy stored in either component drives the conversion of ADP to ATP by respiratory chain enzymes. Here, we examined the J-aggregate-forming capacity of JC-1 to investigate whether mitochondria in mouse and human oocytes and early embryos are homogeneous with respect to $\Delta \Psi \text{m}$. JC-1 is a cationic lipophilic carbocyanine-based dye that accumulates within the mitochondrial matrix where its fluorescence emission characteristics are related to the magnitude of $\Delta \Psi \text{m}$ (Reers et al., 1991, 1995). The formation of J-aggregates within mitochondria, named after Jelley, who described this special type of carbocyanine multimer in 1937, is associated with a large shift in fluorescence maxima of JC-1 to longer wavelengths (Jelley, 1937; Smiley et al., 1991). As a consequence, when mitochondria are examined at appropriate excitation frequencies, low-polarized mitochondria ($\Delta \Psi \text{m} \approx \pm 100 \text{ mV}$) fluoresce green and high-polarized mitochondria fluoresce yellow–red ($\Delta \Psi \text{m} \approx \pm 140 \text{ mV}$), reflecting the formation of JC-1 multimers or J-aggregates detectable in the RITC channel (Reers et al., 1995; Cossarizza et al., 1996). The unique chemistry of this compound has identified heterogeneity in $\Delta \Psi \text{m}$ at the single cell level and regional differences within individual mitochondria of cultured cells (Smiley et al., 1991).

Specificity of JC-1 staining
Here, we show that putative J-aggregate fluorescence occurs in a punctate fashion in the subplasmalemmal and pericortical cytoplasm of maturing oocytes and preimplantation embryos stained with JC-1. However, because of their size (typically <0.5 µm) and occurrence at very high numbers in fully grown oocytes and early mammalian embryos (>85 000/MII mouse oocyte, and >120 000/MII human oocyte), it is virtually impossible to detect the fluorescent signal emitted by individual mitochondria with conventional fluorescence or SLCM. The specificity of R123 and MO staining of mitochondria in maturing mouse oocytes and early human embryos has been confirmed at the TEM level (Van Blerkom and Runner, 1984; Tokura et al., 1993; Van Blerkom et al., 1998, 2000), and in the present study, we tested the specificity of JC-1 for mitochondria under several conditions. Similar to cultured cells in which J-aggregates have been shown to be of mitochondrial origin (Smiley et al., 1991; Reers et al., 1995), mitochondria in human cumulus cells are elongated, fully developed
organelles that can be detected individually with R123 or MO, especially in the extensions and projections elaborated by the cells in culture. JC-1, J-aggregates detected in the RITC channel were localized to cumulus cell mitochondria and failed to develop in cells exposed to FCCP, a proton ionophore that uncouples oxidation and phosphorylation by abolishing the electrochemical gradient that generates $\Delta \Psi_m$. The same findings were obtained for FCCP-treated oocytes, embryos and disaggregated blastomeres at all stages of maturation and preimplantation development, respectively. Mitochondria in blastocyst stage embryos are fully developed, elongated elements, which, owing to the absence of replication during the preimplantation stages, occur in fewer copies per cell than during early development. Rod-shaped elements in trophectodermal cells of mouse blastocysts exhibited intense orange–red fluorescence and their intracellular distribution and geometry are consistent with mitochondria detected with R123. Staining of MII mouse oocytes followed by compartmentalization into mitochondria-rich cytoplasts and mitochondria-depleted karyoplasts, as determined here and previously (Van Blerkom et al., 1998) with R123 and MO, confirmed that J-aggregate fluorescence only occurred in cytoplasts and did not develop in the presence of FCCP. The CBV–MI stage mouse oocyte and the pronuclear human embryo are characterized by a marked perinuclear accumulation of mitochondria (Van Blerkom and Runner, 1984; Van Blerkom et al., 2000). In the present study, staining with R123 confirmed this activity, and with JC-1 the same pattern of intense perinuclear fluorescence was observed only in the FITC channel. These findings offer indirect evidence that JC-1 is mitochondrially associated in oocytes and early embryos.

Whether putative J-aggregate formation or distribution are functions of JC-1 concentration or differential uptake (stage- or time-specific) was examined in parallel studies using R123, MO and JC-1 on the same cohort of oocytes. At different concentrations and exposures, the results in all cases showed largely uniform cytoplasmic JC-1 fluorescence from the earliest time point, with no indication of mitochondrial fluorescence initially appearing at the cell periphery. As expected with organelle-specific probes, at higher concentrations or with longer exposures, the intensity of R123 (Johnson et al., 1980) and MO mitochondrial fluorescence was higher. A similar finding was obtained with JC-1 where the intensity of putative J-aggregate fluorescence appeared to increase at higher JC-1 concentrations, but the occurrence and distribution of J-aggregate fluorescence in the pericortical cytoplasm remained unchanged. However, at the highest JC-1 concentration used in this study, the intensity of cytoplasmic fluorescence in both FITC and RITC channels obscured documentation of putative J-aggregate fluorescence by conventional and SLCM fluorescence microscopy. In our experience, analysis by SLCM was limited to a few scans through an oocyte or embryo before punctate J-aggregate fluorescence was quenched. In this regard, we observed that multiple scans frequently resulted in oocytes and embryos that failed to progress when returned to normal media, suggesting that repeated excitation of JC-1-stained specimens can be developmentally lethal.

In isolated blastomeres obtained from cleavage stage embryos preloaded with JC-1, putative J-aggregate fluorescence appeared (i.e. in the absence of stain) in regions of the cortical cytoplasm that were previously devoid of punctate J-aggregate fluorescence in the intact embryo. A similar conclusion was obtained from blastomere re-aggregation studies in which J-aggregate fluorescence was undetectable in preloaded blastomeres where new intercellular contacts were established, or at regions of contact between individual blastomeres that were allowed to divide undisturbed in culture. This result indicates that putative J-aggregate fluorescence in these cells is not uptake dependent. Because mitochondria in oocytes and early embryos cannot be resolved individually, the possibility that the J-aggregate fluorescence we detected is not mitochondrially associated cannot be excluded. We are unaware of experimental evidence to suggest that (i) J-aggregates develop in, or are associated with, cytoplasmic elements (e.g. Golgi or SER cisternae) other than mitochondria, or (ii) conditions exist within the pericortical cytoplasm of normal oocytes or embryos that could promote spontaneous, non-mitochondrial, JC-1 multimerization. Collectively, findings from intact and experimentally manipulated mouse oocytes and embryos, and correlated fluorescent and TEM images, suggest that clusters of pericortical mitochondria are the source of the observed punctate J-aggregate fluorescence. The absence of such fluorescence in interior portions of the cytoplasm in normally maturing oocytes and developing embryos may result from fluorescence that is below detection, or possibly from mitochondria whose $\Delta \Psi_m$ is insufficient to form JC-1 multimers detectable in the RITC channel under the conditions used in this study.

Possible relationships between J-aggregate formation and mitochondrial function and activity

There is no current evidence to suggest that spatially distinct populations of mitochondria with different metabolic capacities or functions exist in normal mammalian oocytes or early embryos. One study has detected differences in the intensity of JC-1 fluorescence (by SLCM) between human oocytes and preimplantation embryos that appeared to be related to maternal age, and suggested that poor oocyte competence and embryo developmental performance that accompanies increased reproductive age may result from a corresponding decline in mitochondrial metabolic activity (Wilding et al., 2001). Regardless of maternal age, however, no significant or consistent spatial heterogeneity in apparent mitochondrial metabolic activity or $\Delta \Psi_m$ within individual oocytes or early cleavage stage blastomeres was reported in this study.

Whether metabolic level and $\Delta \Psi_m$ in individual oocytes, embryos and blastomeres are associated remains to be determined. However, in other cells, high metabolic activity and high $\Delta \Psi_m$ are not synonymous (Richter et al., 1996; Diaz et al., 1999), and $\rho^0$ cells, which lack mitochondrial DNA and are non-functional in respiration, nevertheless maintain a $\Delta \Psi_m$ (Marchetti et al., 1996; Zamzami et al., 1996). The simplest explanation for the distribution of putative J-aggregate fluorescence described here is one based on proximity to the plasma membrane, where the occurrence of JC-1, J-aggregate-forming pericortical mitochondria could reflect both elevated $\Delta \Psi_m$ and
metabolism (Dedov and Roufogalis, 1999; Garner and Thomas, 1999) that may be associated with a higher ambient oxygen concentration. Intracellular oxygen levels do not necessarily follow simple diffusion kinetics across a plasma membrane because they can be significantly influenced by such factors as the degree of cytoplasmic organization (Aw, 2000) and the occurrence of cytoskeletal-mediated intracellular circulation and convection (Hochachka, 1999; Agutter and Wheatley, 2000). However, maturing oocytes and blastomeres differ from other cells with respect to shape, volume, and, significantly, the presence of tens of thousands of comparatively undeveloped mitochondria (Van Blerkom and Motta, 1979). If mitochondrial polarity and metabolism are related in these unique cells, the occurrence of high-polarized mitochondria in the pericortical cytoplasm may be influenced by higher ambient oxygen in the subplasmalemmal cytoplasm, and these mitochondria may largely support ATP-requiring processes at the plasma membrane level, an activity which may be especially relevant during those stages of development or the cell cycle where a significant portion of the mitochondrial complement is normally internalized to the perinuclear cytoplasm (Van Blerkom, 1991).

Another possible explanation for the distribution of putative J-aggregate fluorescence we observed comes from recent evidence that mitochondria are (i) excitable organelles which can respond to and propagate electrical and Ca\(^{2+}\) signals (Ichas et al., 1997), and (ii) that in differentiated cells, ΔΨm is location dependent. An examination of ΔΨm in four cell types under confluent and dispersed conditions reported that high-polarized mitochondria, determined by the presence of J-aggregates, were specifically localized to cell borders free from intercellular contact (Diaz et al., 1999). The occurrence of high-polarized mitochondria in these regions was suggested to have a role in regulating calcium-induced calcium release (CICR) or other Ca\(^{2+}\) signalling pathways by buffering extra- cellular Ca\(^{2+}\) influxes (Babcock et al., 1997), or in the maintenance of Ca\(^{2+}\)-sensitive elements (Diaz et al., 1999) such as inositol 1,4,5-triphosphate (IP3). It was demonstrated (Ichas et al., 1997) that mitochondrial CICR (mCICR) was a direct consequence of loss of ΔΨm and resulted in a transient Ca\(^{2+}\) release associated with opening of the permeability transition pore complex. Upon pore closure, the proton gradient reforms and ΔΨm is restored. In the same study, analysis of isolated mitochondria showed that mCICR and depolarization could propagate between mitochondria, generating travelling waves of depolarization and Ca\(^{2+}\) release. These investigators proposed that the transmission of electrical and Ca\(^{2+}\) signals between high-polarized mitochondria may amplify Ca\(^{2+}\) signals emitted from other Ca\(^{2+}\) storage elements, such as the SER.

Putative J-aggregate fluorescence was initially present throughout the pericortical cytoplasm of the GV stage mouse oocyte, but as maturation progressed, it became asymmetrically distributed with higher densities detected in the ‘animal’ hemisphere at MII. These changes may reflect spatial remodelling of mitochondria during mouse oocyte maturation that results in a higher density (Van Blerkom and Runner, 1984) or polarized distribution (Calarco, 1995) in the animal hemisphere at MII. At present, we cannot exclude the possibility that persistent intercellular contact at the GV stage results in local differences in JC-1 concentration within the pericortical cytoplasm that may influence the formation or detection of J-aggregate fluorescence by presumably high-polarized mitochondria. However, the findings of Diaz et al. (1999) suggest that loss of intercellular contact could be another factor associated with J-aggregate formation. Our findings with intact and manipulated GV stage mouse oocytes raise the possibility that when and where transzonal cell contact terminates after retraction of transzonal corona cell processes at the initiation of preovulatory meiosis, the occurrence of punctate J-aggregate fluorescence may reflect physiological changes in the pericortical cytoplasm that promote higher states of polarization within the associated mitochondria. Whether putative J-aggregate formation in pericortical mitochondria during the earliest stages of resumed meiosis is indicative of one of the first regulatory events associated with the shift from maternal to oocyte control of development is under investigation.

One study (Ozil and Huneau, 2001) demonstrated that the amplitude of the initial Ca\(^{2+}\) transient that activates the mammalian oocyte has profound developmental consequences. These investigators modulated the amplitude and frequency of the Ca\(^{2+}\) stimulus that parthenogenetically activated rabbit oocytes, and after uterine transfer at the blastocyst stage they assessed the normality of development on day 11.5 of pregnancy. While the dynamics of prematuration development (e.g. morphology and cleavage rate) were not affected by either parameter, developmental performance and the normality of organogenesis were specifically related to the amplitude of the first Ca\(^{2+}\) transient, suggesting that the magnitude of this signal in the early minutes of mammalian oocyte activation is an epigenetic event that has direct downstream developmental consequences. For the mature oocyte, it is unknown whether pericortical domains of putative high-polarized mitochondria could be involved in activation by influencing the amplitude of the first Ca\(^{2+}\) transient. However, if such a capacity exists, ionic or electrical fluxes at the site of sperm attachment may cause a transient depolarization of subjacent, high-polarized (J-aggregate forming) mitochondria resulting in a transient Ca\(^{2+}\) discharge (Loew et al., 1994). The contribution of mitochondrial Ca\(^{2+}\) may enhance the propagation or amplitude of the first Ca\(^{2+}\) wave that normally involves IP3-mediated, Ca\(^{2+}\) mobilization, or influence CICR from small Ca\(^{2+}\) storage vesicles that lie beneath the oolemma (Sousa et al., 1997), or from associated cisternae of the SER. If confirmed, the occurrence of domains of high-polarized mitochondria in the oocyte may have clinical implications with respect to a possible aetiology for the differential
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developmental competence exhibited by morphologically normal preimplantation embryos produced in IVF procedures.

Our findings indicate that the distribution of putative J-aggregate fluorescence in undisturbed and reconstructed cleavage stage mouse embryos preloaded with JC-1 is cell contact-related, with punctate JC-1, J-aggregate fluorescence largely localized to the pericortical cytoplasmin subjacent to the free (apical) margins of blastomeres. For expanded and hatched blastocysts, J-aggregate fluorescence was detected in the trophoectoderm but not in the ICM, although JC-1 fluorescence was clearly observed in both compartments. JC-1 staining of collapsed blastocysts during the re-expansion phase showed J-aggregate formation in unattached cells within the blastocoelic cavity, suggesting that the absence of J-aggregate fluorescence in the ICM is not associated with the availability of the stain within the blastocyst. These findings lead to another speculation concerning possible roles of putative highly polarized mitochondria in early mammalian development that warrants further investigation. It has been suggested that blastomeres with distinct apolar (inside cells) and polar (outside cells) phenotypes in the mouse morula are progenitors of the ICM and trophoderm, respectively (Johnson and Ziomek, 1981a,b). Experimental studies with isolated blastomeres and with embryos reconstructed from inside or outside cells (Fleming and Johnson, 1988) demonstrated that polarity is a metastable phenomenon related to the specificity, geometry (symmetry) and stability of intercellular contact and communication. With respect to the possible occurrence of J-aggregate-forming mitochondria in the early embryo, we are investigating whether their distribution in blastomeres of intact and manipulated mouse embryos is related to, or indicative of, metastable pericortical conditions associated with the generation of polar and apolar cells, and whether their apparent absence in the ICM is associated with extensive intercellular contacts between these cells. Additional studies are also required to determine if (i) differences in J-aggregate fluorescence detected between blastomeres in some human embryos is associated with asymmetrical distributions of mitochondria that occur at the pronuclear stage and which can lead to their disproportionate segregation at the first cleavage division (Van Blerkom et al., 2000) and (ii) whether atypical distributions of punctate J-aggregate fluorescence in GV stage oocytes that fail to mature, or in isolated blastomeres that fail to divide, are indicative of unusual mitochondrial organizations or aberrant cytoplasmic conditions (Matsuyama et al., 2000). Studies of this type may provide new insights into mitochondrial functions and activities in early mammalian development that could have clinical implications in understanding the origins of differential oocyte and embryo competence.

Acknowledgements

We thank Christopher Atkinson for his assistance in these studies and Dr Sayoko Makabe (Toho University School of Medicine) for kindly providing the transmission electron micrograph of the human oocyte.

References


Submitted on February 19, 2001; resubmitted on August 17, 2001; accepted on October 18, 2001.