Fertilization and elimination of the paternal mitochondrial genome

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With rare exceptions, mammalian mitochondria are inherited through the female. This probably serves to minimize lethal cytoplasmic gene competition and to prevent the inheritance of sperm mitochondrial DNA that has been subject to degradation by free radicals. In general, organisms are intolerant of mitochondrial heteroplasmy and, when this occurs in humans, it frequently presents as progressive and lethal bioenergetic or neurological disease. The mitochondria of spermatozoa are specifically destroyed by proteolysis in early embryonic development, in mice at the 4-to-8-cell transition. While there are concerns in human assisted reproduction that microinjection of abnormal or immature sperm cells could lead to lasting harm in the offspring through transmission of abnormal mitochondria, there is no clinical evidence to support this. There is more potential for harm through attempts to ‘rescue’ poor quality oocytes by cytoplasmic or nuclear transfer, as it is not currently possible to control the final fate of the donated mitochondria in relation to nuclear-mitochondrial interactions or the embryonic axes. Moreover, the balance between nuclear and mitochondrial genes and the role of cytoplasmic factors in epigenesis are still poorly understood. The future challenge for biologists is to comprehend the nature of the selective destruction of paternal mitochondria, as it appears to be a species–specific recognition phenomenon.

Key words: embryogenesis/fertilization/maternal inheritance/mitochondrial DNA/mitochondrial transfer

Introduction

This review will attempt to summarize the latest knowledge on the nature of mammalian mitochondrial inheritance and its implications for human assisted reproductive technology. There are comprehensive accounts of the place of the mitochondria in the life cycle (Smith and Alcivar, 1993; Ozawa, 1997; Cummins, 1998a). Epigenesis and genomic imprinting in relation to mitochondrial inheritance will be only briefly touched upon; the interested reader should consult recent reviews (John and Surani, 1996; Latham, 1999).

Mitochondrial inheritance: an overview

Mitochondria exist as a set of semi-autonomous organelles with their own restricted genome of 16.6 kb in humans (much larger
genomes are found in plants and invertebrates). Ancestrally, mitochondria probably derived from endosymbiotic purple bacteria as a means of exploiting oxygen (Margulis and Sagan, 1986; Gray, 1992; Taanman, 1999). In contemporary eukaryotes, mitochondria have sacrificed much of their genome to the somatic cell nucleus and serve as ‘slave’ organelles, carrying out the risky business of oxidative phosphorylation well away from the free-radical-sensitive ‘master’ nuclear genome. Approximately 200 control genes are found in the nucleus, compared with only 37 in the highly compressed genome of the mitochondrion (Shadel and Clayton, 1997; Taanman, 1999). Control is therefore complicated, involving much exchange of information between the nucleus and the many copies of mitochondrial (mt) DNA in each cell’s cytoplasm (Poyton and McEwen, 1996; Shadel and Clayton, 1997). With very rare exceptions, mitochondria and other cytoplasmic factors (e.g. centrioles) are inherited uniparentally in eukaryotic organisms (Birky, 1995; Simerly et al., 1995; Hewitson et al., 1998). For mitochondria, transmission is normally through the female germ cell line, but there are examples of paternal inheritance (for example some conifers in which the male gamete forms a pollen tube). There are some unusual modes of mixed inheritance, for example in mussels, in which paternally derived mitochondria are transmitted to male offspring, but maternal mitochondria pass to both genders (Zouros et al., 1992). However, these seem to be exceptions to the general rule. The mechanisms that prevent dual parental inheritance are extremely diverse and have probably evolved separately many times (Birky, 1995). The evolutionary reasons for this are obscure. It has been suggested (Hurst, 1992; 1995) that it may serve to avoid lethal genome conflict among subservient yet essential organelles. An alternative and perhaps more readily testable hypothesis is that the spermatozoon’s mtDNA is damaged by reactive oxygen species (ROS) during its life and would thus be detrimental to the embryo if allowed to contribute to the zygote (Cummins et al., 1994; Allen, 1996; Cummins, 1998a). The asymmetric life history between maternally and paternally derived mitochondrial DNA could even be central to the evolution of two genders with their complementary life histories (Short, 1998). The ancestral experimentation that resulted in the present combination of meiosis, amphi-mixis and amphi-hygeny among contemporary eukaryotes, thus preceded later developments such as the evolution of genomic imprinting in eutherian mammals (Bell, 1982; Margulis and Sagan, 1986; Latham, 1999).

A healthy complement of founding mitochondria is essential for embryonic growth and fitness. This is apparently accomplished by a stringent genetic bottleneck in early embryogenesis and is possibly reinforced by the selective elimination of oogonia with defective mtDNA (Cummins, 1998a; Jansen and de Boer, 1998). Various lines of evidence indicate that mitochondria in oogonia and the early embryo are quiescent and hence relatively unlikely to engender damaging ROS (Allen, 1996). Indeed replication of mtDNA in the mouse embryo does not start until the egg cylinder stage at day 6.5 (Píko and Matsumoto, 1976; Pikó and Taylor, 1987; Taylor and Pikó, 1995). One report suggests that all mtDNA in the mature human may effectively derive by clonal expansion from a single precursor copy (Blok et al., 1997), but others report higher source numbers (Jenuth et al., 1996). The bottleneck, coupled with stringent selection, also serves to counteract Muller’s ratchet: the inexorable accumulation of deleterious mutations in asexually reproducing organisms (Bergstrom and Pritchard, 1998). The evidence linking oocyte atresia with specific mtDNA defects, as opposed to simple ageing, is scarce (Chen et al., 1994; Keefe et al., 1995; Brenner et al., 1998).
Nevertheless this is an area that deserves more research as it may give a key to the diminishing fertility of the mammalian oocyte with age (Adams, 1984; Jansen and de Boer, 1998).

Many aspects of mitochondrial function that are ‘standard’ in undergraduate texts may now be over-simplifications or simply untrue. For example, the common assertion that mitochondria lack mechanisms to repair oxidative damage to DNA appears to be false, as they possess efficient base and nucleotide excision repair pathways (Bohr et al., 1998; Bohr and Dianov, 1999). It is also commonly stated that mtDNA mutates much more rapidly than nuclear DNA. This is true as a statement of averages, but the mutation rate is actually highly variable across the mitochondrial genome. Some regions show nucleotide substitution rates similar to nuclear DNA, whereas synonymous sites and small rRNAs mutate ~20 times more rapidly and tRNAs ~100 times more rapidly than in their nuclear counterparts (Pesole et al., 1999). The doctrine that mtDNA does not show recombination is also shaky. Mitochondria possess the requisite enzyme systems in vitro (Thyagarajan et al., 1996) and two very recent independent studies have documented recombination in human populations (Eyre-Walker et al., 1999; Hagelberg et al., 1999). As mtDNA is normally sequestered inside a relatively impermeable double capsule, it is difficult to see how recombination could occur without fusion of paternal and maternal organelles early in development (see below). These observations have yet to be absorbed by molecular biologists attempting to reconstruct ancestral human populations based on mtDNA (and Y chromosome) sequences (Seielstad et al., 1998). Assumptions about the steadfastness of the mtDNA and Y chromosome ‘molecular clocks’ are evidently under challenge.

Sperm mitochondrial fate after fertilization

In the majority of mammals, the entire sperm tail enters the oocyte at fertilization. Although this has been known since the last century, it has been overlooked by the present generation of molecular biologists, intent on validating the ‘African Eve’ theory of human origins based on exclusive maternal inheritance of mtDNA (Ankel-Simons and Cummins, 1996). However, except in unusual cases such as inter-species crosses (Gyllensten et al., 1985, 1991; Kaneda et al., 1995; Shitara et al., 1998), the mitochondria apparently do not normally survive.

In most embryos, the sperm midpiece can be seen to degenerate within 1–2 days of fertilization (Cummins, 1998a). This is not merely a temporal decay. In mouse embryos injected with spermatozoa bearing fluorescently labelled mitochondria, intact midpieces have been observed up to the 4-cell stage (day 2) but no later. In some cases, however, mitochondria have persisted for up to 5 days, but only in embryos that had their development arrested at the 4-cell stage or earlier (Cummins et al., 1997). This suggests that disappearance of the mitochondria might be keyed in to critical phases of the second mitotic division, as observed in other mammals (including humans) that have been studied (Cummins, 1998a). Similar results have been obtained using spermatids (Cummins et al., 1998b) and spermatocyte cytoplasm (Cummins et al., 1999), suggesting that whatever destines sperm mitochondria for destruction in the embryo is established by the time cells are committed to spermatogenesis. Kaneda et al. concluded that destruction of the sperm’s mitochondrion probably depends on nuclear-encoded factors and not on recognition of the mtDNA itself; this was because the paternal mtDNA could be detected in inter-specific crosses of *Mus musculus* with *M. spretnus*, but could not be detected in crosses of *M. musculus* with the congenic strain b6.mt(spr), which carries *M. spretnus* mtDNA on a background of *M. musculus* nuclear genes (Kaneda et al., 1995). The same group found that paternally
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derived mtDNA in interspecies crosses does not persist beyond the F1 generation (Shitara et al., 1998), but this has yet to be confirmed by independent investigators.

There are several possible reasons for the lack of persistence of paternal mtDNA (Ankel-Simons and Cummins, 1996; Cummins, 1998a). One is that simple dilution of the 75 or so sperm mitochondrial copies among the 100,000 (or more) of the oocyte makes it very difficult to detect the paternal contribution by current molecular techniques such as PCR, which tends to amplify the dominant DNA sequence selectively in a mixed sample. Alternatively, the sperm mtDNA could be simply degraded by excessive exposure to ROS (Allen, 1996; Aitken et al., 1998) or unequally sequestered to a region of the forming embryo that does not contribute to the inner cell mass (Birky, 1983, 1995). Sperm mitochondria seem inherently dysfunctional as, unlike somatic cell mitochondria, they cannot colonize mitochondrially depleted cell lines (King and Attardi, 1989).

The most convincing evidence for an active as opposed to a passive mechanism of sperm midpiece destruction was reported by Sutovsky (from the Oregon Regional Primate Center, USA) at the 38th Annual Meeting of the American Society for Cell Biology in San Francisco in 1998 (cited by Hopkin, 1999). Sutovsky had previously suggested that spermatozoa could be tagged with ubiquitin and thus targeted for degradation in the zygote by 26S proteasome (Sutovsky et al., 1996, 1997b; Sutovsky and Schatten, 1997). This is an attractive hypothesis, as ubiquitin-mediated degradation is seen in a wide range of cell regulation processes, including the recycling of cell cycle components, class I antigen turnover, receptor-mediated endocytosis, and signal transduction pathways (Hochstrasser, 1996). Fluorescent antibodies to ubiquitin were observed on the developing sperm midpiece in the testis. Although mature spermatozoa do not fluoresce, the signal reappears after the spermatozoon enters the oocyte. Sutovsky therefore postulates that the polypeptide-linked ubiquitin is masked on the spermatozoa by disulphide bonds during epididymal maturation and that these are reduced by high levels of glutathione after oocyte penetration (P. Sutovsky, personal communication). While this intriguing finding needs independent confirmation, it seems a very plausible mechanism. As ubiquitin is likely to be present in the early mitotic phase of spermatogenesis (for cell cycle component turnover), it would entail a simple set of selective steps to enable it to persist on the midpiece and thus ensure later destruction by the embryo. It is still not known what aspect of the mitochondrial sheath would be targeted, but the sperm mitochondria are significantly modified during spermatogenesis and develop some unique haploid-encoded products, including a cysteine-rich selenoprotein capsule protein (Cataldo et al., 1996).

The question remains of how recombination events can (rarely) occur between sperm and oocyte mitochondria. One possibility suggested by Sutovsky (personal communication) is that soon after fertilization a few mitochondria are displaced from the neck region to allow contact between the sperm centriole and the oocyte cytoplasm (Sutovsky et al., 1996, 1997a,b; Sutovsky and Schatten, 1997). In most mammals (but not the murid rodents), microtubule formation catalysed by the sperm centriole serves to bring the male and female pronuclei into apposition and to form the first cleavage spindle apparatus (Simerly et al., 1996; Hewitson et al., 1997). One or more of these early-departing mitochondria might evade proteolysis by fusing with an oocyte mitochondrion to establish a heteroplasmic founder line. Survival would be most unlikely, as the overall contribution of mtDNA to the zygote would be <1 in 100,000 (Ankel-Simons and Cummins, 1996). However this is approximately the same order of magnitude.
Table I. Outcome of mitochondrial transfer to mouse zygotes

<table>
<thead>
<tr>
<th>Mitochondrial source</th>
<th>Technique</th>
<th>Tracking method</th>
<th>Survival of mitochondria</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testis, liver</td>
<td>Microinjection</td>
<td>PCR</td>
<td>No</td>
<td>Ebert et al. (1989)</td>
</tr>
<tr>
<td>Liver, different strain</td>
<td>Microinjection</td>
<td>PCR</td>
<td>Yes</td>
<td>Pinkert et al. (1997)</td>
</tr>
<tr>
<td>Spermatocytes</td>
<td>Microinjection</td>
<td>PCR</td>
<td>No</td>
<td>Cummins et al. (1999)</td>
</tr>
<tr>
<td>Spermatids</td>
<td>Microinjection</td>
<td>PCR</td>
<td>No</td>
<td>Cummins et al. (1998b)</td>
</tr>
<tr>
<td>Spermatozoa</td>
<td>Microinjection</td>
<td>MitoTracker®</td>
<td>No</td>
<td>Rinaudo et al. (1999)</td>
</tr>
<tr>
<td>Human MELAS patient</td>
<td>Microinjection</td>
<td>PCR</td>
<td>Yes, but variable results</td>
<td>Jenuth et al. (1996) (1997)</td>
</tr>
<tr>
<td>Mouse zygotes</td>
<td>Karyoplast/cytoplast fusion</td>
<td>PCR</td>
<td>Yes</td>
<td>Meirelles and Smith (1997 (1998))</td>
</tr>
</tbody>
</table>

PCR = polymerase chain reaction; MELAS = mitochondrial encephalomyopathy with lactic acidosis and stroke-like events.

Experimental mitochondrial transfer

Mitochondrial inheritance has now been studied in a variety of cells and tissues. The results of transfer from various sources to embryos are summarized in Table I. It is clear that mitochondria from all stages of spermatogenesis are unique in failing to persist following transfer. The fate of transferred or fused somatic and zygotic mitochondria is highly variable. This is almost certainly due to fortuitous placing of the mitochondria with relation to the nucleus and to the developing embryonic axes. Mitochondrial replication in cells starts in cytoplasmic regions closest to the nucleus, probably as a result of diffusion or transport of critical control factors (Shadel and Clayton, 1997). It is also now recognized that, like the non-mammalian vertebrates, mammalian oocytes and embryos have well-defined axes: these determine the planes of cleavage and the fate of blastomeres destined to form the inner cell mass, as distinct from the trophoderm (Edwards and Beard, 1997; Antczak and Van Blerkom, 1997, 1999; Van Blerkom, 1998).

The presence of embryonic axes and tissue commitment at such an early stage of development raises a central question about the inheritance of mitochondria, as the next generation can only derive from the germ cell lineage (Weissman, 1891). In Drosophila, the gametes derive from early ‘pole cells’ formed in the embryo. In Caenorhabditis elegans and in zebra-fish, asymmetric segregation of cytoplasm during consecutive cell divisions eventually results in a distinct germ cell lineage. In mice and presumably in all mammals, germ cells appear to be delineated relatively late in development, midway through gastrulation, at ~7.25 days after fertilization (thus <1 day after the beginning of mtDNA replication); they can be detected as large, alkaline-phosphatase cells in the yolk sac close to the forming allantoid (Byskov, 1982). McLaren suggests that germ cell status might be the result of differentiation in response to local signals, possibly from somatic tissues such as the trophoderm and primitive endoderm (McLaren, 1999). In this case, germ cell formation, and hence the transmission of mito-
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Implications for human assisted reproductive technology

There have been concerns expressed that intracytoplasmic sperm injection (ICSI) with abnormal spermatozoa might lead to long-term harm in the offspring through transmission of abnormal mitochondria, although the evidence for this so far is weak (Johns, 1995; Houshmand et al., 1997; Cummins, 1998a). There is evidence that spermatozoa even from normal men contain significant levels of mtDNA deletions (Cummins et al., 1998a) and these may be elevated in men with poor semen quality and infertility (Kao et al., 1995, 1998). On a cautionary note, some authorities consider the evidence linking mtDNA deletions and mutations with ageing (as opposed to disease) to be poor and to be based on non-quantitative measure (Lightowlers et al., 1999). We have found, using competitive PCR, that conventional semi-quantitative PCR approaches (Zhang et al., 1996) can grossly overestimate (up to 1000-fold) levels of deletions in rat mtDNA (Ahmed et al., 1999) and in human mtDNA (D.Mehmet, unpublished observations). On balance, however, there seems to be little risk in using abnormal or even immature sperm cells for ICSI, at least with regard to transmission of abnormal mtDNA. As discussed earlier, the paternal mitochondrial line seems irreversibly committed to suicide in the embryo and any leakage of abnormal mitochondria would be rare and probably rapidly eliminated by natural selection during embryogenesis. There is probably more risk of transmitting underlying genetic disease and fragmented nuclear DNA, as men with severe infertility may possess significantly increased levels of chromosomal and meiotic abnormalities (Retief, 1986; Cummins and Jequier, 1995; Cummins, 1998b; Cummins and Jequier, 1998; Vendrell et al., 1999).

Of greater concern are attempts by clinics to ‘rescue’ poor quality oocytes and embryos...
by cytoplasmic transfer (Cohen et al., 1997, 1998) or nuclear transfer (Zhang et al., 1999). These must be seen as risky and highly experimental procedures. Despite a long history of experimental embryology, we still understand very little about the balance between nuclear and mitochondrial genes and the relative roles of paternally and maternally derived nuclear genes in development (John and Surani, 1996; Narasimha et al., 1997; Latham, 1999). Moreover, as outlined above, it is not currently possible to control the final fate of the donated mitochondria in relation to nuclear–mitochondrial interactions or the embryonic axes, so the outcome of donation would be highly unpredictable. Similar problems beset the creation of heteroplasmic mice by karyoplast and cytoplast fusion, the only good animal models currently available (Meirelles and Smith, 1997, 1998).

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References

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