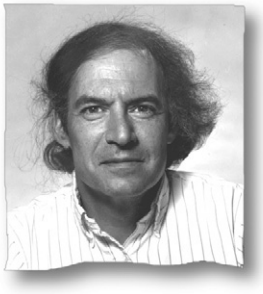
Symposium: Genetic and epigenetic aspects of assisted reproduction

Mitochondria as regulatory forces in oocytes, preimplantation embryos and stem cells



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Abstract

In addition to their role in generating energy (ATP), other possible regulatory functions for mitochondria in the mature oocyte, preimplantation stage embryo and differentiating embryonic stem cell are discussed. The question of the numerical size of a normal mitochondrial complement in the mature oocyte, which has been suggested to be a critical factor in the determination of oocyte and embryo developmental competence, is addressed in the context of mitochondrial DNA copy numbers and the apparent number of organelles detected in living mouse and human blastocysts with mitochondria-specific fluorescent probes. The existence of a spatially stable subplasmalemmal domain of high-polarized mitochondria in the mature oocyte and early embryo is proposed to form a microzone of differential activity that may locally influence the normality of fertilization. High-polarized mitochondria may also influence developmentally significant activities in the peri-implantation blastocyst and differentiating embryonic stem cell. The work discussed indicates that mitochondria may have multiple functions during early development, and specific areas of investigation required to confirm these possibilities are suggested.

Keywords: *ATP, blastocysts, fertilization, high-polarized mitochondria, human, implantation*

Introduction

It might seem that the role of mitochondria in oogenesis, early embryogenesis and stem cells would only be related by the fact that as cells, most of the ATP they require is produced by mitochondrial respiratory activity (oxidative phosphorylation). Indeed, the role of mitochondria in early human development is largely viewed as a respiratory one in which subnormal levels are thought to contribute to common defects and failures during pre-ovulatory maturation, fertilization and preimplantation embryogenesis (Muller-Hocker *et al.*, 1996; Reynier *et al.*, 2001; for reviews, see Christodoulou, 2000; Schon *et al.*, 2000; Cummins, 2002; Brenner, 2004; Van Blerkom, 2004a,b; Dumollard *et al.*, 2007; May-Panloup *et al.*, 2007; McFarland *et al.*, 2007). For somatic cells, ATP production is only one of several important activities in which mitochondria are engaged, and a growing body of evidence suggests that in addition to specialized functions in specific cells, they have more ubiquitous roles as regulatory elements engaged in signal transduction, calcium homeostasis and oxygen sensing (Bunn and Poyton, 1996; Pozzan *et al.*, 2000; Bell *et al.*, 2005; Zimijewski *et al.*, 2005; Gutierrez *et al.*, 2006; Quintero *et al.*, 2006). Here, evidence is presented to indicate similar roles for oocytes, preimplantation stage embryos and possibly, stem cells. The notion of microzonation, in which mitochondria may be functionally compartmentalized within the somatic cell cytoplasm (Aw, 2000), is discussed for developing oocytes and embryos. Some of the concepts presented are speculative, but investigations required for their validation are suggested and confirmation would offer new insight into the role of mitochondria as regulatory forces in early development.

What is a normal mitochondrial complement for the oocyte and how is it related to developmental competence?

Electron microscopic studies of primordial human and mouse oocytes show that at the beginning of oogenesis, the number of mitochondrial progenitors is remarkably small, perhaps as few as 10 per cell (Jansen, 2000). During the relatively prolonged phase of oocyte growth, progressive replication expands the mitochondrial complement to a terminal level present in the pre-ovulatory metaphase II (MII) stage oocyte and preimplantation embryo (Motta *et al*., 2000). The small number of progenitors is one basis for the occurrence of metabolic defects seen during later development (respiratory chain disorders; oxidative phosphorylation diseases) that result from the expansion of pathogenic (mutant) forms that reduce metabolic capacity. A heteroplasmic condition, in which mutant and wild type forms coexist, is probably not atypical for the human oocyte, but may be of no developmental consequence if (i) the proportion of mitochondria with pathogenic mitochondrial DNA (mtDNA) defects, the so-called mutant load, is low, and (ii) the specific mutation does not effect mitochondrial replication. Whether heteroplasmy can be developmentally toxic or ultimately lethal for affected individuals is related to the function or activity level the mutation affects, and the numerical threshold where the mutant load becomes problematic for normal cellular function (Chinnery and Turnbull, 1999; Howell *et al*., 2000; McFarland *et al*., 2007). The recognition that pathogenic mtDNA defects in the fertilizable MII oocyte can have serious downstream consequences is one of the principal reasons for the renewed interest in mitochondrial function in early human development. In addition, the numerical size of the mitochondrial complement has been suggested to be a critical determinant of developmental competence, with subnormal levels related to premature maturational arrest for the oocyte and early demise for the embryo (May-Panloup *et al*., 2007; Shoubridge and Wai, 2007). While electron microscopic studies have revealed stage-specific changes in mitochondrial fine structure during oogenesis and predevelopment (Van Blerkom and Motta, 1979; Motta *et al*., 2000; Sathananthan and Trounson, 2000) and early post-implantation development (Shepard *et al*., 2000), they have been largely uninformative with respect to the numerical size of the mitochondrial complement, especially in the MII human oocyte.

Current estimates of complement size in the mature human oocyte are largely based on determinations of mtDNA copy number and the assumption that each mitochondrion contains one or two genomic copies (Jansen, 2000). However, mtDNA copy numbers show a wide variation between human oocytes from the same and different cohorts; reported mtDNA contents differ by nearly two orders of magnitude, from ~1 × 10^4 to >1 × 10^6 mitochondria/oocyte (see reviews by Brenner, 2004; Van Blerkom, 2004a,b; May-Panloup *et al*., 2007; Shoubridge and Wai, 2007). As noted above, the number of mitochondria in a normal human oocyte is not merely of academic interest, as relative complement size may have direct influences on developmental competence for both oocyte and embryo, especially if certain pathogenic forms occur at high load. At the low end of this range, mitochondrial under-representation has been suggested to create a bioenergetic/metabolic (i.e. ATP) deficit that is unable to promote normal cellular activities such as nuclear maturation to MII, or to be a contributing factor to fertilization failure and early embryo demise (Reynier *et al*., 2001; Brenner, 2004; Shahinaz *et al*., 2006).

Zeng *et al*. (2007) related the normality of pre-ovulatory meiotic maturation in human oocytes with mtDNA copy number and cytoplasmic ATP content. They reported that approximately 50% of human germinal vesicle stage (GV) oocytes that matured to MII *in vitro* exhibited a first polar body but no detectable metaphase spindle. In this study, the meiotic spindle was visualized by polarizing optics that detects microtubular birefringence. Of particular developmental significance was their finding that the average mtDNA copy number (~637 ± 238 K) and ATP content (~1.97 ± 0.38 pmol) of oocytes with spindles was higher than for sibling oocytes without a detectable spindle (average mtDNA copy number, ~491 + 153 K; net ATP content of 1.65 ± 0.32 pmol). They suggested that a low mtDNA copy number was indicative of a reduced organelle complement and a corresponding reduction in cytoplasmic bioenergetic capacity, as indicated by subnormal ATP contents. This notion was suggested further by fertilization results using in-vitro matured MII oocytes with and without detectable spindles. In comparison with oocytes with detectable spindles, intracytoplasmic insemination of oocytes without identifiable spindles was associated with a significantly lower incidence of fertilization and if fertilized, a higher frequency of arrested or abnormal embryonic development during the early preimplantation stages. A similar relationship between mtDNA numbers in human oocytes and outcome after conventional and intracytoplasmic insemination was reported in an earlier study by Santos *et al*. (2006), who found that the mean mtDNA copy number in fertilized human oocytes was ~250 K, while for unfertilized oocytes, the mean number was ~164 K. Shoubridge and Wai (2007) suggested that a mitochondrial complement around 100 K might be normal for the mature human oocyte.

These and other studies (reviewed by Brenner, 2004; Shoubridge and Wai, 2007) indicate that a developmentally significant relationship exists between the apparent size of the mitochondrial complement and oocyte/embryo competence. However, the reported values are difficult to interpret with respect to ATP content or numerical organelle threshold levels that may determine the ability of the oocyte to mature, or the embryo to develop progressively until mitochondrial replication occurs after implantation. For example, the threshold suggested by Zeng *et al*. (2007) to be inconsistent with a detectable MII spindle, fertilization and normal embryonic development is significantly higher than the mtDNA copy number estimated by Santos *et al*. (2006) to be associated with normal fertilization and development. Further complicating the estimation of a 'normal' mitochondrial complement associated with competence is the reported relationship between mtDNA and ATP content. The studies of Zeng *et al*. (2006) indicate that the difference between developmental competence and incompetence for the human oocyte could be as little as 300 fmol, which according to their mtDNA calculations, could result from a difference in mitochondrial numbers between 100 and 150 K.

It has previously been suggested that competence for the human MII oocyte may be associated with a net ATP content around 2 pmol (Van Blerkom *et al*., 1995a). This value was

derived from the measurement of ATP concentrations in normal appearing, uninseminated MII oocytes obtained from gamete intra-Fallopian transfer procedures, or from oocytes that were intentionally uninseminated in conventional IVF cycles. Because quantitative ATP measurements require cell lysis, the concentrations suggested to be associated with normal competence could only be inferred from similar appearing siblings that had positive or negative outcomes. The ATP values found by Zeng *et al*. (2007) to be associated with competence are similar to those reported by Van Blerkom *et al*. (1995a), suggesting that an ATP content of ~2 pmol may be an important threshold. With current estimates of mitochondrial numbers in the mature human oocyte between ~100 K and >600 K, the number of organelles required to generate an average net cytoplasmic content of ~2 pmol is unclear. This conclusion supposes that all mitochondria participate equally in ATP production, but as discussed below, that may not be the case.

Based on reported mtDNA contents in MII human oocytes, mitochondrial proliferation during oogenesis would seem to be highly variable between oocytes, if it is assumed that each mitochondrion contains one or two 16.6 Kb double-stranded, circular genomes (Jansen, 2000; Shoubridge and Wai, 2007). A common explanation for mitochondrial numbers estimated to be in the high hundreds of thousands in the mature human oocyte is that a complement size of this magnitude is the progenitor pool for the individual and is necessary to provide adequate concentrations of ATP to drive early development. From fertilization through the early post-implantation stages, the number of mitochondria/cell is approximately halved with each cell division, until replication begins after implantation. As noted above, subnormal numbers are thought to contribute to a reduced ATP generating capacity that may manifest as maturation failure for the oocyte, poor embryo performance *in vitro*, or impaired implantation potential. However, the notion that high organelle numbers are needed to support early development often fails to consider evidence demonstrating that mitochondria undergo stage-specific structural alterations during the preimplantation stages that are indicative of a corresponding up-regulation of metabolic activity and capacity (see reviews by: Van Blerkom and Motta, 1979; Motta *et al*., 2000). These progressive structural alterations cause mitochondria to change from undeveloped, small spherical elements in the oocyte and cleavage stage embryo, in which a relatively small number of peripherally located cristae surround an electron-dense matrix, to elongated forms during the morula-to-blastocyst transition, where numerous cristae completely traverse a matrix of relatively low electron density, especially in the trophectoderm. A high initial complement size could be needed if ATP output/organelle remained static during early development. However, early metabolic studies in model systems demonstrated that the reduction in mitochondrial numbers/cell during the preimplantation stages is apparently accommodated by increased concentrations of ATP generation/organelle (Brinster, 1971; Van Blerkom *et al*., 1973).

If each mitochondrion contains one or two genomes, then current estimates of a normal mtDNA copy number between ~100 K (Shoubridge and Wai (2007) and ~650 K (Zeng *et al*., 2007) would suggest for a typical 100-cell human blastocyst that each cell should contain approximately 1000–6500 mitochondria (assuming one genome/organelle). Some embryos should contain >10,000 organelles/cell, if estimates of >1,000,000 mitochondrial genomes are valid (Barrit *et al*., 2000; Steuerwald *et al*., 2000; Brenner, 2004). However, electron microscopic analysis of serial thin sections taken at different levels through normal appearing, fully expanded day 5 and 6 human blastocysts show mitochondrial numbers for both trophectoderm and inner cell mass between ~150 and 250 organelles/cell (Van Blerkom, 1993; Makabe and Van Blerkom, 2006). To the best of this author's knowledge, the only systematic count of mitochondria in the mature oocyte has been the single transmission electron microscopic morphometric study reported by Piko and Matsumoto (1976), which indicated that the typical MII mouse oocyte contains ~90,000 mitochondria. This suggests that each cell of the 60-cell mouse blastocyst should contain ~1500 mitochondria.

Quantification of mitochondrial numbers in living mouse blastocysts using organelle-specific fluorescent probes

The question of the numerical size of the mitochondrial complement has been re-examined under the assumption that the number of organelles detectable at the blastocyst stage is largely representative of the complement present in the oocyte at fertilization. Fully expanded, newly hatched mouse blastocysts were stained with mitochondria-specific fluorescent probes such as rhodamine123 (r123), MitoTracker Green, Orange or Red, and JC-1, followed by counting of organelles using serial imaging and conventional epifluorescence and scanning laser confocal microscopy. Embryos were recovered from the uterus on day 4 of pregnancy and allowed to hatch spontaneously *in vitro* prior to analysis. For conventional fluorescence microscopy, embryos were slightly compressed between glass coverslips, which greatly facilitated the individual identification and counting of fluorescent mitochondria, especially in the trophectoderm. Confocal images were taken at consecutive intervals of 1–3 μ while conventional fluorescence microscopy involved serial imaging at depths estimated at ~5 μ. Mitochondrial determinations were made independently by three operators using the same representative digital images produced for each embryo. Inter-operator variability differed by <10 detectable mitochondria/cell.

Figures 1A and **1B** show representative confocal images selected from a section series taken through an entire expanded mouse blastocyst stained with r123. The insert in **Figure 1B** shows the level of resolution obtained that enabled individual mitochondria (arrows) to be identified in a typical trophectodermal cell. r123 was especially useful for detecting trophectodermal mitochondria. **Figure 1A** is a view of cells at the uppermost surface of the embryo, while the image in **Figure 1B** is near the midline. **Figures 1C** and **1D** are confocal images of MitoTracker Orange stained embryos shown at levels similar to those in **Figure 1A** and **1B**. Mitochondria in the inner cell mass (ICM, **Figure 1D**) are readily evident with this fluorescent probe. **Figures 1E** and **1F** are conventional epifluorescent images of a slightly compressed mouse blastocyst stained with the potentiometric mitochondria-specific stain JC-1 and imaged in the fluorescein isothiocyanate (FITC) and rhodamine isothiocyanate (RITC) channels respectively. High-polarized trophectodermal mitochondria appear as distinct yellow (arrows,

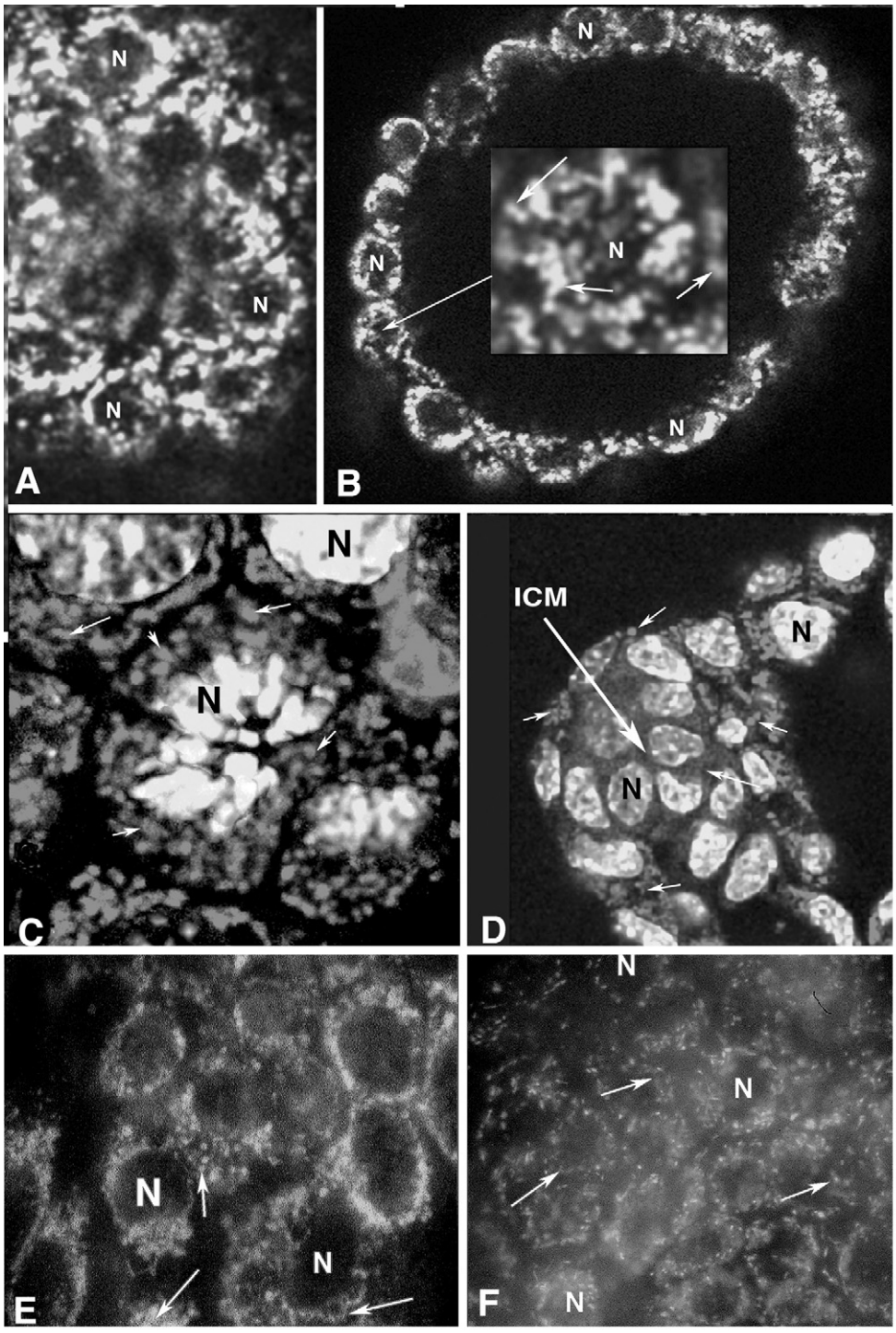

Figure 1. Representative scanning laser confocal images of a section series of rhodamine 123-stained mitochondria in the trophectoderm of a hatched mouse blastocyst taken at the surface (**A**) and midline (**B**) of the embryo. The insert in (**B**) is the cell indicated by an arrow on the left and viewed at the higher magnification used to count mitochondria, arrows pointing to fluorescent mitochondria. (**C**) and (**D**) are representative scanning laser confocal microscopic images of a section series through a hatched mouse blastocyst in which mitochondria (arrows) were stained with MitoTracker Orange. Nuclear fluorescence (N) was obtained by simultaneous staining with 4,6-diamidino-2-phenylindole. (**E**) and (**F**) are conventional epifluorescent images of a hatched mouse blastocyst stained with the mitochondria specific, potentiometric probe JC1 and imaged in fluorescein isothiocyanate (**E**) and rhodamine isothiocyanate (**F**) channels. High polarized mitochondria are indicated by arrows. (Colour figure is available at http://www.rbmonline.com/Article/3087 but readers must be registered and subscribed to view.)

Figure 1E) and red fluorescent rod-like elements (arrows, **Figure 1F**) in the FITC and RITC channels respectively. The number of trophectodermal and ICM cells in each blastocyst stained with r123, MitoTracker, or JC-1, was determined by nuclear counting after staining with the DNA-specific fluorescent probe DAPI, (4,6-diamidino-2-phenylindole) (e.g. N, **Figures 1C** and **1D**). DNA staining accounted for the occasional binucleate cell, and detected chromosomal configurations associated with different stages of the cell cycle. Quantitative information on mitochondria in the ICM was less precise with conventional fluorescence microscopy and for this purpose, numerical estimates were obtained by confocal microscopy after staining with MitoTracker Orange, which is incorporated into all mitochondria, regardless of their transmembrane potential (ΔΨm; see below). Mitochondrial counts using each of the above probes involved at least 10 hatched blastocysts containing an average of 78 (±8) cells.

A similar number of mitochondria/cell was observed with r123, JC-1 and MitoTracker Green, Orange or Red. Estimates of mitochondria/cell were also similar with analysis performed on images obtained by conventional or scanning laser confocal microscopy. The typical mouse trophectodermal and ICM cell contained approximately 125 ± 23 mitochondria. The average number of mitochondria in the fully expanded peri-implantation stage (hatched) mouse blastocyst was ~10,000. To date, nine fully expanded, normal-appearing day 6–6.5 human blastocysts have been examined after the mechanical removal of the zona ($n = 6$) or following spontaneous hatching ($n = 3$). The average number of mitochondria/cell (ICM and trophectoderm) was 151 ± 40 and the average number of cells/embryo was 96 ± 12, indicating that the total number of detectable mitochondria at this stage is ~14,000. Mitochondrial quantitation using individual digital images taken from a serial section series through entire embryo(s) and examined at high magnification could account for overlap between organelles and to a lesser extent, mitochondria that occurred in cross section. Even when the limitations associated with these empirical methods are considered, it seems unlikely that mitochondria could be individually identified and counted in living trophectodermal cells if they occurred at the levels estimated by mtDNA content alone, as noted above.

It could be argued that mitochondria in the blastocyst are heterogeneous in size and that the numbers determined by staining with organelle-specific fluorescent probes do not include those below levels of detection by optical microscopy. However, electron microscopic images for both mouse and human blastocysts show that mitochondria are comparatively homogenous, elongated elements that are several microns in length (Van Blerkom, 1993; Sathananthan and Trounson, 2000; Makabe and Van Blerkom, 2006). The rod-like structures observed by fluorescence microscopy after staining with organelle-specific probes are consistent in shape and size with mitochondrial features determined by electron microscopy. Alternatively, mitochondrial quantification with fluorescent probes may be limited by the level of organelle 'activity' that, if the present values are correct, would suggest that a sizeable proportion of the mitochondria in the oocyte are relatively inactive at the blastocyst stage (dark mitochondria?). Electron microscopic images of mouse blastocysts show a largely uniform mitochondrial fine structure that is consistent with high metabolic activity (Van Blerkom and Motta, 1979). This notion is also precluded by similar numerical findings obtained with fluorescent probes that have different staining properties for mitochondria, including the ability to label mitochondria independent of metabolic activity or the magnitude of their inner membrane potential (ΔΨm) (e.g. MitoTracker Orange), as discussed below. In support of the mitochondrial numbers estimated to populate the cells of the mouse blastocyst are similar levels detected in nascent in-vitro outgrowths of trophectodermal cells (Van Blerkom *et al.*, 2006). In this instance, the identification of individual organelles is greatly facilitated by the highly flattened and expanded nature of these cells (see below, **Figure 4A**, **B**).

The spontaneous loss of mitochondria during the preimplantation stages seems an unlikely explanation for the apparent discrepancy between organelle numbers estimated to occur at fertilization and apparent numbers visually detected at the blastocyst, because degraded forms are not seen by electron microscopy in normal preimplantation stage mouse or human embryos (Motta *et al.*, 2000; Sananthanan and Trounson, 2000). Perhaps the simplest explanation is that approximations of mitochondrial numbers derived from mtDNA copy numbers are overestimates if based solely on the assumption that each organelle contains one or two genomes. Shoubridge and Wai (2007) suggested that unrecognized differences in the efficiency of mtDNA amplification associated with real-time polymerase chain reaction could contribute to significant discrepancies in genomic copy numbers. These factors may explain reported mtDNA copy numbers that differ between studies (and oocytes) by over an order of magnitude. The present findings indicate that current estimates of mitochondrial numbers in MII human oocytes in the mid-hundreds of thousands, to well over one million, may be suspect. However, it is apparent from transmission electron microscopic images of human GV-to-MII oocytes that differences in the apparent density of mitochondria do occur between oocytes from the same cohort (Makabe and Van Blerkom, 2006). Confirmation of electron microscopic findings with more stringently controlled molecular analysis will probably support the notion that some degree of heterogeneity in mtDNA copy and organelle numbers exists within cohorts of developing oocytes. If confirmed, it could suggest that rates of mitochondrial replication may differ substantially between oocytes growing in the same ovary, and raise the interesting question of whether local intra-ovarian or intrafollicular factors influence the regulation of mitochondrial replication during oogenesis.

The physiological significance of differences in the mitochondrial complement, if sizeable, could be associated with bioenergetic threshold effects on critical developmental processes such as rates of cell division. However, two essential questions remain to be answered: (i) what is a normal mitochondrial complement consistent with developmental competence for the oocyte and embryo; and (ii) what is the relationship between this number and the level of ATP production required to drive normal embryogenesis prior to the late onset of mitochondrial replication? These issues could be clinically relevant if mitochondrial proliferation (i.e. rate of replication) during oogenesis changes with advanced maternal age or in younger women is altered by adverse intrafollicular or ovarian conditions that contribute to infertility. For example, could ATP generation capacity below critical (threshold) levels be a factor in intrafollicular gamete degeneration (apoptotic and pathological

cell death)? The notion that numerical mitochondrial under-representation is a direct cause of developmental incompetence for the human oocyte is a controversial one that will be best answered when consensus values for a normal complement and net cytoplasmic ATP concentration are established and related to maturational status, fertilizability, and embryo performance *in vitro*.

Although fluorescence analysis of mitochondria can be highly informative, the accuracy of such quantitative measurements needs to be confirmed by light and transmission electron microscopy using morphometric algorithms. An understanding of the relationship between complement size and bioenergetic status could become especially relevant in the context of non-invasive assays designed to measure metabolic characteristics of cultured human oocytes and embryos. The general intent of these analyses is to define quantitative parameters that may be predictive of outcome and the values used to select specific oocytes for insemination and embryos for transfer. If the size of mitochondrial complement has a direct effect on metabolic parameters intended for selective purposes in clinical IVF, the numerical range of mitochondria that occur in normal appearing MII human oocytes needs to be determined.

Cytoplasmic remodelling and mitochondrial distribution

Developmental significance of mitochondrial malsegregation during cleavage

During early cleavage, disproportionate segregation (i.e. inheritance) of mitochondria occurs between blastomeres. This phenomenon was first described for the human by Van Blerkom *et al*. (2000) and later identified in cleavage stage pig embryos (Katayama *et al*. 2006; Shahinaz *et al*., 2006). In the normally developing cleavage stage human embryo, the segregation of mitochondria between daughter blastomeres was found to be largely equivalent. This conclusion was based on the relative intensity of mitochondrial fluorescence and similar ATP content of blastomeres measured in embryos between the 2–12-cell stages. Disproportionate segregation resulted in significantly reduced mitochondrial fluorescence and ATP content in affected blastomeres. For such cells in intact embryos (absent or minimal fragmentation), where only relative fluorescence intensity could be determined, cell division ceased, or lysis occurred where mitochondrial density indicated by fluorescence intensity was very low. In contrast, sibling blastomeres with normal or above normal intensity continued to divide. For the early human embryo, these findings provided the first direct evidence that the size of the mitochondrial complement in individual blastomeres can differ and be related to bioenergetic status and developmental competence. The cellular basis of symmetrical and disproportionate segregation is discussed below.

In somatic cells, the spatial distribution of mitochondria can change owing to passive relocation from normal intracellular circulation or by active translocation along microtubules where they are vectored to specific cytoplasmic locations. Vectored translocation is a dynamic process of redistribution that accommodates local changes in energy demands, or occurs in response to other intracytoplasmic conditions that may require alterations in density. As first described by Van Blerkom and Runner (1984), a pronounced cytoplasmic remodelling of mitochondria takes place between the germinal vesicle (GV) and metaphase I stages of mouse oocyte maturation, whereby mitochondria are translocated to a perinuclear location along elongated arrays of microtubules that radiate into the cytoplasm from perinuclear microtubular organizing centres (Van Blerkom, 1991). This active remodelling of the cytoplasm results in the formation of a dense perinuclear sphere of mitochondria that persists through the circular bivalent stage and remains detectable during MI, and to a lesser degree, at MII. These authors also reported that perinuclear mitochondrial aggregation recurred during the pronuclear and 2-cell stages and was likely to be cell cycle related. Similar stage-specific mitochondrial translocations have been observed during the in-vitro maturation of porcine (Sun *et al*., 2001) and bovine (Van Blerkom *et al*., 1990) oocytes. Because these studies involved meiotic maturation *in vitro*, the possibility exists that this morphodynamic behaviour is an artefact of in-vitro conditions. However, a similar pattern of mitochondrial redistribution has been reported for *in vivo* matured mouse oocytes retrieved from stimulated follicles at different stages of pre-ovulatory maturation (Tokura *et al*., 1993). Further studies in model systems such as the mouse using transgenic alterations that tag mitochondrial proteins with fluorescent reporters (Misgeld *et al*., 2007) will be important to demonstrate the extent to which stage-specific translocations are in fact a normal aspect of oogenesis and early embryogenesis *in vivo*.

Developmental significance of mitochondrial translocations

Current notions about the function of stage-specific mitochondria redistributions in the oocyte and early embryo have mostly conformed to an interpretation first advanced by Van Blerkom and Runner (1984), who suggested that they largely serve to provide locally elevated concentrations of ATP to support energy-requiring processes associated with nuclear events, such as spindle formation and chromosomal segregation during meiosis and mitosis. However, mitochondria in perinuclear aggregates are low polarized (Van Blerkom *et al*., 2002) and as such, may be less energetic with respect to ATP production than their higher polarized counterparts (see below). If less energetic, a higher density of organelles may be required to raise ambient ATP concentrations. However, it is currently unclear what threshold concentrations of ATP are required to promote normal meiotic and mitotic spindle formation, chromosomal segregation, biosynthetic activities and other cellular processes that produce a competent oocyte and embryo.

Van Blerkom *et al*. (1995a) reported that mouse oocytes matured to MII *in vitro* after treatment at the GV stage with metabolic inhibitors that reduced net cytoplasmic ATP content by approximately 50% of normal levels. In the same respect, Van Blerkom and Davis (2007) recently reported that sperm penetration, cortical granule exocytosis, pronuclear formation and the initial cleavage divisions in the mouse can occur at subnormal ATP concentrations. They suggested that changes in local requirements for ATP within different portions of the cytoplasm of the maturing oocyte and early blastomere may involve transient redistributions of mitochondria that

rebalance supply and demand; this notion was also proposed by Dumollard *et al*. (2003, 2007) with regard to concentrations of free calcium and mitochondrial ATP generation (see below). If changes in mitochondrial organization establish transient microzones of differential metabolism, the dynamics of focal bioenergetic regulation within the cytoplasm may be of greater significance for competence during early development than is the total cytoplasmic ATP content. This may also apply to selection for competence in clinical IVF in which metabolic measurements are based on averages for entire cells or embryos, as discussed below.

Microzonation and mitochondria

In somatic cells, local gradients of O_2, ATP, pH and ions can be created by mitochondrial aggregates and produce focal changes in cytoplasmic physiology that regulate proximal activities (Aw, 2000). For example, local domains or 'microzones' of differential mitochondrial metabolism can cause focal reductions in intracellular pH (pHi) that directly influence cell structure and function by regulating normal cytoplasmic activities and biochemical pathways that occur in proximity to the aggregates. Microzonation is defined as the transient formation and dissociation of mitochondrial clusters and corresponding microdomains of differential metabolism and pH. Aw (2000) proposed that microzonation is a normal dynamic process that 'functionally compartmentalizes' different cellular and biochemical activities within the cytoplasm, such as ATP-dependent enzyme systems (e.g. kinases and ATPase), without the need for membrane partitioning. Taken together, these findings indicate that transient and location-specific changes in mitochondrial density can be important and normal regulatory forces in cells.

Transmission electron microscopic images of mouse and human MII stage oocytes and early cleavage stage embryos indicate that mitochondria are not uniformly dispersed within the cytoplasm, but rather often occur in small metastable clusters (Zamboni, 1971; Van Blerkom and Motta, 1979; Van Blerkom, 2004b; Makabe and Van Blerkom, 2006). This conclusion is supported by studies of living oocytes stained with mitochondria-specific fluorescence probes (Van Blerkom and Runner, 1984; Tokura *et al*., 1993; Van Blerkom *et al*., 2002), such as JC-1 (**Figure 2D**), r123 (**Figure 2E**) and MitoTracker Orange (**Figure 2F**). The stage-specific remodelling of the oocyte and preimplantation embryo cytoplasm, during which non-clustered mitochondria undergo transient, cell-cycle-specific translocation to the perinuclear region, where they accumulate at high density, may have regulatory roles or influences on cytoplasmic activities that are similar to those described for differentiated somatic cells. Although speculative, it is an interesting possibility that microtubule-mediated mitochondria translocations create a level of functional compartmentalization in the oocyte and early embryo that locally regulates biochemical processes and pathways. Microzonation near the oolemma or in perinuclear regions may be particularly relevant in the oocyte and newly fertilized egg, as it could afford a level of autonomous regulation of developmental activities in the largest cells in the body. As discussed below, a similar regulatory role has been suggested for high-polarized mitochondria localized in a subplasmalemmal domain in the oocyte and early embryo.

Small clusters of mitochondria (10–20; Makabe and Van Blerkom, 2006) are a common feature of the pericortical cytoplasm of apparently normal MII human oocytes, (Van Blerkom *et al*., 2002). Mitochondrial clustering in this region may become particularly important during oocyte maturation and early embryonic development, in order to accommodate local increases in the demand for ATP-dependent enzymatic and other biochemical activities. Due to ATP hydrolysis, the pH in the cytoplasm surrounding aggregates of fully developed mitochondria in cultured somatic cells is usually lower (~pH 6.9) than the average cytoplasmic pH (pHi, 7.2–7.3) and as described by Aw (2000), this reduction may serve to promote higher levels of oxidative phosphorylation by supporting a mitochondrial (transmembrane) pH gradient optimized for metabolite transport, accumulation and utilization. Although untested, if similar changes in energy demands are spatially distinct within the oocyte and early embryo, locally elevated concentrations of ATP hydrolysis (by ATPases) that generate H^+ could contribute to the creation or persistence of microdomains of elevated mitochondrial metabolism. This phenomenon may also account for the higher inner mitochondrial membrane potential ($\Delta\Psi m$) exhibited by subplasmalemmal mitochondria in the oocyte and early embryo (Van Blerkom *et al*., 2002, 2003) that in somatic cells, is associated with elevated levels of ATP generation (Gottlieb, 2001).

Although microtubular participation in vectored mitochondrial translocation has been described for the maturing oocyte and early embryo, it is unclear how stage-specific aggregation and dispersal are regulated. For example, why does the dense perinuclear accumulation of mitochondria that arises during the early stages of nuclear maturation largely disperse after the circular bivalent stage in the mouse (Van Blerkom and Runner, 1984)? One possibility under investigation is that the pHi around the internal and external aspects of this relatively dense perinuclear sphere is lowered by the generation of H^+ to levels that shift the dynamic equilibrium (i.e. stability) of microtubules to disassembly. Such a shift could promote the dispersal of mitochondria by diffusion or intracellular circulation. Mitochondrial dispersion may be associated with a focal return to normal pHi concentrations that promote tubulin assembly, microtubular growth, and formation of the meiotic spindles. A similar process may account for the cell cycle specific perinuclear mitochondrial accumulation and dispersal reported for the pronuclear and early cleavage stage mouse, hamster, porcine, bovine and human embryo (Tokura *et al*., 1993; Barnett *et al*., 1996, 1997; Sun *et al*., 2001; Van Blerkom *et al*., 1990, 2000).

The occurrence of abnormal patterns of mitochondrial aggregation in the mature human oocyte in the form of large clusters has been related to poor developmental competence and outcome after IVF (Van Blerkom, 1994; Meriano *et al*., 2001). It is unknown whether clustering of this magnitude creates abnormal intracellular conditions, such 'macrozones' of reduced pHi, which could perturb normal regulatory functions in which mitochondria may participate, but evidence from the mouse and hamster provides some possible clues. Muggleton-Harris and Brown (1988) reported that mitochondria in normally cleaving 2-cell embryos stained with r123 after recovery from the Fallopian tubes showed a relatively homogenous distribution, with perinuclear aggregation detected only during interphase; in contrast, cultured embryos exhibiting a '2-cell block' showed dense mitochondrial aggregates in the perinuclear and pericortical regions. Dispersal of the mitochondrial aggregates

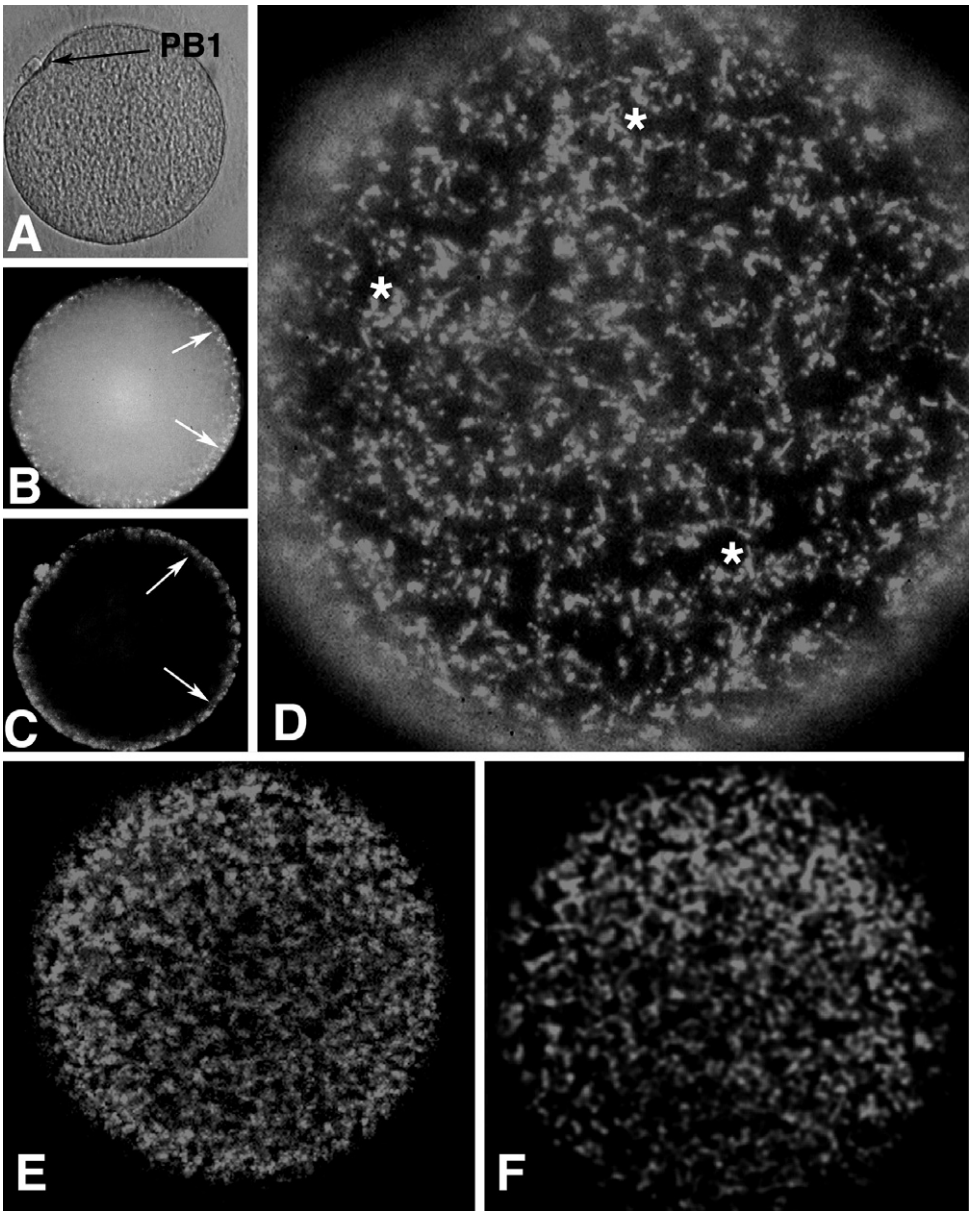

Figure 2. (**A–C**) are representative epifluorescent images of a human metaphase II (MII) stage oocyte (**A**) stained with JC-1. High polarized mitochondria are localized in the subplasmalemmal cytoplasm and fluoresce orange (arrows, **B**) in the fluorescein isothiocyanate channel and red in the rhodamine isothiocyanate channel (arrows, **C**). Low polarized mitochondria are located throughout the subcortical cytoplasm. (**D**) shows a high magnification view of small clusters (asterisks) of high-polarized mitochondria located immediately subjacent to the oolemma. (**E**) and (**F**) are scanning laser confocal images showing the clustered distribution of low-polarized mitochondria stained with rhodamine 123 (**E**) and MitoTracker Orange in the middle of a normal-appearing MII human oocyte. PB1 = first polar body. (Colour figure is available at http://www.rbmonline.com/Article/3087 but readers must be registered and subscribed to view.)

occurred when conditions that reversed the block were introduced. Similar findings for reversibly blocked 2-cell mouse embryos were reported by Bogoliubova *et al*. (1999) and by Barnett *et al*. (1997) for hamster embryos that were transiently blocked at the 2-cell stage by manipulation of the glucose and phosphate content of culture media. In each instance, the dispersal of mitochondria was temporally associated with the ability of the embryo to resume cleavage. Experience suggests that the pronounced redistributions of mitochondria observed in the maturing mouse oocyte are not as evident in the human during maturation from the GV to MII stages *in vitro*. However, dense perinuclear aggregation has been reported for GV stage human oocytes that fail to resume meiosis *in vitro*, and in oocytes that arrested maturation *in vivo* prior to MII (Makabe and Van Blerkom, 2006). In contrast, transient perinuclear mitochondrial aggregation is a normal aspect of early embryonic development in the mouse, hamster, cow, pig and human, as noted above. In these species, stage-specific spatial redistributions of mitochondria appear to be mediated by microtubules that vector mitochondria to the nuclear region (Van Blerkom, 1991; Sun *et al*., 2001).

Differences in the spatial distribution of peripronuclear microtubular arrays have been associated with the relative equivalence of mitochondrial segregation (i.e. inheritance) between blastomeres in the early cleavage stage human embryo (Van Blerkom *et al*., 2000). At the pronuclear stage, a largely spherical peripronuclear distribution of mitochondria leads to comparatively equivalent segregation between blastomeres, while disproportionate mitochondrial inheritance is associated with an ellipsoidal organization in which mitochondrial density around opposed pronuclei is non-uniform. These authors reported that the symmetry of mitochondrial accumulation around opposed pronuclei was related to a corresponding symmetry of microtubular arrays that projected radially into the cytoplasm from perinuclear foci. Unequal mitochondrial inheritance between daughter blastomeres was related to how the meridional plane of the first cleavage division partitioned the embryo with respect to mitochondrial rich and deficient regions. A similar phenomenon was observed at the 2- and 4-cell stages. The consequence of a deficient mitochondrial inheritance was shown by Van Blerkom *et al*. (2000) to be a corresponding reduction in net ATP content that depending upon level resulted in arrested cell division or eventual blastomere lysis, as noted above. A similar correspondence between the spatial distribution of cytoplasmic microtubules in pronuclear eggs and mitochondrial inheritance during early cleavage was reported for the pig (Katayama *et al*., 2006), where different patterns of microtubular and mitochondrial distribution were associated with the normality embryogenesis and the ability of differentially affected embryos to progress through the preimplantation stages.

Transient mitochondrial redistributions are a common strategy used by somatic cells to meet local changes in the demand for ATP. Descriptions of high mitochondrial densities at perinuclear regions in normally developing oocytes and early embryos would be consistent with such a role if the corresponding cellular activities have an elevated demand for ATP and the focal zones are found to be high ATP containing. As noted above, mitochondrial clustering can be associated with a decline in pHi at the periphery of the aggregates to levels that can locally optimize conditions for ATP generation. Whether a similar physiological response occurs in the oocyte and cells of the preimplantation embryo, where perinuclear mitochondrial aggregation appears to be a normal characteristic of their cell cycle, remains to be determined. However, mitochondrial microzonation could be involved in the differential regulation of levels of ATP generation or influence proximal biochemical activities that are pH dependent. The association between competence, cytoplasmic microtubules, and mitochondrial translocations/redistributions appears to be a developmentally significant one that warrants continued study. For example, an intracytoplasmic oxygen gradient may arise if a significant proportion of the oxygen that diffuses into the oocyte is consumed by mitochondria located in subplasmalemmal and pericortical regions. Mitochondria in the interior of the oocyte could experience a comparatively lower ambient oxygen concentration, and similar to metabolic optimization clustering affords in somatic cells (Aw, 2000), mitochondrial translocation may create local conditions that enhance their activity in a comparatively hypoxic milieu.

Mitochondrial polarity (ΔΨm) and microzonation

Mitochondria in mouse and human oocytes and early embryos can be distinguished as two spatially distinct populations by virtue of their level of polarization – the magnitude of the electrical potential across the inner mitochondrial membrane, commonly termed ΔΨm (Van Blerkom *et al*., 2002, 2003, 2006). Differences in transmembrane potential can be detected in living mitochondria with potentiometric fluorescence reporter dyes, and at present, the carbocyanine dye JC-1 remains one of the most specific and informative for this purpose (Reers *et al*., 1995; Salvioli *et al*., 1997). Unlike other fluorescent probes that are responsive to ΔΨm, such as r123 (**Figure 3A**), 10-nonyl acridine orange or MitoTracker Green, where changes in the intensity of monochromic fluorescence are potential related, the fluorescent emission wavelength of JC-1 shifts from green to red with increasing membrane potential (Reers *et al*. 1991). This change results from the potential-driven multimerization of the green-fluorescent JC-1 monomer to multimers, or J-aggregates, which shifts the fluorescent emission to longer wavelengths. For studies of living oocytes and preimplantation stage embryos, JC-1 has been the potentiometric probe of choice because its unique fluorescent characteristics clearly distinguishes cytoplasmic regions containing high- or low-polarized mitochondria. Confirmation of the mitochondrial specificity of JC-1 in general, and its ability to distinguish between high- and low-polarized mitochondria in particular, have been tested in oocytes, preimplantation stage embryos and cultured cells with metabolic inhibitors that differentially effect the magnitude of ΔΨm (Van Blerkom *et al*., 2002, 2003).

An example of high-polarized, J-aggregate positive mitochondria in a cultured mouse fibroblast stained with JC-1 and examined in the RITC channel is show in **Figure 3A**. When examined in the FITC channel, high-polarized mitochondria fluorescence orange-red (Van Blerkom *et al*., 2006; **Figure 3B**). While the fluorescent probes noted above are potential sensitive, subtle changes within subsets of mitochondria in the same cell can be difficult to detect or quantify if based solely on the relative intensity of a monochromatic fluorescent signal (Reers *et al*., 1991). By contrast, differences in fluorescence

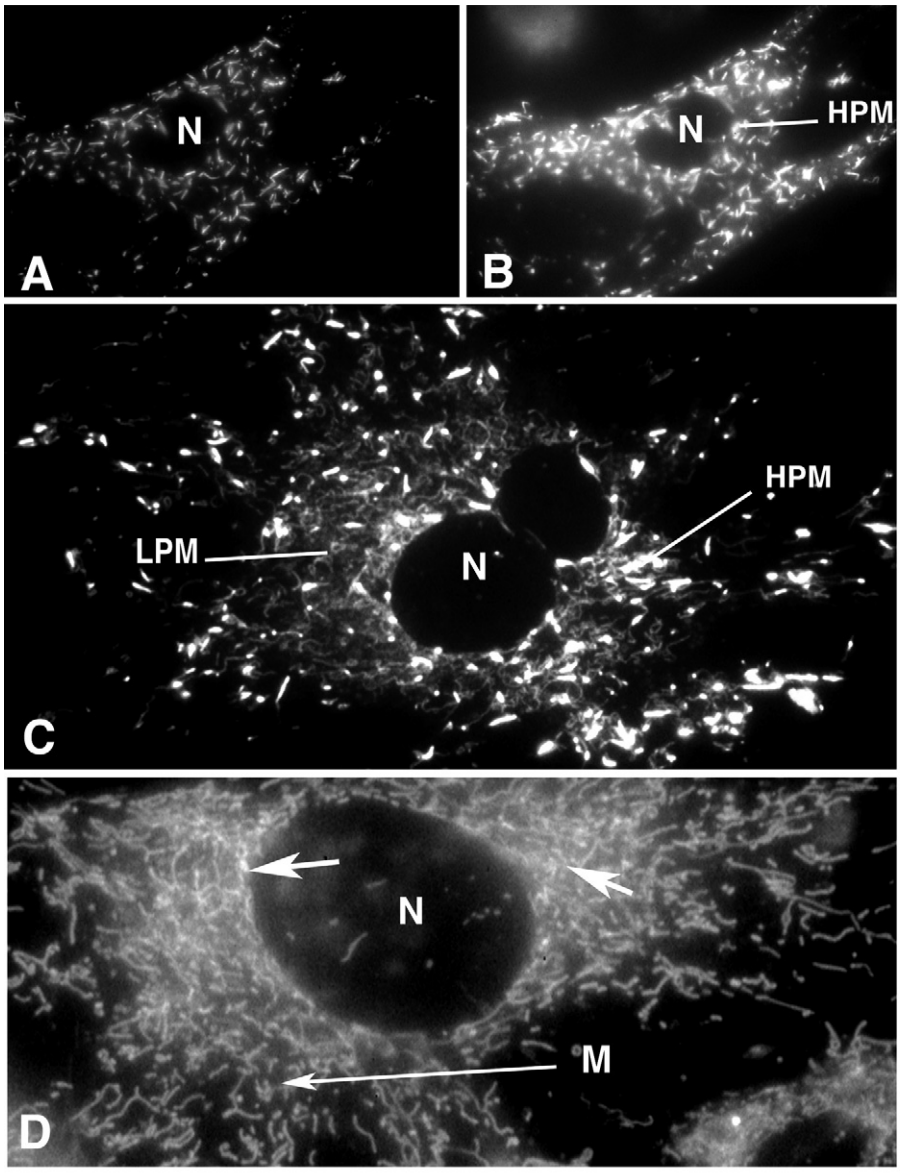

Figure 3. (**A**) and (**B**) show high-polarized JC-1, J-aggregate positive mitochondria in a mouse fibroblast viewed in the rhodamine isothiocyanate (**A**) and fluorescein isothiocyanate channels (**B**). At higher magnification, high- (HPM) and low-polarized (LPM) are evident in the same cell after JC-1 staining (**C**). (**D**) is an epifluorescent image of a mouse fibroblast stained with rhodamine 123 showing the distribution of elongated mitochondria (M) throughout the cytoplasm. Foci of higher fluorescent intensity may be indicative of mitochondria with a higher inner membrane potential (arrows). *N* = nucleus. (Colour figure is available at http://www.rbmonline.com/Article/3087 but readers must be registered and subscribed to view.)

emissions detected with JC-1 facilitate the identification of differential ΔΨm at the cytoplasmic level, and enable the detection of regional differences in ΔΨm within individual mitochondria (Smiley *et al*., 1991). For example, **Figure 3C** shows both high- (HPM, orange-red) and low-polarized (LPM, green fluorescent) mitochondria in the same mouse fibroblast. Upon close inspection of this cell, spots of punctate orange fluorescence probably indicate regions of high polarity within individual mitochondria that, as demonstrated by r123 staining in **Figure 3D**, are highly elongated elements (M). Differences in the magnitude of ΔΨm are less apparent with r123, although foci of higher fluorescent intensity are detectable with this probe (arrows, **Figure 3D**).

Mitochondria in normal cells usually maintain a relatively high polarity in order to establish a transmembrane potential sufficient to drive ATP synthesis by oxidative phosphorylation (Gottlieb, 2001; Ly *et al*., 2003). Typically, the level of this transmembrane potential is consistent with the multimerization of JC-1 into J-aggregates. High ΔΨm is achieved by retaining the permeability transition pore complex in the inner mitochondrial membrane (PTP; also termed the mitochondrial megachannel) in a closed state (Gottlieb, 2001). The physiological significance of ΔΨm and mitochondrial activity has been related to: (i) levels of ATP generation by oxidative phosphorylation, as noted above; (ii) participation in calcium sequestration and release (calcium homeostasis); (iii) rates of protein uptake by and transport within mitochondria; (iv) maintenance of organelle volume homeostasis; and (v) levels of participation in normal intracellular signal transduction pathways (reviewed by Van Blerkom and Davis, 2007). A sudden loss of ΔΨm, when associated with a concurrent release of calcium into the cytosol, has been considered one of the first detectable events in the initiation of the apoptotic cascade, although this may not be a universal characteristic of this cell death pathway (Halestrap, 2006).

Fluctuations in ΔΨm, from high to low and *vice versa*, are normal features of mitochondrial activity resulting from the opening and closing of the PTP. Oscillations in ΔΨm can be influenced or mediated by physiological changes within the cytoplasm, and by external factors such as the extent and nature of intercellular contact. The influence of intercellular contact has been reported to be particularly evident at the margins of cultured cells (Diaz *et al*., 1999) and between blastomeres of early cleavage stage embryos (Van Blerkom *et al*., 2002), where contact was associated with low ΔΨm in mitochondria located immediately beneath the plasma membrane, with a shift to high ΔΨm when contact was terminated. Differences in Ψm detected within the cells of the mouse and human blastocyst, and in stem cell outgrowths derived from the inner cell mass (see below), may be mediated by the type and extent of intercellular contact and communication (Van Blerkom *et al*., 2006). These authors reported that in intact blastocysts, mitochondria in the inner cell mass were low polarized while those in mural trophectoderm were high polarized. The occurrence of high-polarized mitochondria in the polar trophectoderm overlying the inner cell mass was scant. It was suggested that regional and cell-type specific differences in ΔΨm within the blastocyst may reflect corresponding differences in mitochondrial activity that may influence cell function and differentiative behaviour. For the oocyte and early preimplantation embryo, metabolic abnormalities, chromosomal segregation defects (Wilding *et al*., 2001, 2002, 2004), and incipient cell death (Acton *et al*., 2004) have been related to occurrence and cytoplasmic distribution of high- and low-polarized mitochondria.

J-aggregate positive mitochondria are clearly detectable in cultured somatic cells because they are elongated organelles that are usually several microns in length. In contrast, the largely spherical mitochondria in human and mouse oocytes and early blastomeres are between 0.5 and 0.8 μ in diameter (Motta *et al*., 2000), which places them at the limits of resolution obtainable with conventional light microscopic optics. The large size and cytoplasmic density of the oocyte and early embryo also confound detailed study of these organelles in the living state. Consequently, investigations of mitochondrial polarity at these stages are largely examining their behaviour in groups that are sufficient, in aggregate, to produce a collective fluorescent signal that has meaning in a spatial or developmental context. It has been reported that high-polarized mitochondria occur in normal mouse and human oocytes (human, **Figure 2A**) and early cleavage stage blastomeres in a narrow circumferential band just beneath the plasma membrane (**Figure 2B**, **C**). These mitochondria are clearly identifiable by virtue of their orange/red fluorescent emission after staining with JC-1 (arrows, **Figure 2B**, FITC channel; **Figure 2C**, RITC channel), which presumably reflects the occurrence of a transmembrane potential sufficient to promote multimerization into J-aggregates. By contrast, mitochondria throughout the interior of the cytoplasm fluoresce green with JC-1, which is indicative of a comparatively lower state of polarity (**Figure 2B**). The number of high-polarized mitochondria localized in the subplasmalemmal domain has been estimated to represent less than 5% of the total mitochondrial complement present in the MII oocyte (Van Blerkom and Davis, 2006).

As shown by high-resolution fluorescence microscopy (asterisks, **Figure 2D**), and supported by images obtained from serial thin sections examined by transmission electron microscopy (Van Blerkom *et al*., 2002), high-polarized, J-aggregate positive mitochondria occur in the subplasmalemmal cytoplasm in closely spaced clusters. Therefore, at lower magnifications, red fluorescent subplasmalemmal mitochondria are evident owing to their relatively high density within a comparatively narrow spatial domain. The detection of this domain is less evident with other mitochondria-specific dyes that have potentiometric capability, such as r23 or MitoTracker Green and Orange. In these instances, the potential increase in fluorescence intensity between mitochondria in a low- or high-polarized state may be too low to be evident against a monochromic green (FITC channel) or red (RITC channel) background. For example, low-polarized mitochondria that occupy most of the cytoplasm of a MII human oocyte (**Figure 2B**) are clearly detectable after staining with r123 and MitoTracker Orange, as shown in **Figures 2E** and **F** respectively, which are 5 μm scanning laser confocal images taken at the approximate centre of normal appearing MII human oocytes. However, these probes provide no clear indication that high polarity exists in subplasmalemmal mitochondria.

The occurrence of high-polarized mitochondria within a discrete domain beneath the plasma membrane of mouse and human oocytes and blastomeres may be an example of microzonation reflecting differential mitochondrial activity. Current findings suggest that a 'microzone' of high-polarized mitochondria in the subplasmalemmal cytoplasm is: (i) spatially stable and inherited *in toto* between the oocyte and early cleavage stages; and (ii) not renewed with each cell division by the incorporation of formerly low polarized mitochondria (Van Blerkom and

Davis, 2006). These studies also indicated that the presence of this microzone was developmentally significant. For example, spontaneous fragmentation that produces numerous small fragments that decorate the exposed surface of a blastomere does not significantly reduce cell size or volume, but the corresponding J-aggregate positive mitochondria are eliminated from the cell during the formation of these structures (Van Blerkom and Davis, 2006). For some blastomeres, virtually all of the high-polarized mitochondria were observed in a single, focal cluster of small fragments, suggesting that the entire domain could be expelled from a blastomere, even if the degree of fragment formation is considered minor. Arrested cell division for the affected cell was the only microscopically detectable consequence associated with the loss of subplasmalemmal high-polarized mitochondria. A similar developmental effect was observed for intact cleavage stage human embryos, where the inheritance of the subplasmalemmal domain between blastomeres was asymmetrical. In these cases, most of the high-polarized mitochondria were spontaneously partitioned into one blastomere at the first cell division. The affected cell remained undivided while those with a domain developed progressively. Thus, elimination from the blastomere by minor fragment formation or disproportionate inheritance during cleavage appear to be naturally occurring perturbations in the distribution of high-polarized mitochondria with developmental consequences confined to a particular blastomere(s) and related to the degree of loss. The failure to restore or re-establish this domain in the affected cell(s) supports the notion that the corresponding mitochondria are numerically finite and spatially stable (Van Blerkom and Davis, 2006).

Differential ΔΨm and compartmentalization of mitochondrial function

Spatial stability and inheritability of a subplasmalemmal domain of high-polarized mitochondria are not direct evidence for specialized functions or activities that may be compartmentalized within the cytoplasm; however, they do suggest the possibility that activity levels may differ from their lower polarized counterparts. Dumollard *et al.* (2003) reported that levels of mitochondrial ATP generation in the mouse oocyte are up- or down-regulated by coincident changes in ambient concentrations of free calcium that in turn, are largely controlled by proximal elements of the smooth-surfaced endoplasmic reticulum (SER) that store and release this cation. They proposed that ionic cross talk between mitochondria and SER could rapidly adjust ATP production to changing focal energy demands without the necessity of a global up-regulation of mitochondrial metabolic activity, which could have negative consequences on competence if corresponding cytoplasmic levels of reactive oxygen species generated by mitochondria also increased. Micro-regulation of ATP production has been observed in cultured cells (Aw, 2000), and would seem to be especially relevant in the oocyte because it affords the largest cell in the body a similar measure of local control. Local control would also have the benefit of optimizing the supply of ATP to meet differential focal demands, as ATP is normally consumed within a few microns of the site of generation and has a relatively short half-life. The rapid turnover of ATP in the mature mouse oocyte is indicated by a comparatively precipitous decline in net cytoplasmic content measured in the presence of metabolic inhibitors (Van Blerkom *et al.*, 2003). Thus, maintenance of a normal average cytoplasmic ATP content for the MII mouse oocyte of ~700 fmol (Van Blerkom *et al.*, 1995a) would seem to involve ATP production by the entire mitochondrial complement, of which the vast majority are characterized as low polarized on the basis of their JC-1 emission wavelength.

Van Blerkom and Davis (2007) suggested that the contribution of high-polarized mitochondria to the net cytoplasmic ATP content of the oocyte and early embryo may be relatively marginal, but could be highly relevant in the context of their spatial localization in the subplasmalemmal cytoplasm. In this regard, the capacity of the more numerous, but lower polarized mitochondria to up-regulate ATP production in response to local ionic changes (e.g. calcium) may be comparatively lower than for high-polarized forms. A higher density of organelles may be required to affect a significant increase in ambient ATP concentrations and transient perinuclear mitochondrial aggregation in the oocyte and early embryo may be an adaptive reaction to a reduced: (i) responsiveness to metabolic regulatory signals; or (ii) capacity to generate energy. In contrast, high-polarized mitochondria that stably surround aggregates of SER cisternae in the pericortical and subplasmalemmal cytoplasm may be more responsive to small changes in ambient calcium. Their unique spatial co-localization with the SER, as well as proximity to other sources of calcium in the subplasmalemmal cytoplasm (Sousa *et al.*, 1996,1997), may permit a rapid, focal up-regulation of ATP production in response to coincident changes in the levels of this cation that occur at fertilization.

Could compartmentalization of high-polarized mitochondria within the subplasmalemmal cytoplasm facilitate fertilization?

As suggested above, the occurrence of high-polarized mitochondria stably confined to a narrow domain (~5 μ) beneath the plasma membrane could facilitate the fertilization process by enabling the up-regulation of ATP generation in response to an acute increase in ambient calcium. A focal increase in ATP availability could be necessary to: (i) drive morphodynamic activities associated with penetration and sperm nuclear migration into the ooplasm (Van Blerkom *et al.*, 1995b); (ii) remodel the oolemma and subplasmalemmal cytoskeletal elements after penetration; or (iii) provide levels of phosphate required by kinases whose activity may be co-ordinately up-regulated during fertilization. Mitochondria are also involved in the regulation of cytoplasmic calcium homeostasis owing to their ability to sequester and release this cation in response to various stimuli that include changes in ambient calcium levels (calcium-induced, calcium release pathway) and electrical fluxes (Ichas *et al.*, 1997). At fertilization, transient increases in free calcium originate from the SER, specialized calcium storage granules in the subplasmalemmal cytoplasm (Sousa and Tesarik, 1996, 1997), and possibly, from other mitochondria, through the mitochondrial, calcium-induced, calcium-release signalling pathway (mCICR). The level of mitochondrial participation in the regulation of calcium is a function of ΔΨm (Harris, 1979; Loew *et al.*, 1994) suggesting that the high polarized forms may be more active in their response to changes in ambient calcium than those showing a lower transmembrane potential after JC-1 staining.

It has recently been reported that sperm penetration and cortical granule exocytosis in the mouse was restricted to regions subplasmalemmal cytoplasm were mitochondria were high polarized (Van Blerkom and Davis, 2007). Experimentally induced reductions in ΔΨm reversibly blocked these processes, as shown by penetration and cortical granule exocytosis following the restoration of normal polarity. Of particular interest was the finding that the net average cytoplasmic ATP content was well below normal during penetration and cortical granule exocytosis, despite the reappearance of a high polarity. These results suggest that local levels of mitochondrial activity, including ATP synthesis and participation in calcium homeostasis in the subplasmalemmal cytoplasm, may be more critical to the success of the earliest stages of fertilization than overall mitochondrial activity within the cytoplasm. This interpretation may explain penetration failure by conventional IVF in human oocytes with a scant, discontinuous, or absent domain of subplasmalemmal high-polarized mitochondria (Jones *et al*., 2004).

Here, it is suggested that the unique distribution of high-polarized mitochondria in the oocyte and newly fertilized embryo could represent a type of functional compartmentalization that may be uniquely adapted to meet the specific spatial requirements of the oocyte and early embryo for ATP. Compartmentalization of mitochondrial activity may also extend to their participation in the regulation of free calcium concentrations. Fully developed mitochondria are excitable organelles that can release calcium in response to external ionic and electrical signals (including calcium; Loew *et al*., 1994; Ichas *et al*., 1999) and it has been proposed that during fertilization, the transient release of calcium from high-polarized mitochondria may occur in response to electrical and ionic fluxes associated with sperm penetration (Van Blerkom *et al*., 2003). If confirmed, release of calcium from this domain might be expected to require a transient depolarization or reduction in ΔΨm. Indeed, fully developed mitochondria undergo frequent and periodic oscillations in ΔΨm associated with the release or uptake of calcium in response to cytoplasmic (Harris, 1979; Loew *et al*., 1994) and extracellular signals (Diaz *et al*., 1999). The possibility that normal sperm penetration and cortical granule exocytosis require subplasmalemmal mitochondria to be high polarized and capable of undergoing transient reductions in ΔΨm was investigated in zona-free mouse oocytes (Van Blerkom and Davis, 2007) exposed to the following inhibitors: (i) the proton ionophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), which dissipates the pH gradient that maintains ΔΨ; and (ii) bongkrekic acid, which prevents opening of the megachannel and keeps mitochondria in a persistently high-polarized state (Stoner and Sirak, 1973). Under these conditions, the oolemma was densely decorated with firmly attached spermatozoa, but penetration and cortical granule exocytosis did not occur until the inhibitory effects of FCCP and bongkrekic acid were reversed, and subplasmalemmal mitochondria returned to high polarity in the case of FCCP, or were capable of reducing polarity after the elimination of bongkrekic acid.

Mitochondria and signal transduction

The above results indicate that the morphologically underdeveloped mitochondria of MII mouse and human oocytes have physiological properties and responses to inhibitors that are similar to their fully developed somatic cell counterparts. The possibility that they may also participate in intracellular signalling comes from recent findings showing that reactive oxygen species (ROS) generated by active mitochondria, such as superoxide, are involved in multiple cell signalling pathways (termed redox signalling pathways) that control rates of cell proliferation and other cellular activities, including molecular responses to hypoxia (reviewed by Bunn and Poynton, 1996; Bell *et al*., 2005). The cellular response to hypoxia is one of the normal redox-signalling pathways in which mitochondria participate as the oxygen sensing organelles of cells. The conversion of mitochondrial superoxide to peroxide by cytosolic superoxide dismutase is one of the first steps in the activation of the hypoxia inducible transcription factor pathway (HIF; Bruick, 2003; Bell *et al*., 2005; Gutierrez *et al*., 2006). When activated, the HIF pathway up-regulates the expression of at least 28 genes, including proteins directly involved in angiogenesis such as VEGF (vascular endothelial growth factor; Semenza, 1999).

Protein components of the HIF pathway (e.g. HIF-1α) have been identified by immunofluorescence in the oocyte and granulosa cells of human pre-ovulatory follicles at both the protein (Van Blerkom and Trout, 2007; Van Blerkom, unpublished) and mRNA concentrations (Van Blerkom, 2002). However, whether this pathway is functional in the oocyte, as it appears to be in the blastocyst stage embryo (Harvey *et al*., 2004), is unknown. Because of their close proximity to the oolemma and oxygen that diffuses into the oocyte, the extent to which subplasmalemmal mitochondria are responsive to changes in ambient oxygen and involved in redox signalling has been examined. In this regard, preliminary findings indicate that nitric oxide (NO) produced by the cumulus granulosa cells that surround the oocyte has a depressive effect on the magnitude of ΔΨ in subplasmalemmal mitochondria. If NO competes with oxygen by virtue of its ability to bind cytochrome C oxidase, the terminal acceptor in the electron transport chain, it may regulate mitochondrial participation in redox signalling pathways and levels of ATP generation in a manner similar to those reported for somatic cells (Erusalimsky and Moncada, 2007). At present, a reduction in the depressive effects of NO on ΔΨm appears to be associated with: (i) the degree of LH-induced expansion of the cumulus oophorus during the terminal stages of pre-ovulatory maturation; and (ii) a progressive shift to high polarity within the subplasmalemmal domain of the mouse oocyte after ovulation. If confirmed, such findings could support the notions of mitochondrial microzonation and functional compartmentalization discussed above.

Mitochondria as regulatory forces in the preimplantation stage embryo

The spatial distribution of high-polarized mitochondria in the early preimplantation stage embryo, and cell-type specific differences detected at the blastocyst stage, have been suggested to indicate differential regulatory roles for these organelles during early development (Van Blerkom *et al*., 2002; 2006). Diaz *et al*. (1999) reported that the polarity of mitochondria at the margins of cultured cells was influenced by the extent and type (gap junctions) of intercellular communication. ΔΨm was lower where cells were in contact and higher (J-aggregate

forming) at the free margins. A similar finding was reported for cleavage stage mouse embryos where the magnitude of ΔΨm, indicated by the fluorescence emission wavelength of JC-1 stained organelles, was associated with cell contact (Van Blerkom *et al*., 2002). The notion that mitochondrial polarity and related levels of activity are influenced by the nature and extent of intercellular contact and communication during early development was investigated in intact normal and delayed implanted mouse blastocysts, and trophectodermal and inner cell mass outgrowths (e.g. **Figure 4A**) derived from these embryos (Van Blerkom *et al*., 2006). In the intact blastocyst, ICM mitochondria were found to be low polarized while those in the mural trophectoderm were high polarized. Mitochondria in trophectodermal outgrowths were largely high polarized in cells that were loosely associated with their neighbours, and low polarized where the density of cells was markedly higher (asterisk, **Figure 4B**). The ΔΨm in ICM cells that proliferated *in vitro* was also related to the extent of intercellular contact, with high polarity occurring in low density regions (arrows, **Figure 4C**) and low polarity occurring were these stem cell-like cells were in higher density.

Whether cell-type specific differences ΔΨm within the intact blastocyst are: (i) related to the type and extent of intercellular contact and communication; (ii) can be related to differences in mitochondrial function or activity; and (iii) influence cell behaviour (e.g. rate of cell division) or development (e.g. gene expression) are under investigation. However, the up-

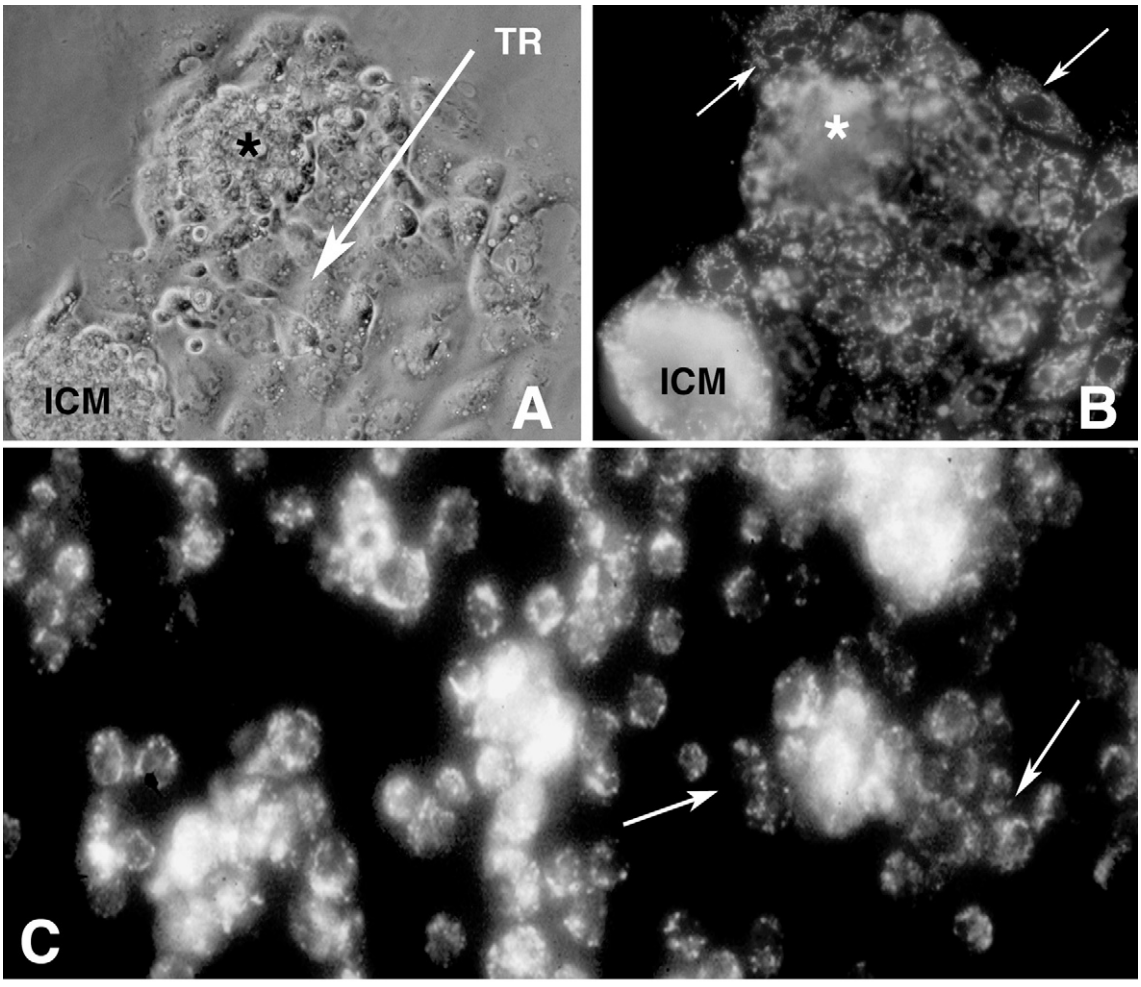

Figure 4. JC-1 staining of mouse blastocyst outgrowth (**A**) shows low-polarized mitochondria localized primarily to the inner cell mass (ICM) region, while bright orange fluorescent rods in the outgrowing trophectoderm (arrows, TR, **B**) demonstrates the presence of J-aggregates which are indicative of high-polarized mitochondria. Isolated explants of the mouse ICM proliferating into cells resembling stem cells are shown in (**C**) after staining with JC-1. The presence of high and low-polarized mitochondria (arrows) is related to the density of intercellular contact. Cells in relatively loose association are primarily J-aggregate positive (arrows), indicating high-polarity, while those in close association largely fluoresce green, indicating a lower polarity. The asterisks in **A** and **B** indicate areas of high density outgrowth for the trophectoderm, i.e. multilayered, where the corresponding mitochondria in individual cells could not be counted. The asterisk in **B** refers to a dense cluster of outgrowing ICM cells where the corresponding mitochondria were low polarized. This figure was adapted from a similar image presented in Van Blerkom *et al*. (2006). (Colour figure is available at http://www.rbmonline.com/Article/3087 but readers must be registered and subscribed to view.)

regulation of the synthesis and secretion of VEGF and leptin, two potent angiogenic growth factors (see review by Van Blerkom, 2002), of which the former is known to be regulated by the HIF pathway (Semenza, 1999), occurs in portions of the trophectoderm (Krussel *et al*., 2001; Cervero *et al*., 2005; Hwu *et al*., 2006). The potential role of high-polarized mitochondria as oxygen sensors, whose response to acute hypoxia in the peri-implantation blastocyst may be to facilitate the transcription of genes that can stimulate angiogenesis at site of implantation, warrants investigation. In the same context, whether low ΔΨm in the ICM can be related to the inability to respond to hypoxia, or to the expression patterns of specific genes that may influence the behaviour and function of cells within the ICM, could provide important clues regarding their unique ability to proliferate as 'undifferentiated' stem cells, as discussed below.

Mitochondria and stem cells

At present, the extent to which mitochondria influence the behaviour or developmental state of stem cells remains to be investigated. However, there are some early reports suggesting roles in cellular activity and function that are indicative of a regulatory capacity. Chung *et al*. (2007) demonstrated that mitochondrial oxidative phosphorylation was required for cardiac differentiation of stem cells. Disruption of mitochondrial oxidative phosphorylation prevented stem cell differentiation into energetically competent, contracting cardiomyocytes, leading these authors to conclude that mitochondria-dependent energetic circuits are critical regulators of de-novo cardiogenesis. Lonergan *et al*. (2007) suggested that the ability of embryonic stem cells to translocate mitochondria, especially to the perinuclear region, indicates normal differentiative capacity, while those without this capacity may be approaching or entering developmental senescence. The inability to spatially remodel mitochondria in these cells may also be associated with reduced ATP generative capacity.

While at an early stage of investigation, these findings offer promising leads related to mitochondrial activities that may participate in the regulation of stem cell homeostasis or differentiation. As noted above, highly proliferative stem cell-like outgrowths of mouse ICM show different levels of mitochondrial polarity that are related to the degree of intercellular contact. The mitochondria in cells within high-density portions of the outgrowths are primarily low polarized but as the density and extent of intercellular contact lessens, the cells contain both low and high-polarized forms (e.g. **Figure 4C**). Mitochondria in isolated cells and cells that had few intercellular contacts are primarily high polarized. Similar to other cultured cells (Diaz *et al*., 1999), the magnitude of mitochondrial polarization observed in ICM outgrowths was dynamic and changed in relation to cell density. Preliminary observations of human embryonic stem cell cultures suggest that spontaneous differentiation into cells with phenotypic characteristics of neurons, or contractile activity consistent with myocytes, occurred in regions of relatively low cell density (Van Blerkom, unpublished). If this phenomenon is confirmed, and stem cells are shown to shift ΔΨm to a higher state prior to induced or spontaneous differentiation, it could indicate that ΔΨm may be a factor in the pathway leading to differentiation. Likewise, it remains to be seen whether keeping stem cell mitochondria in a constitutively low polarized condition could maintain phenotypic homeostasis, i.e. in an undifferentiated state.

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