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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 *Paramecium* (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 homoplasmy (single haplotype), but this might be over-emphasized. Heteroplasmy (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 components mutate up to 100 times more rapidly than nuclear DNA (Pesole *et al*., 1999). Contrary to many texts, mitochondrial DNA has a range of endogenous repair mechanisms only slightly more restricted than nuclear DNA (Croteau *et al*., 1999). Many reports have been published on the detection of mtDNA adducts as a result of cumulative oxidative damage with age (Beckman and Ames, 1996).

Evolution of mitochondria

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 (Berner, 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 $OH\cdot$, $O_2^{-}\cdot$ and H_2O_2, 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 10^7 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., 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 radical-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 organisms contains many active mitochondrial genes, together with truncated or rearranged sequences homologous to mtDNA (Thorsness and Weber, 1996). There are many possible mechanisms and pathways for exchange to have occurred. These include temporary openings in the mitochondrial membranes during fusion and/or budding processes, degradation followed by release of mtDNA to the cytoplasm, illicit use of nucleic acid or protein import machinery, or fusion between nuclear and mitochondrial membranes. Similar unusual events may help to explain recent evidence for rare mtDNA recombination events in human populations (Awadalla *et al*., 1999; Eyre-Walker *et al*., 1999a; Hagelberg *et al*., 1999), which have challenged the accuracy of molecular clocks used for taxonomic reconstruction (Strauss, 1999). Moreover, recent work using long polymerase chain reaction (PCR) has identified paternal mtDNA in abnormal (polyploid) human embryos (St John *et al*., 2000a,b). Studies in yeast have shown that the rate of transfer of mitochondrial gene-sized DNA fragments to the nucleus is approximately equivalent to the rate of spontaneous mutation of nuclear genes (Thorsness and Weber, 1996). This history of active interchange between the nuclear and mitochondrial genomes has implications for the clinical investigation of mitochondrial diseases. It is important to distinguish between pathogenic mutations in the mtDNA itself as opposed to 'fossil' nuclear pseudogenes (Wallace *et al*., 1997; Herrnstadt *et al*., 1999). The bottom line is that for human (and probably most vertebrates), the long processes of evolution appear to have whittled mtDNA down to a bare minimum, with little room for error except in the hypervariable 'control' region used for taxonomic purposes. The lack of tolerance for mutations, deletions or heteroplasmy probably explains why natural selection exerts such extreme controls against variation or heteroplasmy within the organism. Indeed, birds appear to be even less tolerant of mtDNA mutations than mammals (Stanley and Harrison, 1999). On a cautionary note, heteroplasmy may be more widespread than usually reported: a recent study documents high levels of heteroplasmy in human hair roots (Grzybowski, 2000).

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 spermatozoa 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 heteroplasmy 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 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

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 homoplasmy, supporting the general hypothesis that selective pressure acts to minimize heteroplasmy (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 (Blok *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 ρ°, 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 heteroplasmy 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 microcell 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 nucleocytoplasmic 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 passaged 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 heteroplasmic 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; Gershon, 1999; 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; Korpelainen, 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).

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 (Martinou, 1999). Homeostasis between cell death and cell proliferation probably relies on heterodimerism 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 (Karnikova *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 (Folgerø *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).

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 cytoplast 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 (Barritt *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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