

## Focus on ART

**Mitochondria in human oogenesis and preimplantation embryogenesis: engines of metabolism, ionic regulation and developmental competence**

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**Abstract**

**Mitochondria are the most abundant organelles in the mammalian oocyte and early embryo. While their role in ATP production has long been known, only recently has their contribution to oocyte and embryo competence been investigated in the human. This review considers whether such factors as mitochondrial complement size, mitochondrial DNA copy numbers and defects, levels of respiration, and stage-specific spatial distribution, influence the developmental normality and viability of human oocytes and preimplantation-stage embryos. The finding that mitochondrial polarity can differ within and between oocytes and embryos and that these organelles may participate in the regulation of intracellular  $Ca^{2+}$  homeostasis are discussed in the context of how focal domains of differential respiration and intracellular-free  $Ca^{2+}$  regulation may arise in early development and what functional implications this may have for preimplantation embryogenesis and developmental competence after implantation.**

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**Introduction**

In clinical *in vitro* fertilization (IVF), current investigational efforts are directed to understanding why a high proportion of oocytes result in developmentally incompetent embryos. Studies of embryo performance during the preimplantation stages show high frequencies of abnormal development and early demise, with further losses seen after uterine transfer as measured by outcome per embryo. There is a growing consensus of opinion that much of this embryonic wastage originates from oocyte chromosomal and subtle cytoplasmic defects whose adverse developmental consequences are not expressed until well after fertilization. The notion that mitochondrial dysfunctions or abnormalities in the oocyte may be a critical determinant of human embryo developmental competence has gained currency from recent studies in which defects at the structural and mitochondrial DNA (mtDNA) levels have been identified. Likewise, the number of mtDNA copies has been shown to differ between human oocytes (in the same cohort) by over an order of magnitude, and for the early embryonic stages developmentally significant differences in mitochondrial numbers between blastomeres can result from disproportionate inheritance during the cleavage

stages. Structural, spatial and genetic dysfunctions that affect the capacity of mitochondria to produce ATP by oxidative phosphorylation could have pleiotropic effects on early human development that, as described below, may include the normality of spindle organization and chromosomal segregation, timing of the cell cycle, and morphodynamic processes such as compaction, cavitation and blastocyst hatching. Mitochondrial dysfunctions that may initiate or contribute to the activation of apoptosis have also been suggested to be a proximal cause of human oocyte wastage and early embryo demise. Because the normalcy of critical nuclear and cytoplasmic activities may be determined by mitochondria, it is not surprising that their role in early human development as related to outcome in IVF treatments has become a subject of clinical and basic research interest (Christodoulou 2000, Howell *et al.* 2000, Jansen 2000*a,b*, Cummins 2002, 2004, Brenner 2004, Chinnery 2004, Eichenlaub-Ritter *et al.* 2004). From a basic science viewpoint, the extent to which mitochondria contribute to or actually determine oocyte and embryo competence must be better understood if proactive clinical therapies such as oocyte mitochondrial donation/replacement (Cohen *et al.* 1997) are to

be considered acceptable treatments for certain types of infertility in which a mitochondrial association has been clearly identified (Brenner 2004, St John *et al.* 2004).

## Mitochondria as genetic forces in early human development

### **Mitochondrial transmission across generations is uniparental**

It has long been known that the human mitochondrial genome is 16 560 kb of double stranded DNA that encodes 13 proteins in the respiratory chain, and 22 unique transfer RNAs and 2 ribosomal RNAs (Clayton 2000, Trounce 2000). Although not without some controversy (Cummins 2004), mitochondria are inherited through the maternal lineage with paternal mitochondria arriving at fertilization targeted for destruction primarily by ubiquitin-dependent proteolysis (Schwartz & Vissing 2002, 2003, Johns 2003, Sutovsky 2004). The maternal transmission of mitochondria between generations is the genetic basis for the inheritance of certain debilitating or ultimately lethal metabolic disorders in the human (Chinnery & Turnbull 1999, Christodoulou 2000, Leonard & Schapira 2000, Chinnery 2004), and heterogeneity in mtDNA is used in forensic medicine to assist in the identification of individuals and in anthropology to trace the origins and geographical dispersal of populations.

All of the mitochondria in the mature oocyte (metaphase II, MII stage) arise from the clonal expansion of an extremely small number of organelles present in each primordial germ cell that after colonization of the forming ovary, expands by mitosis to form numerous progeny that can be identified as 'nests' of primordial oocytes (Makabe & Van Blerkom 2004). By some estimates (Jansen 2000b), <10 mitochondria may be the progenitors of the tens to hundreds of thousands of organelles present in the human oocyte at fertilization. That mitochondrial transmission across generations is uniparental and that procreation requires their significant numerical expansion presents some potentially unique biological challenges. For example, it has been argued that this uniparental replication might be expected to follow the ultimately lethal consequences of Muller's ratchet hypothesis (Muller 1964, and concisely discussed by Jansen 2000a), whereby species extinction is an inevitable consequence of asexual reproduction owing to the accumulation of deleterious mutations by random genetic drift. Indeed, because of maternal inheritance and high mutation frequency, the human mtDNA should be prone to Muller's ratchet. Counteracting this natural entropic tendency to mutational degradation and extinction is the severe reduction in maternal germline mtDNA copy number in the primordial germ cell, a phenomenon generally known as the 'mitochondrial bottleneck' (Bergstrom & Pritchard 1998). As a result of the reduction in progenitor organelles, the accumulation of mtDNA mutations is diminished with

certain mutations, such as those that could adversely affect replication or metabolic capacity being eliminated by natural selection or 'dying out' through oogenesis (Hoekstra 2000). However, others have argued that there is not a single mitochondrial bottleneck at the outset of oogenesis, but rather an active selection process that occurs throughout oogenesis and early embryogenesis and involves multiple stage-specific bottlenecks and differential patterns of mitochondrial segregation (Howell *et al.* 2000). The occurrence of individuals with maternally inherited metabolic diseases (OXPHOS diseases) resulting from known mtDNA mutations demonstrates that (a) the bottleneck is not an effective natural means of eliminating oocytes carrying potentially lethal mitochondrial genetic defects and (b) that developmental competence does not require that the mitochondrial complement be genetically normal or even capable of normal levels of respiration (oxidative phosphorylation).

### **Mitochondrial transmission between generations is not necessarily monogenomic: homoplasmy and heteroplasmy**

As maternally inherited organelles, the mtDNA genotype(s) in the embryo is largely determined by what existed in the few mitochondria contained within the primordial germ cell and resulting primary oocyte. If the expansion of the mitochondrial population during oogenesis involves an identical genome, the MII oocyte and resulting embryo would be expected to be homoplasmic. Heteroplasmy occurs when two or more different mitochondrial genotypes occur in the same cell, whether a primordial germ cell, oogonia, oocyte or blastomere. Heteroplasmy *per se* does not imply an adverse condition if the mtDNA mutations are benign with respect to function, but can become problematic and have cytopathological consequences if the mutant form(s) has reduced respiratory capacity and occurs at toxic levels (mutant load). While heteroplasmy as a factor in human infertility or early embryo demise is a current issue in reproductive medicine (Brenner 2004, Cummins 2002, 2004), it is necessary to note that adverse developmental consequences of mtDNA mutations become relevant only when they affect mitochondrial activities (e.g. replication and respiration) at levels that are inconsistent with cell survival or normal function (Christodoulou 2000). Indeed, the threshold levels at which OXPHOS diseases become clinically significant are usually quite high (Chinnery & Turnbull 1999, Leonard & Schapira 2000, Trounce 2000).

Heteroplasmy, detected by highly sensitive analysis of mtDNA in the oocytes of certain women, has been related to infertility by virtue of the occurrence of certain mtDNA genotypes such as the 'common deletion' mtDNA 4977, which in one report was proposed to increase with maternal age and negatively affect competence in women >40 years old (Keefe *et al.* 1995). However, in a recent review of the relationship between competence and the

various types of mtDNA mutations detected in human oocytes obtained by ovarian hyperstimulation for IVF, Brenner (2004) found no compelling evidence to suggest that any occurred at loads which could compromise outcome. This is not to say that mtDNA is unimportant in the establishment of competence or as an etiology of infertility but rather, that additional investigation is needed to validate such interpretations, especially if proactive therapies (e.g. cytoplasmic transfer) are contemplated in an IVF treatment cycle. In this respect, Brenner (2004) described some promising leads related to point mutations in the control region of the mitochondrial genome responsible for replication. These mutations seemed to increase in frequency in the oocytes of certain women, especially those of advanced reproductive age and, if confirmed, could be an important and unrecognized factor in outcome because mitochondrial replication does not begin until after implantation. Therefore, replication defects would not be expected to compromise preimplantation embryogenesis, but depending upon mutant load, could manifest as post-implantation demises described as chemical (transient elevation of human chorionic gonadotropin levels) or anembryonic pregnancies (no fetal pole detected by ultrasonography).

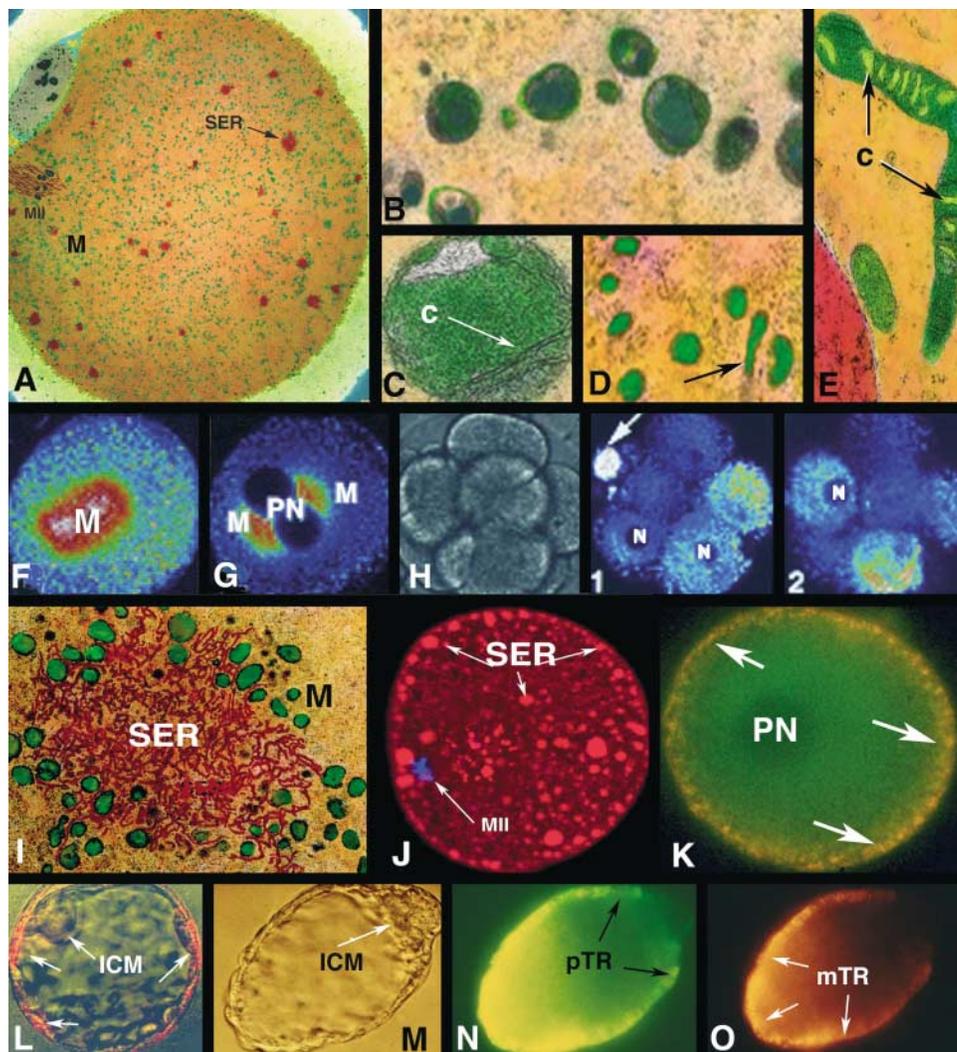
Experimental approaches to the question of whether specific mtDNA defects in human oocytes cause postimplantation demise require some formidable challenges to be overcome. For example, are unused blastocysts from IVF programs, even if available, suitable material to screen for mtDNA defects and should the trophoblast and inner cell mass (ICM) be analyzed separately? For patients with a history of repeated chemical or anembryonic pregnancies, is it ethical to use IVF protocols to generate multiple blastocysts such that some could be transferred or cryopreserved while others are used for mtDNA analysis? If specific mtDNA mutations that affect competence during the pre- and postimplantation stages are clearly identified, screening and ethical issues become moot as it would be expected that the same protocols used for embryo biopsy and preimplantation genetic diagnosis as applied to chromosomes and nuclear DNA (Verlinsky & Kuliev 2000) would be applicable to mtDNA. However, an assessment of whether a particular mtDNA mutation could influence competence and outcome requires the ability to accurately quantify the mutant load. This is especially evident when it is considered that some mtDNA-related OXPHOS diseases clinically manifest only when a genetic defect occurs at high load (Christodoulou 2000), while for others the severity of the clinical symptoms is proportional to the mutant load (Dahl *et al.* 2000). Whether the finding that mtDNA copy numbers that can vary by over an order of magnitude between MII oocytes in the same cohort (see below) presents another challenge for the application of mitochondrial analysis in clinical IVF, remains to be determined.

## Mitochondria as metabolic forces in early development

### *Mitochondrial fine structure and metabolic activity*

It has long been known from transmission electron microscopy (TEM; Sotelo & Porter 1959, Baca & Zamboni 1967) that mitochondria in mammalian oocytes and early embryos have a unique fine structure in which a spherical profile, dense matrix and relatively few cristae are indicative of an undeveloped state (for reviews see Van Blerkom & Motta 1979, Makabe & Van Blerkom 2004). What makes recent TEM studies of human oocytes and embryos clinically relevant is the possibility that structural abnormalities detected in certain infertile women could be associated with mitochondrial dysfunctions that reduce their metabolic activity and may, therefore, be an important etiology of oocyte or embryo incompetence (Motta *et al.* 2000, for review). This may be especially relevant in women of advanced reproductive age as reported by Muller-Hocker *et al.* (1996).

Similar to other mammals (Van Blerkom & Motta 1979), mitochondria in fully-grown human oocytes are the most abundant organelles detected by electron microscopy (Fig. 1A) and occur as spherical/ovoid elements <0.5 µm in diameter (Dvorak *et al.* 1987). Typically, these mitochondria contain only a few short cristae that rarely penetrate an electron-dense matrix (Fig. 1B and C). This phenotype persists through the cleavage and late morulae stages of human embryogenesis *in vitro* before a gradual transition to an elongated form with a matrix of low-to-moderate electron density is observed (arrows, Fig. 1D and E). An increased number of lamellar cristae that completely traverse the inner mitochondrial matrix is generally characteristic of mitochondria actively engaged in ATP production by oxidative metabolism, and this profile represents the predominant form seen at the blastocyst stage in most mammals. For the human preimplantation embryo developing *in vitro*, serial section TEM analysis has shown that at the blastocyst stage virtually all cells contain (albeit in different proportions) both undeveloped and well-developed mitochondria (Fig. 1D). Unlike the situation that prevails in other mammals such as the mouse and rabbit (Van Blerkom & Motta 1979), for the human blastocyst the fully developed mitochondrial phenotype shown in Fig. 1E may predominate in some cells and be comparatively scarce in others (Sathanathan *et al.* 1993, Van Blerkom 1993). It is not known whether the apparent cell-specific differences in the state of mitochondrial differentiation observed in human blastocysts are related to the conditions of culture and therefore not representative of the *in vivo* situation. Alternatively, they could be a normal aspect of early human development and represent developmentally significant differences in mitochondrial activity within the embryo, perhaps related to differential cell function, as discussed below for the mouse blastocyst.



**Figure 1** A–E and I are transmission electron micrographs that have been colorized to enhance the visualization of certain subcellular components. (A) Mitochondria (M, blue) and complexes of smooth-surfaced endoplasmic reticulum (SER, red) in a metaphase human oocyte. The association between mitochondria and the SER is shown at high magnification for a single complex in I. Differences in mitochondrial fine structure seen in human oocytes (C) and cleavage (B) and blastocyst stage embryos (D,E) indicate a progressive transformation to forms presumed to be more active in respiration. c, cristae, MII, metaphase II spindle. These pictures are adapted from Makabe & Van Blerkom (2004). (F, G) Scanning laser confocal microscopic images of a human pronuclear (F,G) and a normal appearance 8-cell embryo (H) stained with mitochondrial-specific fluorescent probes (H1,2). A symmetrical distribution of peri-pronuclear (PN) mitochondria (M) is shown in a fully compiled image (F) and 5  $\mu$  section (G). Differences in the mitochondrial segregation between blastomeres during cleavage present as differential intensities of fluorescence (H1,2) and can be traced back to asymmetric peri-pronuclear aggregation at the one-cell stage. The arrow in H1 denotes the second polar body. These pictures are adapted from Van Blerkom *et al.* (2002). (J) A compiled 15  $\mu$  scanning laser confocal microscopic image showing spherical complexes of SER in a living MII human oocyte stained with an SER-specific probe. An arrow indicates the MII chromosomes. (K–O) Conventional epifluorescent microscopic images showing red J-aggregate fluorescence in a human pronuclear (PN, arrows, K) and blastocyst-stage (arrows, L) embryo, and in a peri-implantation, day 5.5 (M) mouse blastocyst observed in the FITC (N) and RITC (O) channels after staining with JC-1. The potential developmental relevance of pericortical J-aggregate fluorescence to high polarized mitochondria in the oocyte and early embryo, and differential J-aggregate fluorescence between the mural (mTR) and polar trophoderm (pTR) and inner cell mass (ICM) are discussed in the text. Amy Jones provided image K (see also Jones *et al.* 2004). Green fluorescence in this image is derived from JC-1 monomeric staining in low polarized mitochondria. Yellow fluorescence in N results from signal cross-over from the RITC channel.

The undeveloped morphology of oocyte and early embryo mitochondria has been traditionally interpreted in the context of low respiratory activity. However, there are two important factors to consider for the human oocyte: (a) if the number of mitochondria and mtDNA copies are synonymous (Cummins 2002), the apparent mitochondrial

complement in human oocytes may number in the hundreds of thousands and (b) levels of mitochondrial ATP generation are likely to be demand-related and in part determined by stage-specific exogenous conditions. For example, owing to the absence of an intrafollicular vasculature, the oocyte may reside in a near anoxic

environment for most of its life and energy demands may be minimal or supplemented with ATP produced by glycolysis or imported from exogenous sources such as the associated granulosa cells (Albertini 2004), whose cytoplasmic extensions penetrate deeply into the ooplasm and communicate directly with the oolemma by means of gap junctions (Motta *et al.* 2003, Makabe & Van Blerkom 2004). Changes in the level of oocyte metabolism could occur during follicular growth as the fluid-filled antrum develops and perfollicular blood flow rates increase significantly, which may increase the intrafollicular concentration of dissolved oxygen available to the oocyte during the preovulatory period (see review by Van Blerkom 2002). If the cumulus and coronal cells are significant sources of ATP for the oocyte, increased mitochondrial ATP generation may occur during meiotic maturation to compensate for the loss of contact with the surrounding somatic cells that occurs at the resumption of meiosis.

Stage-specific spatial remodeling of the maturing oocyte cytoplasm is also associated with mitochondrial redistributions that suggest that ATP production may occur at different levels within the ooplasm. Transient and focal upregulation of mitochondrial respiration may be an important mechanism by which differential intracytoplasmic energy demands in the maturing oocyte and early embryo are met without involving the entire mitochondrial complement (Van Blerkom & Runner 1984). Indeed, Dumollard *et al.* (2004) demonstrated that ATP supply and demand are tightly coupled in the MII mouse oocyte and newly fertilized egg, suggesting that up- or down-regulated mitochondrial activity is likely stage-specific and differentially localized within the cytoplasm during early development. According to Dumollard *et al.* (2004), the underdeveloped morphology of oocyte and early embryo mitochondria in the mouse may serve to limit oxidative phosphorylation capacity/organelle and consequently reduce the potential for generating reactive oxidative species at levels where oxidative stresses could either compromise mitochondrial function or perhaps initiate apoptosis (Liu & Keefe 2000, Liu *et al.* 2000). The question of how mitochondrial activity is regulated may be of particular interest in clinical IVF if oocytes with very different numerical complements occur within cohorts (see below). For example, could premature arrest of preovulatory meiotic maturation or of early embryogenesis be associated with a mitochondrial complement that is insufficient to supply nascent ATP demands, especially if stage-specific demands are differentially located within the cytoplasm? At the other extreme, could oocytes with unusually high numbers of mitochondria be unable to tightly regulate focal supply and demand and, as a result, generate reactive oxidative species at levels that become developmentally toxic after fertilization (Van Blerkom 2004)?

Many studies, some beginning in the 1930s (Boeil & Nicholas 1939) and continuing during the subsequent decades (Fridhandler 1961, Brinster 1967, Stern *et al.* 1971, Ginsberg & Hillman 1973, 1975a, Biggers & Borland

1976, Gott *et al.* 1990) up to the present time (for review see Biggers 2004), have examined metabolic pathways of ATP generation during early mammalian embryogenesis. It is clear that mitochondrial oxidative metabolism is a major contributor of ATP during the entire preimplantation stage, with over 85 % of all ATP produced in the mouse blastocyst derived from mitochondria (Benos & Balaban 1983). Trimarchi *et al.* (2000) estimated oxygen consumption by individual mouse embryos and described a progressive increase in uptake from the one-cell to the blastocyst stage such that expanded mouse blastocysts consumed 60–70 % more oxygen for mitochondrial oxidative phosphorylation than did cleavage stage embryos (30 %). The progression of preimplantation development occurs against a background of nonreplicating mitochondria whose numbers/cell would be expected to be halved with each cell division. Stage-specific changes in mitochondrial fine structure to forms more consistent with high respiratory activity have traditionally been assumed to compensate for the continual decline in mitochondrial numbers at the blastomere level. In the early embryo, these morphological changes largely occur in concert with increased demands for ATP needed to support stage-specific cellular biosynthetic activities, plasma membrane production and developmentally critical morphodynamic processes such as (a) formation of a fluid-filled blastocoel cavity that begins with cavitation at the morula stage and continues through the phase of blastocyst expansion and (b) emergence of the embryo from within the confines of the zona pellucida during the so-called hatching stage that precedes implantation.

The extent to which fine structural defects in mitochondria are associated with arrested or abnormal patterns of human embryo development warrants investigation, especially when it is considered that poor outcomes in clinical IVF may be associated with certain cleavage-stage embryo phenotypes (Veeck 1999). A similar situation occurs at the end of the preimplantation stage as indicated by the variety of blastocyst phenotypes that often show significant defects in ICM or trophoblast development (Van Blerkom 1993, Veeck & Zaninovic 2003). At present, our understanding of mitochondrial fine structure in human oocytes and preimplantation-stage embryos is limited to a very few studies that present images from selected thin sections. For the human, the relationship between metabolism and competence is an emerging one, and it may be timely to consider systematic fine structural analysis of the arrested or seemingly abnormally developing human embryos that are usually discarded by IVF programs, as well as presumably normal embryos that may be donated to research.

### ***Is human oocyte and embryo competence related to the size of mitochondrial complement?***

Traditional estimates of mitochondrial numbers in oocytes by TEM have used representative sections whose selection for numerical analysis is determined by a morphometric

algorithm. While laborious, this approach is capable of offering reasonably accurate values assuming that mitochondria are relatively uniformly distributed throughout the cytoplasm. With this method, between 120 000 and 350 000 mitochondria have been estimated to occur in MII human oocytes (Jansen 2000b, Cummins 2002). However, when mtDNA copy numbers are determined by polymerase chain reaction methodology using probes for specific genes such as ATPase 6 (Van Blerkom 2004) or directed to specific sequences (Brenner 2004), the number of human mitochondrial genomes in MII oocytes from the same or different cohort(s) has been reported to differ by well over an order of magnitude, ranging from a low of approximately 20 000 to well over 800 000 (Chen *et al.* 1995, Steuerwald *et al.* 2000, Reynier *et al.* 2001, Barrit *et al.* 2002, Van Blerkom 2004). If the current consensus that each oocyte mitochondrion contains a single genome is accurate (Cummins 2002), this rather astonishing and unexpected variation in mtDNA numbers between similarly appearing oocytes raises some fundamental questions about how competence may be determined well before fertilization.

Reynier *et al.* (2001) proposed that premature arrest of preovulatory meiotic maturation and fertilization failure after conventional IVF may be directly related to low mtDNA numbers, especially in the 20 000 to 60 000 range, if low copy numbers are associated with a reduced metabolic capacity. Van Blerkom *et al.* (1995) measured net cytoplasmic ATP levels in cohorts of unfertilized and uninseminated MII human oocytes obtained from women undergoing IVF and GIFT procedures and reported that the ATP content of equivalently appearing oocytes could differ by an order of magnitude. Whether a developmentally significant relationship exists between ATP content and mitochondrial or mtDNA copy numbers remains to be determined, and the simplest interpretation of very high mtDNA copy numbers is that each mitochondrion contains more than a single genome. However, it is difficult to determine the actual number of organelles by electron microscopy and mtDNA copy number by PCR in the same oocyte because these procedures use mutually exclusive protocols. One approach currently under investigation in our laboratory uses quantitative mitochondria-specific fluorescence (Van Blerkom *et al.* 2000) to provide an initial distinction between oocytes that can be used to select specimens for electron microscopy and mtDNA determination. If these studies confirm that the size of the mitochondrial complement and mtDNA copy numbers are related, it may be possible to establish a normal complement size consistent with competence. While incompetence may be understood in the context of sub-normal levels, the existence of supranormal complements presents potentially different types of metabolic issues and questions.

Early cleavage arrest in the homozygous embryos of certain mutant mouse strains (Ginsberg & Hillman 1975b) has been related to unusually high levels of mito-

chondrial oxidative phosphorylation and correspondingly high cytoplasmic ATP levels. A situation where mitochondrial ATP supply exceeds cellular demand could be toxic if associated with elevated levels of oxidative free radical production which could cause irreversible nuclear and mtDNA damage leading to cytoplasmic deterioration, mitochondrial disruption and eventually death by degenerative or apoptotic processes (Liu & Keefe 2000, Liu *et al.* 2000). Whether a similar situation can occur in the human owing to high levels of ATP generation (Van Blerkom *et al.* 1995) that may be associated with unusually high mitochondria/mtDNA copy numbers warrants further study. The importance of this research would be demonstrated if meiotic spindle defects and errors in chromosomal segregation that result in lethal aneuploidies and other chromosomal disorders common in human oocytes and early embryos could be directly associated with differences in mitochondrial complement size, mtDNA copy numbers and cytoplasmic mechanisms that regulate mitochondrial activity (Schon *et al.* 2000, Dumollard *et al.* 2004, Eichenlaub-Ritter *et al.* 2004).

### ***Spatial remodeling of mitochondria is a common aspect of early mammalian development***

Studies of several mammalian species have shown that mitochondria undergo stage-specific changes in distribution during oocyte maturation and early embryogenesis. During maturation of the mouse oocyte *in vitro*, mitochondria translocate to the perinuclear region along microtubular arrays extending from perinuclear microtubular organizing centers (Van Blerkom 1991) to form a sphere of organelles that encloses the condensing bivalent chromosomes and, later, the nascent metaphase I and II spindles (Van Blerkom & Runner 1984, Tokura *et al.* 1993). After fertilization in the mouse (Van Blerkom & Runner 1984), hamster (Bavister & Squirrell 2000) and human (Van Blerkom *et al.* 2000), mitochondria migrate to the perinuclear region, again by a microtubule-mediated process, to form a condensed aggregate surrounding the opposed pronuclei. A similar transient perinuclear accumulation occurs in each blastomere during the early cleavage stages. It is generally thought that spatial remodeling of mitochondria may allow higher ambient levels of ATP to occur in regions of the cytoplasm where stage-specific activities may have higher energy demands (Van Blerkom & Runner 1984, Barnett *et al.* 1996).

Evidence that mitochondrial inheritance between cells may be a critical determinant of human embryo viability was reported by Van Blerkom *et al.* (2000), who described the consequences of disproportionate mitochondrial segregation during cleavage. These authors found that the pattern of mitochondrial inheritance between the 2- and 12-cell stages could be related to the geometry and symmetry of the perinuclear mitochon-

drial accumulation at the one-cell stage. An examination of the developmental progression of individual embryos with cleavage divisions producing blastomeres of uniform size showed that different patterns of mitochondrial segregation had different consequences for the resulting blastomere(s) and for the ability of the embryo to develop progressively *in vitro*. Digital imaging and quantitative analysis of mitochondria stained with organelle-specific fluorescent probes by scanning laser confocal microscopy enabled (a) the geometry of pronuclear mitochondrial aggregation to be determined in fully-compiled (symmetrical distribution, Fig. 1F) and individual (Fig. 1G) images and (b) related to the intensity of mitochondrial fluorescence in each blastomere during cleavage. For example, the normal appearing 8-cell embryo shown in Fig. 1H had an asymmetric peri-pronuclear distribution of mitochondria at the pronuclear stage. Differences in the relative intensity of mitochondrial fluorescence between blastomeres were detected in the intact embryo (Fig. 1H1 and 2) and confirmed by disaggregation into individual blastomeres and reanalysis (Van Blerkom *et al.* 2002). These authors measured the ATP contents of each blastomere and related the levels to previously quantified mitochondrial fluorescence and reported that disproportionate mitochondrial inheritance resulted in blastomeres between the 2- and 12-cell stages with very different capacities to generate ATP. Sibling embryos with a symmetrical pronuclear aggregation (e.g. Fig. 1F,G) showed a uniform pattern of mitochondrial segregation and relative metabolic equivalence between blastomeres. At the blastomere level, the consequences of disproportionate mitochondrial segregation ranged from arrested cell division to abrupt blastomere swelling and lysis which these investigators suggested were directly related to mitochondrial content and ATP generating capacity.

If aberrant segregation patterns were confirmed to be a critical determinant of human embryo competence, the development of noninvasive methods for the clinical IVF laboratory would be warranted. Such methods would need to be noninvasive, take advantage of the ability of mitochondria to autofluoresce at particular wavelengths (infrared) and be of sufficient sensitivity to detect differences between blastomeres that are developmentally significant. From a basic science viewpoint, it would be relevant to investigate how different patterns of perinuclear mitochondrial aggregation are established, especially in light of the findings by Van Blerkom *et al.* (2000) that perinuclear mitochondrial geometry may be mediated by the distribution of corresponding arrays of microtubules which, in turn, may be influenced by intrinsic (ATP production, pH<sub>i</sub>; Van Blerkom 2002) or extrinsic (pH<sub>i</sub>; Gaulden 1992) factors.

### **Mitochondria as regulatory forces in early development**

In addition to ATP production, mitochondria in somatic cells (Pozzan *et al.* 2000) are directly involved in the regulation of intracellular free Ca<sup>2+</sup> by virtue of their ability to sequester and release this cation in response to a variety of signals including (a) electrical fluxes (Ichas *et al.* 1997), (b) Ca<sup>2+</sup> itself, by means of Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release (CICR) pathways (Duchen 2000) and (c) molecular signals associated with the activation of the apoptotic pathway (Berridge *et al.* 1998). The signal for CICR can originate from storage sites such as the smooth surfaced endoplasmic reticulum (SER), specialized storage granules or from Ca<sup>2+</sup> released by other mitochondria (mCICR; Rizzuto *et al.* 1994, Babcock *et al.* 1997, Duchen 2000, Hajnoczky *et al.* 2000). Focal changes in cell physiology, including localized changes in ambient Ca<sup>2+</sup> levels, have been shown to up- or down-regulate respiratory activity in the corresponding mitochondria. This is an important means by which somatic cells undergoing spatial remodeling or morphodynamic alterations involving mitochondrial redistribution can keep ATP supply and demand in balance at different locations within the cytoplasm (Aw 2000). There is growing evidence that mitochondria in oocytes and early embryos are involved in and subject to the same regulatory forces that operate in somatic cells (Rutter & Rizzuto 2000), especially with respect to levels of intracellular Ca<sup>2+</sup> and their respective storage elements such as SER (Sousa *et al.* 1996, Liu *et al.* 2001, Tesarik 2002, Van Blerkom *et al.* 2002, 2003, Dumollard *et al.* 2003, 2004).

TEM analysis of normal MII human oocytes demonstrates that mitochondria are never far from interconnected networks of SER (Fig. 1A). Indeed, these networks exist as spherical domains of cisternae in which mitochondria surround or are contained within their matrix (Fig. 1I), or both (Van Blerkom 2002, Makabe & Van Blerkom 2004). For the entire oocyte, TEM methodology cannot efficiently determine the distribution and geometry of the SER system. However, studies of human oocytes using ER-specific fluorescent probes derived from the 'Dil family' of lipophilic carbocyanine analogs (Molecular Probes, Eugene, OR, USA) demonstrate that virtually every discrete SER complex can be imaged by scanning laser confocal microscopy, and that their relative density and distribution within the ooplasm can be determined. In this respect, it is reassuring that the spherical SER complexes detected in sections of Dil-stained oocytes (15 µm; Fig. 1J) have the same relative geometry and distribution as those identified in TEM images that are colorized in order to enhance the visualization of these elements (Fig. 1A and I).

Several studies have investigated the relationship between mitochondrial respiration and levels of intracellular-free Ca<sup>2+</sup> in MII oocytes and newly fertilized eggs.

A direct role for mitochondria in the regulation of intracellular free  $\text{Ca}^{2+}$  was indicated in the study of Liu *et al.* (2001) who showed that by segregating mouse oocyte mitochondria and SER into separate compartments, the former was involved in the clearance of  $\text{Ca}^{2+}$  released by the latter, thus mediating the establishment of  $\text{Ca}^{2+}$  oscillations during activation. These authors also proposed that mitochondrial  $\text{Ca}^{2+}$  sequestration and release were related to their metabolic activity. More recently, Dumollard *et al.* (2003, 2004) reported the following for unfertilized MII and newly penetrated mouse oocytes: (a) mitochondrial oxidative metabolism is a major and developmentally critical source of ATP at these stages, (b) high ATP turnover rates at MII suggest that mitochondrial ATP production and utilization are closely balanced and (c) sperm-triggered  $\text{Ca}^{2+}$  oscillations are transmitted to mitochondria and directly stimulate (up-regulate) mitochondrial respiration in concert with these ionic transients. The importance of these findings is related to the suggestion that by limiting the up-regulation of  $\text{Ca}^{2+}$  to specific cytoplasmic regions in which developmental activities occur that have transiently higher ATP demands, a relatively constant net level of cytoplasmic mitochondrial oxidative metabolism can be maintained in the oocyte that meets differential energy demands while reducing the potential for generating damaging or toxic levels of reactive oxygen species (Dumollard *et al.* 2004).

As noted previously, stage-specific changes in mitochondrial distribution occur during oocyte maturation, pronuclear formation and early cleavage. If the SER co-translocates with mitochondria, a metabolic regulatory pathway may exist in which transient domains of differential ionic signaling could upregulate mitochondrial metabolism in order to support location-specific developmental processes. Developmental processes that may have higher energy demands and involve regional domains of SER-mitochondrial complexes could include germinal vesicle breakdown, formation of the first and second metaphase spindles, pronuclear evolution and movement, and the establishment of the first mitotic spindle. Another possible consequence of establishing differential ATP generating domains is that clusters of mitochondria with different levels of oxidative phosphorylation can locally change physiological characteristics such as intracellular pH (Aw 2000). These focal changes could, in turn, influence other cytoplasmic activities, such as the capacity to promote microtubular polymerization (Van Blerkom *et al.* 2000). This notion is currently under investigation in our laboratory and, if supported experimentally, could offer insight into how certain developmental processes are compartmentalized and focally regulated in a stage-specific manner during early mammalian development.

### **Is mitochondrial polarity ( $\Delta\Psi_M$ ) related to competence?**

Mitochondrial polarity is a measure of the inner mitochondrial membrane potential ( $\Delta\Psi_M$ ) and differences in magnitude have been related to levels of respiration and the ability of these organelles to participate in the regulation of  $\text{Ca}^{2+}$  homeostasis. Mitochondrial respiration involves outward proton pumping across the inner mitochondrial membrane that creates a proton gradient that has two components, a  $\Delta\Psi_M$  and a pH gradient, with the energy stored in either component driving the conversion of ADP to ATP by respiratory chain enzymes. The relationship between  $\Delta\Psi_M$  and oxidative metabolism first described by Mitchell & Moyle (1967) has been studied in somatic cells with  $\Delta\Psi_M$ -sensitive fluorescent reporter probes. One particular molecule, 5,5',6,6'-tetrachloro-1,1,3,3'-tetraethylbenzimidazolycarbocyanine iodide (JC-1), has become widely used to assess the relative magnitude of  $\Delta\Psi_M$  since its specificity as a polarity reporter was first described by Reers *et al.* (1991) and shown by Smiley *et al.* (1991) to be able to detect domains of high and low polarity within individual mitochondria. At relatively low potentials (<100 mV), JC-1 usually exists as a monomer with green fluorescence detected in the fluorescein isothiocyanate (FITC) channel. However, as the potential increases (>140 mV) JC-1 monomers multimerize to form metastable stacks or arrays termed J-aggregates (Reers *et al.* 1995), named after Jelley (1937) who first described the physical and chemical properties of these unusual crystal-like carbocyanine multimers. JC-1 multimerization shifts the fluorescence emission maxima to longer wavelengths such that high or hyperpolarized mitochondria in cultured somatic cells appear in the rhodamine isothiocyanate (RITC) channel as intense orange-to-red fluorescent rods.

The unique capacity of JC-1 to report differences in  $\Delta\Psi_M$  has been used in a variety of studies of somatic cells under normal and experimental conditions, and more recently, for mammalian oocytes and embryos to examine the association between developmental competence and mitochondrial activity (Wilding *et al.* 2001, 2002, 2003, Van Blerkom *et al.* 2002, 2003, Acton *et al.* 2004, Jones *et al.* 2004). In MII mouse and human oocytes and embryos (Ahn *et al.* 2002, Van Blerkom *et al.* 2002, 2003, Jones *et al.* 2004), the highest intensity of J-aggregate fluorescence is reported to occur in the pericortical/subplasmalemmal cytoplasm (arrows, Fig. 1K). If mitochondria with different polarities are compartmentalized within the oocyte, the apparent pericortical distribution of high-polarized mitochondria is of particular interest if related to metabolic or ionic activities, because it suggests a mechanism by which domains of differential function could be established. Although this is an appealing notion from a development viewpoint, whether the distinct pericortical complexes of SER and putative high-polarized mitochondria detected in human oocytes (Van Blerkom

*et al.* 2002) are functional in this regard remains to be determined. However, studies designed to test this hypothesis need to consider the findings of Van Blerkom *et al.* (2003) and Dumollard *et al.* (2004), who demonstrated that owing to reversal of ATP synthase activity under conditions of electron transport inhibition, a  $\Delta\Psi_M$  sufficient to maintain J-aggregate formation can persist in mouse oocytes, despite a significant reduction in mitochondrial ATP generating capacity. Therefore, high levels of mitochondrial respiratory activity and hyperpolarization detected by J-aggregate formation are not necessarily synonymous. Indeed, it has been suggested that mitochondria which are actively involved in ATP synthesis undergo continuous  $\Delta\Psi_M$  dissipation, which should be reflected by an average low- rather than high-polarized condition (Diaz *et al.* 1999).

While it is unclear whether high mitochondrial polarity and elevated respiratory activity are related during early development, there is some evidence that differences in  $\Delta\Psi_M$  may be related to competence. Ahn *et al.* (2002) reported that thawed 2-cell mouse embryos that failed to divide or that developed abnormally showed a cryopreservation-associated loss of mitochondrial hyperpolarization in the subplasmalemmal domain. Jones *et al.* (2004) found that cryopreservation of MII human oocytes was accompanied by loss of the hyperpolarized pericortical mitochondrial domain that characterized the fresh oocyte. Thawed oocytes also exhibited a significantly reduced capacity to up-regulate levels of intracellular free  $Ca^{2+}$  in response to  $Ca^{2+}$  ionophore stimulation. The studies of Wilding *et al.* (2001, 2002, 2003) on human oocytes and embryos produced by IVF suggest that differences in the ratio between high and low polarized mitochondria may reflect aberrant mitochondrial distributions in the oocyte and metabolic defects in the embryo that could result in lethal chromosomal segregation errors. Acton *et al.* (2004) reported that differences in mouse embryo competence were related to the ratio of high-to-low polarized mitochondria that, in turn, was related to whether fertilization occurred *in vivo* or *in vitro*. Furthermore, these authors reported that an increased ratio of high-to-low polarized mitochondria was associated with increasing degrees of fragmentation during cleavage of human embryos after IVF. Van Blerkom *et al.* (2002) and Jones *et al.* (2004) noted that within cohorts, some human oocytes exhibited no detectable J-aggregate fluorescence while for others, intense fluorescence occurred throughout the cytoplasm. In the mouse, intense cytoplasmic J-aggregate fluorescence was associated with abnormally elevated levels of intracellular  $Ca^{2+}$  in activated oocytes (Van Blerkom *et al.* 2003). Whether the differences in  $\Delta\Psi_M$  are directly related to competence or are consequences of other developmentally lethal perturbations needs further investigation. For example, Jones *et al.* (2004) suggested that positive outcomes after cryopreservation may be derived from those relatively few MII human oocytes in which cortical domains of mitochondria remained hyperpolarized

after thawing, while loss of hyperpolarization in 2-cell mouse embryos was coincident with changes in cell membrane fluidity and subplasmalemmal actin microfilament integrity (Ahn *et al.* 2002).

At present, the relationship between mitochondrial function, intracellular  $Ca^{2+}$  and developmental competence may be one that warrants detailed analysis with respect to  $\Delta\Psi_M$ . Ozil & Huneau (2001) demonstrated that experimentally reducing the mobilization of intracellular free  $Ca^{2+}$  to levels slightly below those measured at oocyte activation had lethal downstream consequences first detected during organogenesis, i.e. after implantation. Indeed, embryo performance *in vitro* during the preimplantation stages was unremarkable with no evident morphological indications that lethal defects would occur several days after uterine transfer. The possibility that differences in the state of mitochondrial polarization between MII human oocytes is a factor in outcome after embryo transfer, is an intriguing one, especially in view of current pregnancy results with thawed oocytes that demonstrate high frequencies of postimplantation failure (Jones *et al.* 2004).

If spatial differences in  $\Delta\Psi_M$  within oocytes and blastomeres are developmentally relevant, how do domains of high and low polarized mitochondria develop? Diaz *et al.* (1999) showed that whether mitochondria located beneath the plasma membrane of cultured cell lines were high or low polarized was directly influenced by the presence or absence of intercellular contacts. Van Blerkom *et al.* (2002) described very similar findings for mouse and human oocytes and cleavage-stage embryos. For example, J-aggregate fluorescence did not develop in pericortical regions in experimentally manipulated oocytes where patches of cumulus and corona cells remained intact, but did so after their physical elimination. For cleavage-stage mouse embryos, low polarized mitochondria predominated at regions of intercellular contact. When embryos were disaggregated and individual blastomeres repositioned, formerly J-aggregate-negative cortical domains were J-aggregate-positive, and vice versa. In the same study, these authors reported that the ICM in both expanded human (Fig. 1L) and mouse blastocysts contained low polarized mitochondria. More recent studies with peri-implantation mouse blastocysts (J Van Blerkom, H Cox & P Davis, unpublished observations) found that the highest levels of J-aggregate fluorescence occurred in the abembryonic mural trophoctoderm and diminished significantly towards the polar trophoctoderm, where J-aggregate fluorescence was undetectable or scant (Fig. 1M and O). The ICM and polar trophoctoderm both showed low polarized mitochondria (Fig. 1N). These authors suggested that cell- and location-specific differences in  $\Delta\Psi_M$  seemed to be related to the nature and extent of intercellular contact, with the abembryonic mural trophoblast in the preimplantation mouse embryo containing the only cells devoid of such contacts.

A possible developmentally significant relationship between high  $\Delta\Psi$  and cell-specific function was indicated

in the study mentioned above (J Van Blerkom, H Cox & P Davis, unpublished observations) by two dynamic processes localized to the abembryonic trophoblast: (a) the elaboration of highly motile finger-like projections and (b) the formation of enlarged cellular protrusions. The trophoblastic projections are thought to be cytoplasmic extensions involved in the initial stages of embryo contact with and invasion into the endometrial epithelium (Bergstrom & Nilsson 1976), and the enlarged cellular protrusions are likely indications of transformation of abembryonic mural trophoblast into nondividing, polyploid giant cells (Maris *et al.* 1988). While these studies suggest that differential mitochondrial polarity and cell function/activity may be related during embryogenesis, confirmation will require more critical studies in which cell and embryo fate is determined when  $\Delta\Psi_M$  is experimentally manipulated. Nevertheless, if additional research continues to support the notion that mitochondrial polarity, respiration and focal regulation of intracellular  $Ca^{2+}$  are related in important ways during early development, it may offer a new basis for understanding how competence is established in the oocyte and maintained in the embryo. For clinical IVF, it may be relevant to ask whether intrafollicular factors or conditions that can affect competence (Van Blerkom 2002) do so by influencing these activities.

### Concluding comments

In many respects, studies of the mitochondria in early mammalian development have raised more questions than have been definitively answered, although their long known role in ATP production in the oocyte and embryo remains intact. Issues related to complement size, spatial distribution, fine structural organization, mtDNA integrity and copy number, high and low polarity, participation in ionic regulation and inheritance patterns offer promising leads for additional investigation and possible applications in clinical IVF. In particular, study of their stage-specific reorganization and association with other cytoplasmic components such as the SER should determine whether they are (a) directly involved in the focal or global cytoplasmic activities that determine normal competence or result in demise and (b) which of their many nonrespiratory functions in somatic cells also pertain to the oocyte and preimplantation-stage embryo. Continued research will also yield the type of basic scientific information that will be necessary to demonstrate that controversial procedures applied to certain infertile women, such as donor ooplasmic transfusion (Cohen *et al.* 1997), actually treat mitochondrial defect(s) that have been assumed to occur in the recipient oocyte and are associated with cleavage-stage demise (Brenner *et al.* 2000, Brenner 2004). Clinicians and patients need to know whether these or other similar manipulations (e.g. direct mitochondrial transfer, Van Blerkom *et al.* 1998) are safe for the intended child

and can, therefore, be used effectively in the treatment of infertility or to 'correct' oocytes with known mtDNA defects, such as those associated with OXPHOS diseases (Brenner 2004, Cummins 2004, St. John *et al.* 2004). Although anticipated, whether mitochondrial research will also yield methods that can be used routinely in the clinical IVF laboratory to select for high competence oocytes and early embryos remains to be determined.

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