Mitochondria: potential roles in embryogenesis and nucleocytoplasmic transfer

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This review examines current understanding of mammalian mitochondria and mitochondrial DNA in the light of new reproductive technologies. Mitochondria are central to ageing, apoptosis, metabolism and many diseases. They are controlled by a dual genome system, with cooperation between endogenous mitochondrial genes and mitochondrial genes translocated to the nucleus over the course of evolution. This translocation has been accompanied by extreme compression of the mitochondrial genome, with little tolerance for mutations or heteroplasmy (multiple genomes). The highly compact mitochondrial genome appears to be maintained by a stringent numerical bottleneck in embryogenesis and oogenesis, followed by clonal expansion from a highly selected subset of precursor molecules. The dual nature of control between nucleus and cytoplasm sets up potential conflicts, which are normally resolved by natural selection. Such potentially opposing interests and mechanisms are probably partly to blame for the poor rates of success in cloning animals by nuclear transfer. The ability to construct cell systems and animal embryos with novel combinations and permutations of nuclear and cytoplasmic genes will provide powerful tools for examining these fundamental biological questions. Clinically, attempts to ‘rescue’ abnormal human oocytes or embryos by cytoplasmic transfer risk complex and unpredictable outcomes emerging from disharmonious nuclear–cytoplasmic interactions.

Key words: ageing/apoptosis/cloning/cytoplasmic transfer/mitochondrial DNA

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Introduction

In this review, the role of mitochondria in reproduction will be examined, with particular emphasis on the implications for cloning technology and manipulation of embryos by nuclear and cytoplasmic transfer. Readers who are interested in learning more about the rapidly growing field of mitochondrial genetics are referred to recent reviews on mitochondrial DNA (mtDNA) in reproduction and the life cycle (Cummins, 1998); in disease and ageing (Lightowlers et al., 1997; Ozawa, 1997; Zeviani and Antozzi, 1997; Wallace, 1999), and on the regulation of transcription and replication (Shadel and Clayton, 1997).

Mitochondria were first described by Altmann in 1890 (see Graff et al., 1999), and 70 years later were shown to contain their own DNA (Nass and Nass, 1963). The essential role of these organelles in a variety of physiological processes is now recognized: controlling oxidative energy supply in normal and pathological physiology, embryonic development, apoptosis, and general body ageing. Besides the production of ATP in oxidative phosphorylation (OXPHOS), mitochondria control several fundamental metabolic pathways including the synthesis of amino acids, folic acid, haem, nucleotides, pyrimidines, phospholipids and uric acid (Enriquez et al., 1999b). Mitochondrial proliferation, differentiation and local tuning carry on largely independent of (though ultimately subservient to) the host cell cycle (Enriquez et al., 1999b). These processes are modulated by thyroid hormone, along with general body metabolism (Enriquez et al., 1999a).
Dual control of mitochondria

Mitochondrial function is normally controlled by a combination of nuclear and mitochondrial genes. Generally, this proceeds amicably, but sometimes conflict occurs. This potential for dissonance between gene sets obviously has very ancient origins as it is also seen in living protists such as *Paramaecium* (Ruiz and Beisson, 1980; Ruiz and Knowles, 1980). The potential for conflict has important implications for newly evolving technologies such as cloning, as unpredictable phenomena can emerge in living systems from disharmony between multiple gene sets (Hurst *et al*., 1996). This is clearly seen from the emerging work on genome imprinting, where certain genes influencing placental and embryonic development are expressed or suppressed according to whether they pass through paternal or maternal gametogenesis (Latham, 1999). The separation of effects according to parental origin is not necessarily limited to genetic elements. Differential control of maternally and paternally inherited cytoplasmic organelles can also occur for other cell elements such as the centrosomes, the microtubule organising centres of the cell (Wu and Palazzo, 1999).

Mitochondria are generally thought to exist in the body at a high level of homoplasm (single haplotype), but this might be over-emphasized. Heteroplasm (multiple haplotypes) may be more common than suspected, as most studies rely on fingerprinting techniques that cannot distinguish different types (Grzybowski, 2000).

The complete mitochondrial sequence is now known for 58 chordate and 29 non-chordate species, with some remarkable parallels and similarities (Boore, 1999). The genome is generally a closed circular molecule with little redundancy, although linear forms with telomere-like terminations are known (Nosek *et al*., 1998). Mitochondrial DNA is tightly linked to the electron transport system and is thus vulnerable to damage—some more common than suspected, as most studies rely on fingerprinting techniques that cannot distinguish different types (Grzybowski, 2000).

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The current consensus is that mitochondria originated in an endosymbiotic relationship between the ancestors of eukaryotic cells and α-proteobacteria. There is a certain degree of irony in that the closest living relative to mitochondria appears to be *Rickettsia*, an obligate intracellular parasite of crab lice that causes human typhus (Andersson *et al*., 1998; Gray, 1998; Gray *et al*., 1999). In this endosymbiotic process, the proto-eukaryotes acquired the capacity to use oxygen for energy production by OXPHOS. There was a pronounced and extended rise in atmospheric oxygen over the period 375 to 275 million years ago, possibly through the increased burial of organic carbon (Bernier, 1999). Mitochondria enabled the eukaryotes to prosper by exploiting this dangerous and highly reactive element. OXPHOS is much more efficient at producing energy than, for example, glycolysis or the citric acid cycle (Lodish *et al*., 1995); thus organisms capable of using this pathway must have received an enormous selective advantage. However, this resulted in a trade-off between the benefits of OXPHOS, set against the risk of elevated free radical production. Oxygen can become partly reduced in mitochondria to form reactive oxygen species (ROS), some of which, such as OH·, O2− and H2O2, are potentially highly mutagenic and carcinogenic (Nohl, 1986; Halliwell, 1999). Each of the mitochondria in the human body, which together occupy up to 25% of the total cytosol (Lodish *et al*., 1995), produces around 107 ROS per day (Max, 1992). These radicals cause much of the ‘wear and tear’ of ageing (Holliday, 1995). A form of control over uncontrolled ROS production by programmed cell death (apoptosis) thus probably arose at the same time as the endosymbiotic event. Mitochondria still play a central role in the apoptotic cascade through the release of cytochrome c (Green and Reed, 1998), a topic discussed further below under ‘Mitochondria and apoptosis’. The risks of ROS production to the host genome are so great that no OXPHOS occurs in the nucleus. The process is relegated to the cytoplasm, where the mitochondria have been reduced to disposable elements. Apoptosis furnishes the organism with a facility for cell death should discordant cellular events threaten the integrity of the system. The need for conformity among mitochondria is probably a major driving force ensuring uniparental inheritance and low levels of heteroplasmy (Birky, 1995; Hurst, 1995; Cummins, 1998).

Genome conflict and cooperation in mitochondrial evolution

The idea that genomes act as consortia, with varying levels of cooperation and conflict, is not new. From the perspective of ‘Darwinian Medicine’, conditions that are presently regarded as ‘diseases’, together with the responses to them (for example, fever), may be manifestations of early stages of colonization or exploitation by microorganisms (Nesse and Williams, 1995). Newly acquired diseases—such as syphilis in Europe in the Middle Ages—tend to be the most virulent (Diamond, 1997), but natural selection gradually levels out conflicts until varying degrees of cooperation eventually emerge. Intercalation of mobile genetic elements is common in bacteria and protozoa. The ubiquitous presence of mobile eukaryotic genetic elements in metazoa was established by Barbara McClintock in work that won her the Nobel Prize in 1983 (Lodish, *et al*., 1995). A significant proportion of the eukaryotic nuclear DNA comprises ‘foreign’ intron sequences in the form of transposons and retrotransposons (Lodish *et al*., 1995; Carvalho and Clark, 1999). Much of this ‘noise’ in the genome appears to be neutral in terms of its impact on fitness (Kimura, 1983). It is generated by a variety of DNA turnover mechanisms (Dover, 1993), but there is some evidence (in *Drosophila*) that intron size may be subject to pruning by natural selection (Carvalho and Clark, 1999). Estimates of how much of the eukaryotic genome is ‘foreign’ vary. For example, retro-elements alone (genome sequences generated by reverse transcription from RNA) make up at least 10% of the mammalian genome (Löwer *et al*., 1996). As much as 3% of the human genome is inactive viral ‘fossil’ DNA including, surprisingly, HIV-like genes that probably date back 30 million years (Yang *et al*., 1998).
al., 1999). Such invasive DNA must threaten genomic integrity. In response, organisms have evolved a variety of epigenetic defence mechanisms such as imprinting, paramutation and gene silencing, which will inevitably compromise attempts to clone animals from somatic cell nuclei (Wolffe and Matzke, 1999). This is not the place to elaborate further on the lateral movement of genes between organisms; the interested reader is referred to a recent book reviewing the topic (Syvanen and Kado, 1999). It is also worth noting that, with the unfolding of the Human Genome Project, mtDNA, being relatively small, compact and fully sequenced, is a useful test-bed for bioinformatic tools (see for example http://www3.ebi.ac.uk/Research/Mitbase/mitbase.pl) (Anderson et al., 1981; Andrews et al., 1999; Attimonelli et al., 1999; Curole and Kocher, 1999).

During the course of vertebrate evolution, most mitochondrial genes have ‘jumped’ to the nucleus. In mammals, only 37 genes remain in 16.7 kb of DNA, apparently the bare minimum needed to encode for the dangerous, free radically-generating business of electron transport. This reduction is extreme, and the resulting genome has no introns: some genes even overlap. By comparison, yeast mtDNA is much larger (78 kb) and plant forms are gigantic—up to 2.5 × 10^6 bp in muskmelons (Lodish et al., 1995). The history of translocation can be guessed from some existing phenomena. In yeast, for example, mtDNA sequences are involved in the repair of double-stranded nuclear DNA breaks (Richetti et al., 1999). This is a continuing process of colonization of nuclear by mitochondrial DNA. The nuclear genome of most eukaryotic animals from somatic cell nuclei (Wolffe and Matzke, 1999), which have challenged the accuracy of molecular clocks used for taxonomic reconstruction (Strauss, 1999).

Mitochondrial inheritance

The view that spermatozoa are mere vectors for paternal DNA is over-simplistic. In most mammals, but not murine rodents (Manandhar et al., 1998), the spermatozoon also transmits the centrosome that acts as a template for the microtubule assembly for pronuclear juxtaposition and the first spindle apparatus of the zygote (Hewitson et al., 1999). The spermatozoon also transmits some unique molecules such as ‘oscillin’ that triggers calcium oscillations and oocyte activation (Dale et al., 1999; Swann and Parrington, 1999) and paternal tubulin (Simerly et al., 1999). Despite misinformation in many texts, the spermatozoon also carries in its full complement of midpiece mitochondria, but these are normally eliminated in early embryogenesis in a species-specific manner (Ankel-Simons and Cummins, 1996; Shoubridge, 1999; Cummins, 2000a). How spermatozoo are recognized as ‘self’ is not clear, but ubiquitination of sperm midpiece proteins during spermiogenesis opens the way for later recognition and destruction by embryonic proteasomes (Sutovsky et al., 1999; Cummins, 2000a). Possible targets for ubiquitination are the unique sperm mitochondrial capsule selenoprotein—a modified form of glutathione peroxidase with structural function (Cataldo et al., 1996; Cummins et al., 1998). Other potential sperm recognition targets include the mitochondrial protein prohibitin (Choongkittaworn et al., 1993; Berger and Yaffe, 1998; Sutovsky et al., 1999). This is one of a family of proteins with roles in senescence and tumour suppression that are also implicated in the control of ovarian granulosa cell apoptosis (Thompson et al., 1999). Foreign sperm mtDNA, or mtDNA in spermatozoa from construct mice with a differing nuclear genotype from the recipient can survive in embryos, but in an erratic and unpredictable manner (Gyllensten et al., 1991; Kaneda et al., 1995; Shitara et al., 1998).

The evolutionary reasons for uniparental inheritance of mitochondria (and other cytoplasmic organelles) are obscure, and the mechanisms by which it is achieved are extraordinarily diverse (Birky, 1995). The most likely hypothesis is that heteroplasm in cytoplasmic genes such as mtDNA sets up potential tensions between nuclear and mitochondrial control elements that will reduce fitness and will be selected against by natural selection. Thus, elimination of one set of cytoplasmic elements that will reduce fitness and will be selected against by natural selection. Thus, elimination of one set of cytoplasmic genes at fertilization avoids the possibility of lethal genome conflict discussed above (Hurst, 1994; Hurst and McVean, 1996). The debate over the strict imposition of uniparental inheritance of mtDNA has been re-kindled by observations on recombination in mtDNA in human populations discussed above (Awadalla et al., 1999; Eyre-Walker et al., 1999a,b; Hagelberg et al., 1999; Macaulay et al., 1999). Moreover, the inability of sperm mitochondria to survive in experimental transfer situations does
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not necessarily prove that there is an insurmountable barrier; other factors such as reduced redox potential may prevent sperm mitochondrial survival (Van Blerkom et al., 1998).

The mitochondrial bottleneck

Mitochondria pass through a stringent genetic bottleneck during transmission in the female life cycle. Clonal expansion from this bottleneck acts to maintain homoplasy, supporting the general hypothesis that selective pressure acts to minimize heteroplasm (Cummins, 1998, 2000a,b). However, as has been pointed out (Smith et al., 2000), there are several periods during which restriction of copy number could occur. These are during the replication and migration of primary germ cells (PGC); during oogenesis; during early embryogenesis and during the commitment of embryonic inner-cell-mass elements to form PGC. The human oocyte has around 100 000 mitochondria, each with a single copy of mtDNA (Chen et al., 1995; Jansen and de Boer, 1998). In the mouse, transcription of mitochondrial mRNA starts at the 2-cell stage (Taylor and Pikó, 1995), but replication of mtDNA does not occur until the egg cylinder stage (Pikó, 1975; Pikó and Matsumoto, 1976; Ebert et al., 1988). At this point, there are 910 cells (Hogan et al., 1986), so the pre-migratory mouse germ cells probably receive 10–100 copies of mtDNA (Jansen and de Boer, 1998). In other mammals, including humans, replication probably commences at the hatched blastocyst stage (Van Blerkom, 1989; Smith and Alcivar, 1993). This numerical bottleneck in the germ cell lineage, perhaps coupled with selection against defective mtDNA, ensures that the oocyte receives a highly homogeneous population of mtDNAs by clonal expansion (Marchington et al., 1997; Cummins 2000b). One estimate is that the effective founder population of mtDNA in mammals could be as small as a single copy (Blob et al., 1997), while another estimate for the mouse suggests around 200 (Jenuth et al., 1996). The final disposition of mitochondria in tissues is not random, but can show tissue-specific and age-related selection for different mtDNA genotypes in experimental heteroplasmic animals (Jenuth et al., 1997). This is discussed further below. It emphasizes the unpredictable interactions that can develop between nuclear and mitochondrial genomes.

One long-term evolutionary implication for the ‘bottleneck’ theory is that numerical restriction, coupled with clonal expansion and rigorous selection against defective mtDNA, can serve to counterbalance Müller’s ratchet. This is the inexorable accumulation of defective mutations in asexually reproducing life forms (Bergstrom and Pritchard, 1998). This also helps explain the rapidity with which novel mitochondrial genotypes can become fixed in populations (Ashley et al., 1989).

Control of mitochondrial function

Control of mitochondria is complex and involves cooperation between nuclear and mitochondrial genomes (Poyton and McEwen, 1996; Scarpulla, 1997; Surpin and Chory, 1997; Enriquez et al., 1999b). What remains of the genome in the organelle is a 16.5 kb fragment of DNA existing as several copies packed into nucleoids within the matrix. Only 37 genes remain, coding in part for 13 proteins of the electron transport chain, 22 tRNA and two rRNA species. The majority of the genes, along with pseudogenes and extra copies, are in the nucleus, where they evolve at the slower rate of nuclear genes (Collura and Stewart, 1995). Proteins required for mitochondrial function are synthesized in the cytosol and imported into the mitochondrial matrix with the aid of chaperones (Shadel and Clayton, 1997). This is an energy-dependent process driven by ATP consumption and the electrochemical potential across the inner mitochondrial membrane (Lodish et al., 1995; Neupert, 1997).

The rapid evolution of parts of the mitochondrial genome in concert with its partner nuclear genes—especially by synonymous base pair mutations (Pesole et al., 1999; Saccone et al., 1999)—has resulted in a high degree of species-specificity for many nuclear–mitochondrial interactions. Thus, mtDNA from chimpanzees, bonobos and gorillas can substitute for human mtDNA in human mitochondrial-free cell lines. However, mtDNA from orang-utans and Old and New World monkeys and from lemurs cannot (Kenyon and Moraes, 1997). When ape mtDNA is introduced into human cells carrying either no mtDNA or with mutated forms, only those cells with a total absence of mtDNA can be re-populated. However, exogenous human mtDNA is successfully incorporated and maintained in these cells (Moraes et al., 1999). Whether this concept can be applied to oocytes and embryos has yet to be fully tested, and will be further discussed below.

Much work on the complex control of normal and pathological mitochondrial function comes from the discovery that cell lines can be constructed devoid of endogenous mtDNA (King and Attardi, 1989). These cells, designated p°, can be re-populated with xenogeneic mitochondrial lineages. These and other studies show that survival of mitochondrial genotypes is critically dependent on the nuclear background (Hayashi et al., 1991; Dunbar et al., 1995; Holt et al., 1997; Hao et al., 1999; Vergani et al., 1999). It has been shown recently that rat mtDNA can restore translation but not respiration in mtDNA-depleted mouse cell lines (Yamaoka et al., 2000). Thus, the need for harmony between nuclear and mitochondrial genes appears to vary in stringency, with the ability to assemble respiratory complexes being most sensitive. Such hybrid cell lines can tolerate heteroplasm for many generations, only to show sudden and unpredictable shifts that may be associated with selection or with somatic karyotype alterations (Lehtinen et al., 2000). These findings reinforce the need to understand the complex interactions between nuclear and mitochondrial genomes. One technique which shows great promise in this area is the use of micropellet transfer of cytoplasts containing selected intact chromosomes to mitochondrial-free cell lines (Barrientos and Moraes, 1998; Wu and Palazzo, 1999).

Finally, it is worth emphasizing that nucleocyttoplasmic incompatibility is not limited to mitochondria. There is a minimal cytoplasmic volume required to support sperm decondensation and completion of fertilization (Wakayama and Yanagimachi, 1998). There are many other examples of incompatibilities between nucleus and the cytoplasm, or in nuclear programming by the cytoplasm. For example, in the inbred DDK mouse strain, an embryonic lethal phenotype is caused by incompatibility between a maternal factor of DDK origin and a paternal gene of non-DDK origin (Pardo-Manuel de Villena et al., 1999). Genome imprinting affects such interactions, through conflicting tensions between imprinted genes differentially methylated when passed through paternal or maternal gametogenesis (Latham, 1999).
Role of mitochondria in disease

The link between mitochondria and disease was first established in 1962 (Luft et al., 1962), and since then more than 50 different mitochondrial mutations have been linked to human disease (Larsson and Clayton, 1995; Wallace et al., 1995). Mitochondrial dysfunction is also associated with a variety of common bioenergetic disorders ranging from neurodegeneration to heart disease, diabetes and diabetes mellitus (Graff et al., 1999). There are also strong links between mitochondrial disease and oxidative stress-limiting control mechanisms (Melov et al., 1999). Most mitochondrial diseases lead to early death, and may even manifest as Sudden Infant Death Syndrome (Opdal et al., 1999), but there are intriguing hints that certain mutations can segregate differentially on a tissue-specific basis (Chinnery et al., 1999). This unpredictable interaction between mtDNA mutations and higher-order selective pressures can have implications for using mtDNA lineages in tracing human genealogies, as inheritance is not necessarily neutral (Wallace et al., 1999). A number of pharmacological and genetic strategies have been proposed for treating mitochondrial disorders (Graff et al., 1999), but these are still largely conjectural or on a cell-culture basis (Taylor et al., 1997; Murphy and Smith, 2000). Two recent reports also show that it is possible to produce genetically modified chimeric mice by introducing mutant mtDNA into embryonic stem cells (Levy et al., 1999), or by direct microinjection into zygotes (Irwin et al., 1999). These will be valuable model systems for the development of therapies for mitochondrial diseases (Taylor et al., 1997).

Mitochondria and reproductive ageing

On a more prosaic level, accumulation of deletions and rearrangements in mtDNA is implicated in general body ageing. The importance of mitochondrial maintenance in normal ageing was first postulated in the early 1980s (Miquel et al., 1980) and later elaborated (Linnane et al., 1989). Since then, many reports have shown age-related links between accumulation of deletions and mutations in mtDNA, accompanied by an inevitable decline in neuromuscular efficiency (Ozawa, 1997; Wallace, 1997; Cortopassi and Wong, 1999). The mitochondrial theory of ageing has a number of competitors, however, and is not universally accepted as few valid quantitative data exist (Holliday, 1995; Lightowlers et al., 1999). There are conflicting reports about the rate of accumulation of damage, as measured by adduct formation and repair (Croteau et al., 1999). One study (unconfirmed) showed that mtDNA may undergo extensive fragmentation with age, so that wild-type molecules decline to 11% of the total (Hayakawa et al., 1996). Ageing is also accompanied by large accumulations of point mutations in the mtDNA region responsible for the control of replication (Michikawa et al., 1999). There is no consensus on the mode of accumulation of damaged mtDNA (Croteau et al., 1999). The original theory postulated a form of ‘wear and tear’ model (Miquel et al., 1980). However, it was also suggested (de Grey, 1997) that mitochondria with reduced respiratory function are less liable to lysosomal degradation, because of reduced free radical production—a suggestion subject to recent dispute (Gershon, 1999; Kowald, 1999). One possibility is that defective mitochondria might proliferate because of reduced ATP production, leading to enhanced proliferation through reduction of ATP-driven negative feedback on replication (Enriquez et al., 1996; Hofhaus and Gattermann, 1999). The mitochondrial ‘wear and tear’ theory is supported, however, by evidence of impaired mtDNA repair mechanisms in diseases that show premature ageing, such as xeroderma pigmentosum (Driggers et al., 1996) and Down syndrome (Druzhyna et al., 1998). Moreover, mitochondrial variants and maternal genetic effects are strongly associated with human longevity (De Benedictis et al., 1999; Korpeilainen, 1999). It seems likely that mitochondria, control of ROS and life expectancy may be linked through common genetic systems controlling trade-offs between life span and reproductive output (Kirkwood and Kowald, 1997).

As mitochondria are clearly implicated in general ageing processes it is logical to suspect that mtDNA may also play a specific role in female reproductive ageing (Jansen, 1995; Janny and Ménézo, 1996; Jansen and de Boer, 1998; Kirkwood, 1998). A nested PCR strategy amplifying two-thirds of the mitochondrial genome has also been used to study human oocytes and embryos (Barritt et al., 1999b). These authors found rearrangements in 50.5% of the oocytes, declining significantly to 32.5% in the embryos, but there was no relation to maternal age. For patients with a mutation causing Kearns–Sayre syndrome, the same team also found significantly fewer mutations in embryos compared with oocytes (Brenner et al., 1998). This strongly implicates a role for mtDNA in determining oocyte fertilizability and embryo development. Once menopause occurs, there is an increase in the general ovarian levels of mtDNA deletion (Kitagawa et al., 1993; Suganuma et al., 1993). It has also been found (Keefe et al., 1995) that oocytes from older women were more likely to contain detectable levels of mtDNA deletions. Others (Muller-Hocker et al., 1996) found evidence of age-related increases in mitochondrial volumes in human oocytes, but were unable to relate this to changes in mtDNA or measurable OXPHOS capacity. Ageing in all mammals studied results in reduced oocyte fertility and increased levels of abnormal development (Foote, 1975; Adams, 1984). Moreover, age-related impaired protein synthesis and mitochondrial function play a role in the increased aneuploidy rates through altered maturation kinetics and spindle formation (Eichenlaub-Ritter, 1998).

Mitochondria and apoptosis

Apoptosis is the process of programmed cell death, either as a part of normal tissue differentiation, or as a means of eliminating defective cells. Caspases, a family of cysteine-dependent aspartate-specific proteases, are central to the control of this. There are two main groups. Initiator caspases, such as caspase-8 and caspase-9, function to activate other caspases. Executor caspases, such as caspase-3, −6 and −7, are responsible for dismantling cellular proteins.

There are at least three cellular control mechanisms (Mehmet, 2000). First, the plasma membrane can release proteins that trigger activation of caspases. In this process, an apoptosis-inducing signalling complex recruits caspase-8 after the binding of specific ligands oligomerizes ‘death receptors’. Second, the endoplasmic reticulum can independently activate caspase-12 following the disruption of ionic balance (Nakagawa et al., 2000).
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Third, and relevant to this review, mitochondria trigger apoptosis through disruption of redox potential, electron transport, OXPHOS and ATP production. Apoptosis results from a cascade initiated by caspase-9, activated when cytochrome c is released into the cytoplasm from the space between the inner and outer mitochondrial membranes.

Thus, leakage from dying mitochondria is an important event that triggers the cascade leading to apoptosis (Shimizu et al., 1999). This probably dates back to the ancestral endosymbiotic event, allowing 'the fundamental framework for bacterial warfare to be incorporated into the cell death mechanisms used by animal cells' (Green and Reed, 1998). This process is modulated by two sets of proteins. The Bcl-2 family inhibits cytochrome c release (Dell’Orco et al., 1996), whereas proteins that promote cell death (Bax and Bak) stimulate opening of a voltage-dependent voltage channel (porin) causing water to enter the mitochondrion. This swells and dies, allowing intra-organelle factors to escape (Martinoiu, 1999). Homeostasis between cell death and cell proliferation probably relies on heterodimerization between Bcl-2 and Bax (Kroemer et al., 1997). These events lead to caspase activation with secondary endonuclease activation and consequent DNA fragmentation (Zamzami et al., 1997; Trbovich et al., 1998). There is, however, recent evidence that apoptosis induced by the p53 transcription factor can occur despite the lack of cytochrome c release into the cytosol, possibly by modulating mitochondrial membrane potential via ROS release (Li et al., 1999).

Oogenesis

It is now clear that there is considerable plasticity and capacity for self-repair in the mammalian oocyte. There are well-defined cytoplasmic axes and gradients (Antczak and Van Blerkom, 1997; Edwards and Beard, 1997). These help to determine cleavage planes and the fate of components in later embryonic development (Gardner, 1997). Moreover, these axes appear to be predetermined by follicular factors such as blood supply and oxygen availability during follicle growth (Van Blerkom, 1998). At ovulation, oocytes contain around 100 000 mitochondria, but these are structurally undifferentiated and generate low concentrations of ATP in oocytes and early embryos, compared with later stages (Dvorak and Tesarik, 1985; Van Blerkom, 1989). The mitochondria undergo marked microtubule-mediated redistribution in the maturing oocyte and early embryo, presumably in response to localized energetic needs (Muggleton-Harris and Brown, 1988; Pozo et al., 1990; Barnett et al., 1996; Van Blerkom et al., 1998). Disruption of this process adversely affects chromosomal organization and segregation (Van Blerkom, 1991). There is also significant fusion between mitochondria at around the time of ovulation, so that overall numbers are reduced by about one-third (Cran, 1987; Smith and Alcivar, 1993). There are significant differences in net ATP content between oocytes, and within and between individuals, that reflect later embryo developmental potential (Van Blerkom et al., 1995). Moreover, completion of maturation is critically dependent on a correct nucleocytoplasmic volume ratio (Karlikova et al., 1998). This possibly affects the ability to support normal chromosomal segregation (Gaulden, 1992). Reduced ATP content produced by uncoupling OXPHOS in oocytes does not affect fertilization, but reduces later embryo development rates (Van Blerkom et al., 1995). Declining mitochondrial function in older women may also contribute to declining fertility (Keefe et al., 1995), and this would be consistent with the general role of mitochondria in life span determination discussed earlier (Kirkwood and Kowald, 1997).

Almost all oocytes are eliminated by apoptosis during atresia in fetal and adult life. One essential unresolved question is whether selection based on mtDNA plays any role in this (Jansen and de Boer, 1998; Short, 1998; Krakauer and Mira, 1999; Shoubridge, 1999; Cummins, 2000b). If there were such selection, it would then serve as a fail-safe mechanism to reinforce the power of the germline bottleneck to select for mitochondrial uniformity and integrity in the oocyte and embryo. Given the quiescent picture of the oocyte’s mitochondria discussed above, it is difficult to see how this could work, and the oocyte itself is insulated in a cocoon of granulosa cells from the earliest days of ovarian development. However, there are several working hypotheses that could be tested. The critical phase to examine would probably be the period when primordial oocytes enter the FSH-responsive growth phase, which is when apoptotic atresia commences (Morita and Tilly, 1999). First, the ovary may be testing for general levels of oxidative capacity in terms of ATP production, as this would determine the ability to grow in response to FSH (Van Blerkom et al., 1995). While meiotic arrest in oocytes is maintained by high cAMP concentrations (Downs et al., 1989), aberrant cAMP concentrations can also accelerate apoptosis in mature follicles (Amsterdam et al., 1999). Second, the ovary may rely on tonic calcium concentrations as a measure of oocyte fitness: calcium homeostasis and mitochondrial metabolism are closely interwoven (McCormack and Denton, 1993), and mosaic patterns of free calcium alterations and mitochondrial damage can be seen in neurodegenerative states (Itoh et al., 1996). This would in a sense foreshadow events that follow fertilization, where impaired calcium signals are implicated in abnormal embryo development and death (Tesarik, 1999). Third, the ability of the growing follicle to respond to FSH by oestrogen production, via the mitochondrial cytochrome P450 side-chain cleavage enzymes, may be impaired. However, this is strictly a function of the granulosa cells and not the oocyte itself (Stocco, 1999). Fourth, there may also be mitochondrial involvement in the production of meiosis-activating sterols (Byskov et al., 1999).

Various studies are beginning to resolve some of these questions. One group (Zhang et al., 1999) studied meiotic maturation in human oocytes reconstructed by germinal vesicle transfer between women of different age groups (>38 and 31 years old respectively), and found normal maturation rates and meiotic chromosomes. Given the quiescent nature of the oocyte’s mitochondria, the significance of this observation for evaluating later nuclear–mitochondrial interactions is, as yet, unclear.

Embryogenesis

The importance of mitochondrial health for the embryo is obvious, and most mitochondrial mutations outside the hypervariable D-loop are probably eliminated by embryo death (Wallace et al., 1995). The importance of cytoplasmic factors, and cytoplasmic ATP for growth and development have been reviewed (Smith and Alcivar, 1993; Van Blerkom et al., 1998). Compared with
most cell systems, the exact OXPHOS energetic requirements for various embryo stages are not particularly well characterized across mammals (Barnett and Bavister, 1996). However, among the species that have been studied, such as bovine (Thompson et al., 1996), rodent (Brison and Leese, 1991; Leese, 1991) and human (Leese et al., 1998), there is generally a shift in ATP production from oxidative metabolism to glycolysis during the first three to four cell divisions. This may anticipate low partial pressures of oxygen in the uterine cavity. Indeed, optimal oxygen concentrations during embryogenesis appear to be critical for normal embryogenesis, and atmospheric levels (21%) may even inhibit normal development to the blastocyst stage. The importance of these shifts in embryo metabolism, with a shift from pyruvate to glucose metabolism, is now recognized by the new multi-phase human embryo culture media (Gardner et al., 1998). Moreover, there are clear differences between in-vivo- and in-vitro-produced embryos, particularly as development proceeds. In cattle, for example, aerobic glycolysis is 2-fold higher in in-vitro-produced embryos compared with those produced in vivo (Khurana and Niemann, 2000). It is not clear what role mitochondria play in these developments. The mtDNA does not replicate in early embryogenesis, although both nuclear and mitochondrial genes commence transcription from the 2-cell stage in the mouse. By the blastocyst stage there is 30-fold increase in nuclear-encoded respiratory chain peptides, accounting for 3.5–7% of the total protein synthesis (Taylor and Pikó, 1995). Significant variations have been found in embryonic metabolism and development rates in relation to oxygen concentration in congenic mouse constructs, where the mtDNA differs from that of the nucleus (Nagao et al., 1997, 1998b). Moreover, subsequent bioenergetic performance as adults can be impaired in such animals (Nagao et al., 1998a). This re-emphasizes the need for congruence between nuclear and mitochondrial genes in development. Elucidating such interactions between mitochondrial and nuclear genomes and embryo metabolism is critical if embryo growth rates are to be improved in vitro. It may also help in understanding the puzzling observations on altered ruminant fetal growth patterns following brief periods of embryo culture in vitro (Thompson et al., 1995). Death of embryos is accompanied by apoptosis and the activation of death regulatory proteins, but the role of mitochondria in this is not yet well defined (Jurisicova et al., 1998).

Implications for infertility treatment

Male subfertility and sperm dysfunction are associated with defective mitochondrial function and reduced copy numbers (Folgerù et al., 1993; St John et al., 1997; Kao et al., 1998; Wei and Kao, 2000). Concerns have therefore been raised that intracytoplasmic sperm injection (ICSI) with dysfunctional spermatozoa might result in the evasion of embryonic recognition mechanisms and transmission of abnormal paternal mtDNA, with subsequent detrimental effects on the embryo (Lestienne et al., 1997). Others (St John et al., 2000b) have suggested that spermatozoa from such men may be particularly susceptible to free radical attack associated with reduced inner mitochondrial membrane potential. There is a recent report that paternal mtDNA can be detected in abnormal (polyploid) human embryos (St John et al., 2000a); moreover there is a correlation between levels of fragmenting DNA, measured by COMET and terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling (TUNEL) assays, and dysfunctional mitochondria in human spermatozoa (Donnelly, personal communication). Dysfunctional spermatozoa may also evade normal apoptotic surveillance systems in the testis, and thus transfer damaged DNA, with negative effects on embryonic development (Sakkas, 1999; Sakkas et al., 1999). These observations, taken together with the claimed potential for recombination between paternal and maternal mtDNA, either directly or via a nuclear route (Awadalla et al., 1999), suggest that the use of severely dysfunctional spermatozoa for ICSI should be regarded with caution. Despite these concerns, there is no clinical evidence yet that paternal mitochondria survive following ICSI (Houshmand et al., 1997; Danan et al., 1999).

Mitochondria: inheritance, embryogenesis and cloning

Implications for cloning, cytoplasmic transfer and cell fusion

Experimental chimeras have been around for nearly 40 years (Tarkowski, 1998), but the recent development of cloning from somatic cell nuclei has revolutionized experimental embryology (Wilmut et al., 1997; Wakayama et al., 1998). The term ‘clone’ derives from the Greek ‘klon’, a twig or slip. Merriam-Webster (http://www.m-w.com/home.htm) defines it as ‘the aggregate of the asexually produced progeny of an individual’. Strictly speaking, the animals that have been produced by somatic cell nuclear transfer over the past couple of years are not true clones, but are better described as ‘genomic copies’ as they are mosaics of cytoplasmic and nuclear elements from differing sources (Campbell, 1999). There are many possible permutations. Generally, to arrive at a combination that will support embryo development, the recipient cytoplasm is an activated enucleated oocyte (Campbell, 1999; Wakayama and Yanagimachi, 1999). However, there are several possibilities for a source of introduced genetic material: nuclear transfer alone; transfer of a karyoplast (nucleus plus a small amount of cytoplasm and associated mitochondria); or complete fusion with a somatic cell. One would predict varying outcomes for the fate of introduced mitochondria, depending on chance association with existing oocyte polarity and axes (Edwards and Beard, 1997) or proximity to the eventual nucleus. This is true for mice when karyoplast or cytoplast transfer is used to produce heteroplasmy. In such constructs there is high variability in the mitochondrial genotypes of the progeny. There is evidence of occasional stable heteroplasmy, probably resulting from mitochondrial fusion (Meirelles and Smith, 1997, 1998). It is known that mitochondrial proliferation in general occurs first in those closest to the nucleus (Shadel and Clayton, 1997), and of course proliferation itself does not normally start until the hatched blastocyst or egg cylinder stage, as discussed earlier. While ‘Dolly’ the sheep was created by fusing a mammary fibroblast with an enucleated oocyte (Campbell et al., 1996), there is no evidence that the transferred mitochondria survived (Evans et al., 1999), and this is a surprisingly common finding. Thus, others (Takeda et al., 1999) found that the recipient oocyte mitochondria dominated in cloned cattle. In addition, low levels of transmission of mtDNA were observed in cows produced by cytoplast–blastomere fusion, but this level varied markedly according to the stage of development of the donor cell (Steinborn et al., 1998a,b). Varying levels of heteroplasmy were also found
in cloned cows produced by blastomere transfer (Hiendleder et al., 1999). It is worth reiterating that heteroplasmy may be naturally higher than hitherto suspected (Grzybowski, 2000).

This field is changing rapidly as new models are explored, and one group (Takeda et al., 2000) has found preferential replication of RR strain mtDNA in heteroplasmic embryos produced by fusion with C57BL.6 strain mouse embryos. At the time of completing this review, the only clear picture that emerges is that the outcome and eventual dominance or disappearances of different mitochondrial genotypes are difficult to predict from the nuclear genome.

Cytoplasmic transfer can be used to create experimental heteroplasmic ‘transmitochondrial’ mice (Irwin et al., 1999). This approach has recently been advocated as a means of achieving pregnancy for women with repeated failed IVF due to poor oocyte or embryo quality. The concept is one of ‘rescuing’ embryos by improving the quality of the cytoplasm (Cohen et al., 1997, 1998; Alikani et al., 1999; Huang et al., 1999; Lanzendorf et al., 1999). Similar interventions have been reported to remove abnormal or fragmenting cytoplasm (Alikani et al., 1999) or to restore euploidy by removal of excess pronuclei (Cohen et al., 1994). The birth of children has been reported following transfer of donor cytoplasts (Cohen et al., 1998). In the first case studied, analysis of the mtDNA showed that the fetus reverted to maternal type (Cohen et al., 1997), while an unconfirmed report indicated that children born may be heteroplasmic (Barratt et al., 1999a), a surprising finding given the enormous natural selective pressures that have evolved apparently to eliminate or minimize this state, as discussed earlier.

One clear message emerging from the animal cloning work is that it is extremely inefficient: only 1–2% of nuclear transfer clones survive to birth. There is unexpectedly high embryonic wastage at all stages and marked variation from normal body size, together with growth and immune system abnormalities in many of the survivors (Campbell, 1999; Wakayama and Yanagimachi, 1999). Some of these anomalies may be related to adverse epigenetic effects of culture conditions (Young et al., 1998; Sinclair et al., 1999), or to problems with genome imprinting (Latham, 1999). However, incompatibility between nuclear and cytoplasmic genes is also likely (Gartner et al., 1998). Only further research will clarify this puzzling and rather alarming series of outcomes, if reproductive as opposed to therapeutic cloning is to come of age (Australian Academy of Science, 1999).

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Note added in proof

A recent comprehensive evaluation of the human mitochondrial genome found no evidence that recombination has played any significant role in the evolution of human mtDNA and gives support to the idea of a relatively recent African origin of Homo sapiens with a common origin at 52,000 ± 27,500 years ago (Ingman et al., 2000). However, the detection of an extinct lineage of mtDNA in an anatomically modern Australian Aboriginal individual from Lake Mungo, dated to ≈60,000 years ago, challenges this conclusion (Adcock et al., 2001).

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