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Fate of microinjected spermatid mitochondria in the mouse oocyte and embryo

James M. Cummins[1], Teruhiko Wakayama[2] and Ryuzo Yanagimachi[2]
School of Veterinary Studies, Murdoch University, Murdoch, Western Australia, and Department of Anatomy and Reproductive Biology, University of Hawaii Medical School, Honolulu, Hawaii, USA

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Summary

Mouse round spermatids labelled with MitoTracker were microinjected into Sr^{2+}-activated mouse oocytes. The labelled mitochondria were tracked up to the morula/blastocyst stage using fluorescence microscopy. The overall incidence of embryos with labelled mitochondria fell from 80% in the 1-cell zygote to 25% in 2-cell, 9% in 4-cell and ~1% in 8-cell or later stages. Thus it appears that almost all round spermatid mitochondria finally disappear from embryos during the 4-cell to 8-cell transition, as happens for mature spermatozoa (Cummins *et al. Zygote* 1997, **5**: 301–8). The spermatid mitochondria remained tightly bound together during this process. In contrast, labelled primary spermatocyte and cumulus mitochondria dispersed rapidly throughout the oocyte cytoplasm within 3 h. We hypothesise that spermatid mitochondria may be bound together by cytoskeletal elements produced in the early haploid spermatid. These elements, together with terminal differentiation of the sperm mitochondria, may be central to the processes by which the embryo 'recognises' the sperm mitochondria and inhibits inheritance of paternal mitochondrial DNA. These results suggest that round spermatid injection for infertile men will not pose a significant risk to offspring by transmitting abnormal mitochondrial genomes.

Keywords: Cytoplasmic inheritance, Embryo, Midpiece, Mitochondrial DNA, Spermatid

Introduction

In a previous paper we described the fate of MitoTracker-labelled microinjected mouse sperm components in embryos and non-activated oocytes (Cummins *et al.*, 1997). Briefly, mouse sperm mitochondria largely disappear during the 4- to 8-cell transition (day 3 of development), but persist for up to 5 days in non-activated oocytes and in arrested 2- and 4-cell embryos. This confirms previous structural and immunocytochemical observations in the rat (Szöllösi, 1965; Shalgi *et al.*, 1994), hamster (Hiraoka & Hirao, 1988), rabbit (Bedford, 1972) and cattle (Sutovsky *et al.*, 1996). paternal mitochondria appear to be specifically targeted for destruction, as other thiol-rich sperm components such as the perforatorium and axonemal coarse fibres persist to the blastocyst stage and possibly later (Cummins *et al.*, 1997).

The mechanisms by which the mitochondria of one parent (usually paternal but occasionally maternal) are eliminated are obscure and extremely diverse in eukaryotic organisms (Birky, 1995; Ankel-Simons & Cummins, 1996). The nearly universal existence of this phenomenon suggests there must be good evolutionary reasons for avoiding heteroplasmy from biparental inheritance of cytoplasmic genes. Hurst (1992) has suggested that this set of strategies serves to minimise the chances of lethal conflict between differing sets of subservient cytoplasmic genomes. Certainly in humans, individuals born heteroplasmic for mitochondrial DNA are rare and this condition is usually associated with early death due to mitochondrial disease arising as *de novo* mutations in the oogonial stem cell line (Shoffner & Wallace, 1990; Ozawa, 1997).

All correspondence to: J.M. Cummins, PhD, Division of Veterinary and Biomedical Sciences, Murdoch University, Murdoch, Western Australia 6150. Telephone: +61–8–9360 2668. Fax: +61–8–9310 4144. e-mail: cummins@central.murdoch.edu.au.
[1]School of Veterinary Studies, Murdoch University, Murdoch, Western Australia 6150.
[2]Department of Anatomy and Reproductive Biology, University of Hawaii Medical School, Honolulu, Hawaii 96822, USA.

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Moreover the rapid fixation of neutral mutations between generations suggests that there is stringent selection for a limited set of mitochondrial genomes during oogenesis (Ashley *et al.*, 1989). Estimates of the number of precursor genomes in this 'restriction-amplification bottleneck' (Linnane *et al.*, 1992) range from a few hundred in mice (Jenuth *et al.*, 1996) down to a single mitochondrion in humans (Blok *et al.*, 1997).

In mice it seems that recognition of mitochondria and their subsequent destruction by proteolytic embryonic factors is based not on the mitochondrial DNA (mtDNA) itself, but on nuclear-encoded proteins of the sperm mitochondrial sheath or associated elements (Kanada *et al.*, 1995). This was studied using a combination of *Mas spretus* sperm and *Mus musculus* oocytes, where the 'foreign' mtDNA could be detected in embryos by the polymerase chain reaction (PCR). In contrast, when the males were gene constructs carrying *M. spretus* mtDNA on a nuclear background of *M. musculus* genes the 'foreign' mtDNA could not be detected. While this intriguing observation needs to be repeated, it points to unique recognition mechanisms in the early embryo. One possibility (still to be confirmed) is that elements of the mitochondrial sheath are tagged by ubiquitin during the second cell cycle and destroyed as part of the second true cell cycle of the embryo (Sutovsky *et al.*, 1996).

If recognition and elimination of the paternal mitochondria are based on recognition by the embryo of elements of the sperm midpiece, then the microinjection of round spermatids, where these elements may not have yet fully formed, may allow paternal mtDNA to evade detection and elimination. This is potentially a concern for human infertility treatment, as the dominance of maternal transmission of mtDNA means that male fitness is irrelevant to the evolutionary 'interests' some of the mitochondrial genome, and some mitochondrial disorders (including perhaps some forms of infertility) are more severe and more prevalent in men than women (Johns, 1996; Cummins, 1997). There is indeed an example of bi-parental mtDNA inheritance in the mussel, where paternal mitochondria get inherited by male – but not female – offspring whereas maternal mitochondria are transmitted to both genders (Sutherland *et al.*, 1998). Where men with spermatogenic arrest are being treated for infertility by round spermatid injection there is therefore a remote possibility that this will allow abnormal mtDNA to colonise the embryo and thereby lead to increased embryonic mortality or even to mitochondrial disease in offspring (Cummins & Jequier, 1995). To address this question in an animal model we therefore studied the outcome of microinjection of labelled round spermatid mitochondria into activated mouse oocytes.

Materials and methods

Animals and culture techniques

The animals used were sexually mature (3 months old) B6D2F$_1$ mice (C57BL/6J female × DBA/2 male hybrids). Culture techniques were as previously described (Cummins *et al.*, 1997). CZB medium (Chatot *et al.*, 1989), supplemented with 5.56 mM D-glucose and 5 mg/ml bovine serum albumin (BSA; fraction V), was used for culture after microinjection. For preparing cells and oocytes before and during microinjection, modified HEPES-CZB was used (Kuretake *et al.*, 1996). This was buffered with Hepes (20 mM), a reduced amount of NaHCO$_3$ (5 mm) and contained polyvinyl alcohol (PVA: cold-water soluble; MW 10×10^5) instead of BSA to reduce stickiness. All cells used were washed individually in Hepes-CZB before injection.

Preparation and activation of oocytes

B6D2F$_1$ females, 6–12 weeks old, were each injected with 5 IU pregnant mare serum gonadotrophin (PMSG, Sankyo, Japan) followed 48 h later by 5 IU human chorionic gonadotrophin (hCG, Everedy Drugs, NY). Oocytes were collected by rupturing the oviducts about 15 h after hCG injection. They were treated with 0.1% bovine testicular hyaluronidase in Hepes-CZB to remove the cumuli (300 USP units/mg: ICN Biochemicals, Costa Mesa, CA), and then rinsed and kept in CZB for up to 3.5 h at 37 °C under 5% CO$_2$ in air. In one experiment the cumulus cells were rinsed and kept in CZB medium for injection into oocytes. Oocytes were activated by 1 h treatment with 10 mM SrCl$_2$ in Ca^{2+}-free CZB medium. They were microinjected within 30 min of activation. This treatment activates a very high proportion of mouse oocytes without the inconsistent effects of other parthenogenetic agents such as ethanol and thimerosal (Bos-Mikich & Whittingham, 1995).

Preparation of spermatids and spermatocytes

Spermatids and spermatocytes were prepared from the testes of male B6D2F$_1$ mice (Ogura *et al.*, 1993; Kimura & Yanagimachi, 1995*a*, *b*; Sasagawa *et al.*, 1998). The testicular capsule was removed, and the seminiferous tubules rinsed briefly in Hepes-CZB medium. These were then transferred to fresh medium and minced with fine iridectomy scissors. Round spermatids and primary spermatocytes were identified on the basis of size and nuclear conformation, using Hoffman interference optics and an inverted microscope (Ogura *et al.*, 1994; Kimura & Yanagimachi, 1995*a*, *b*; Sasagawa *et al.*, 1998). They were stained with MitoTracker fluorochrome (Molecular Probes, Eugene, OR)

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for 5 min at a final concentration of 4 μM as previously described for mature sperm (Sutovsky *et al.*, 1996; Cummins *et al.*, 1997), and then washed in CZB medium before injecting them into oocytes. MitoTracker was prepared from 1 M dimethylsulphoxide (DMSO) stock and stored desiccated at –20 °C. A Leitz micromanipulator was used, equipped with a Piezo-micropipette driving unit (Model PMM-01 by Prima Meat Packers, Tsuchiura City, Ibaraki-ken, Japan). The micropipettes (~7 μm tip diameter) were pulled from borosilicate glass capillary tubing (1.0 mm outside diameter, 0.75 mm inside diameter; Sutter Instruments, Novato, CA) and contained a mercury column to maximise penetration efficiency (Kimura & Yanagimachi, 1995*a*, *b*).

Table 1 Survival of activated oocytes following microinjection

Microinjection protocol	Survival (%)
Stained spermatids	447/478 (93%)
Unstained spermatids	54/56 (96%)
Oocytes stained before activation and then injected with spermatids	35/38 (92%)
Oocytes stained after activation and then injected with spermatids	19/20 (95%)
Stained spermatocytes	48/48 (100%: survival 1–3 h only)
Stained cumulus cells	80/86 (93%: survival 1–3 h only)

Injection of spermatocytes and cumulus cells

Spermatids were injected in seven separate trials (Tables 1, 2). In two experiments MitoTracker-labelled primary spermatocytes and cumulus cells (recovered from the oocyte preparation dishes) were injected into activated oocytes. The plasma membranes of these cells were broken mechanically immediately before injection. While careful timing of injection will allow primary spermatocytes to complete meiosis and even achieve syngamy in oocytes (Sasagawa *et al.*, 1998), in these studies we were only interested in the early events so we examined the oocytes at 1, 2 and 3 h.

Observations on oocytes and embryos

In all 750 oocytes were injected. Some of these were checked 1–3 h after microinjection to study early events while the oocyte cytoplasm was recovering. Otherwise embryos were checked on day 2 and early on day 3 (1-cell and 2-cell), late on day 3 (4-cell and 8-cell) and on day 4–5 (8-cell and morula).

The following control experiments were carried out to see whether MitoTracker staining had any side effects on embryo development: (1) Oocytes (38) were

Table 2 Persistence of MitoTracker-stained spermatid mitochondria in Sr^{2+}-activated oocytes and embryos

	Day 1				Day 2–3 early				Day 3 late				Day 4–5				Total (normal only)			
Stage	0	+	++	+++	0	+	++	+++	0	+	++	+++	0	+	++	+++	0	+	++	+++
1-cell (n = 6)	13 20%	9 14%	13 20%	30 46%	4 100%	0	0	0					9	0	0	1	13 20%	9 14%	13 20%	30 46%
																	Total positive 80%%			
2-cell (n = 6)					56 59%	6 6%	17 18%	16 17%					1	0	0	0	56 59%	6 6%	17 18%	16 17%
																	Total positive 25%			
4-cell (n = 7)									89 91%	1 1%	4 4%	4 4%	2	0	0	0	89 91%	1 1%	4 4%	4 4%
																	Total positive 9%			
8-cell (n = 5)									70 99%	0	0	1 1%	7	0	0	0	70 99%	0	0	1 1%
																	Total positive 1%			
Morula/ blastocyst (n = 5)													40 98%	0	0	1 2%	40 98%	0	0	1 2%
																	Total positive 2%			

n, number of replicate trials.
0, no staining detectable; +, single faint points of fluorescence; ++, patches of intermediate intensity; +++, bright arrays of fluorescence.

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stained with MitoTracker before Sr^{2+} activation and spermatid injection. (2) Oocytes (20) were stained with MitoTracker after activation and before spermatid injection. (3) Oocytes (56) were injected with unstained spermatids and then activated.

Fluorescence microscopy

Fluorescence microscopy was carried out as previously described (Cummins *et al.*, 1997). Oocytes and embryos were mounted on glass slides under coverslips supported by wax dots and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). The preparations were sealed with nail varnish and stored in the dark at 4 °C before studying with fluorescence microscopy. They were examined using a Zeiss model 14 microscope with a high-pressure mercury vapour lamp (HBO 100 W) and epifluorescence illumination as well as transmitted light illumination (Cummins *et al.*, 1986). The filter combination used a pass band filter transmitting 395–425 nm wavelength light (UV: filter number 48.77.18) with a long wave pass filter (LP450). Photomicrographs were made using Kodak Tmax P3200 high-speed film. The negatives were scanned and enhanced using Adobe PhotoShop and Macintosh computers and printed out on a Kodak 8650 PS dye sublimation laser printer.

Results

Oocyte survival

Results are presented in Table 1. Staining with MitoTracker clearly had no effect on survival and activation. Overall 707 of 750 oocytes (94%) survived the microinjection procedure as judged by cytoplasmic integrity at 1–3 h. Activation, demonstrated by extrusion of the second polar body, was generally more than 90%. Control oocytes injected with non-labelled spermatids developed normally, with 30 of 54 (55%) developing to morulae or blastocysts.

Spermatid injection

Initially the spermatid cytoplasm was oval in shape after extrusion through the 7 μm micropipette (Fig. 1). After about an hour it rounded up (Figs. 2, 3) and the mitochondria remained closely associated with the nucleus. After formation of the male pronucleus and during subsequent cell division the mitochondria almost invariably coalesced into a tight bundle (Figs. 8, 10, 12), and very rarely into two patches. The staining intensity tended to decline with time (Table 2), so that while in the 1-cell embryos 46 of 52 had bright (+++) fluorescence this declined to 16 of 39 (41%) in the 2-cell and 4 of 9 (45%) in the 4-cell embryos. The final fate of mitochondria appeared generally to be small points of fluorescence within single cells, although in one case a bright cluster of mitochondria was seen in a day 4 morula (Table 2, Fig. 12).

The results are presented in Table 2 and graphically in Fig. 13. A total of 380 embryos was studied of which 360 were judged to be developing normally at the time of observation: 67 of the 447 (15%) oocytes that survived the initial microinjection were discarded due to degeneration during the first 2–3 days of culture. Of embryos checked at the 1-cell stage (day 1), 80% showed positive staining for labelled mitochondria. This declined to 25% for 2-cell embryos and 9% for 4-cell embryos. Staining largely disappeared at this stage, although in two cases (one 8-cell and one morula) there were positive groups of brightly staining mitochondria in single cells (Fig. 12).

Spermatocyte and cumulus cell injection

A very different picture appeared when labelled spermatocytes or cumulus cells were injected. In these cases the labelled mitochondria started out as a large, brightly stained cluster of mitochondria surrounding the nucleus (Figs. 5, 6). By 3 h after microinjection the mitochondria generally dispersed widely throughout the cytoplasm of the oocyte. This rapid dispersal of labelled mitochondria to a range of focal planes within the oocyte made them difficult to identify individually and to photograph and the oocyte appeared as a brightly fluorescent cell contrasting with the dark, unlabelled polar bodies.

Discussion

These results show that spermatid mitochondria are, if anything, less viable in the oocyte and embryo than those of mature spermatozoa. This would therefore seem to alleviate the concerns that the microinjection of immature germ cells in the treatment of human male infertility might lead to mitochondria disease in offspring (Cummins, 1997; Houshmand *et al.*, 1997).

However, we must be cautious about assuming absolutes for such a dynamic and little-understood biological system. While it is generally assumed that mitochondrial segregation is random at cell division and that replication occurs independently of the cell cycle, in fact there is a complex species-specific and even cell-specific system of communication between the mitochondrial and nuclear genomes (Poyton & McEwen, 1996). In intraspecific cybrid fusions between primate and human cell liens Kenyon & Moraes (1977) showed that mtDNA from chimpanzee, bonobo and gorilla – but not from more distantly related primates –

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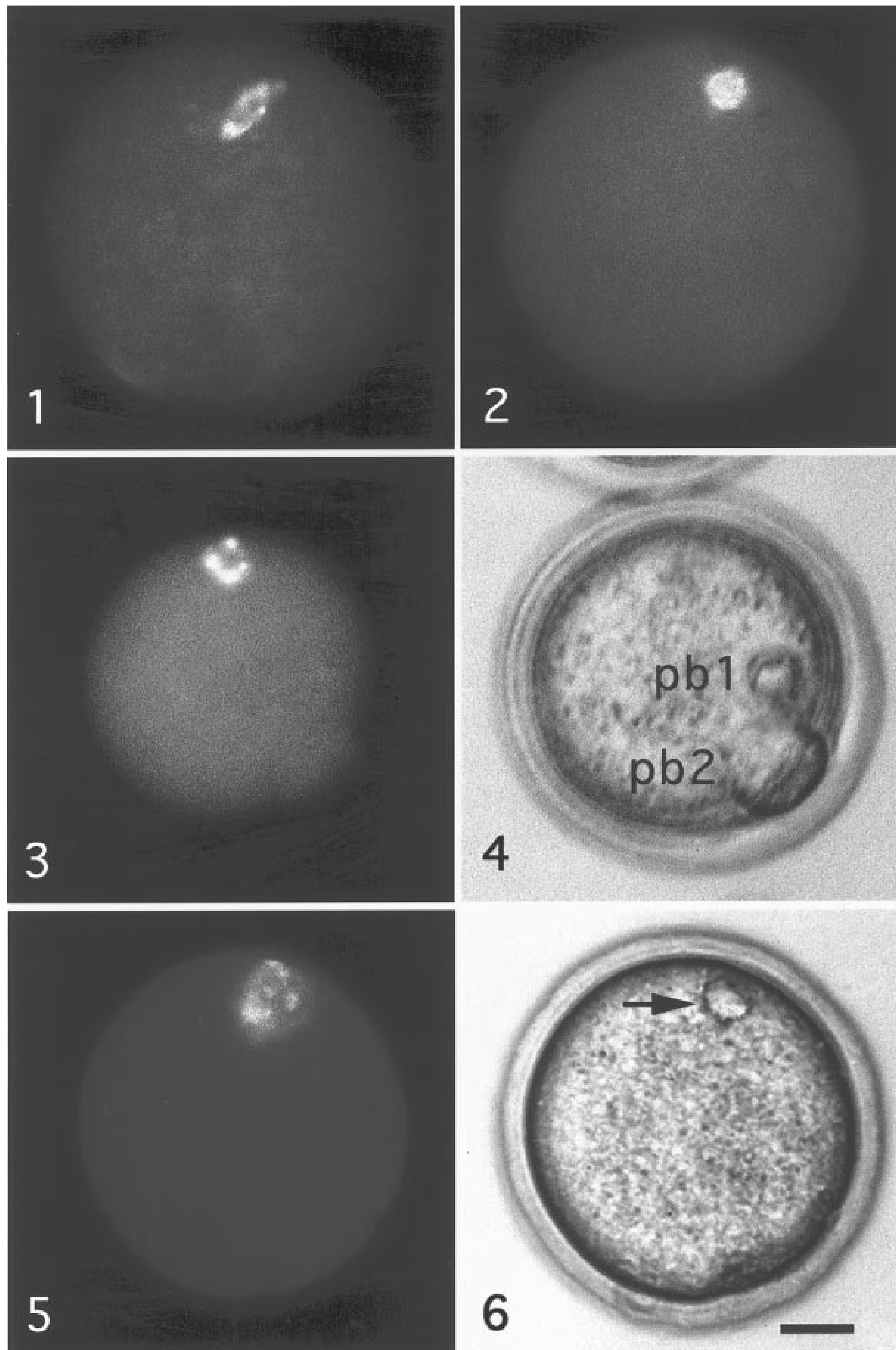

Figures 1–6 All figures are to the same magnification. The scale bar (in Fig. 6) represents 20 μm. **Figure 1** Shortly after spermatid injection the mitochondria appears as an oval patch of fluorescence due to compression in the microinjection needle. **Figure 2** By 3 h after injection the mitochondria round up and remain closely associated with the spermatid nucleus (not visible). **Figure 3** After extrusion of the second polar body and formation of the pronuclei the spermatid mitochondria remain in a tight cluster, either close to the male pronucleus or, as here, peripherally located in the ooplasm. **Figure 4** The same oocyte as Fig. 3 by phase contrast microscopy to demonstrate the polar bodies (pb1, pb2; in a different focal plane). **Figure 5** Oocyte 1 h after injection with a MitoTracker-labelled primary spermatocyte. The labelled mitochondria are starting to disperse. The spermatocyte is much larger than the spermatid (compare with Figs. 1–3). **Figure 6** The same oocyte as Fig. 5 photographed by phase contrast microscopy, demonstrating the intact spermatocyte nucleus (arrow).

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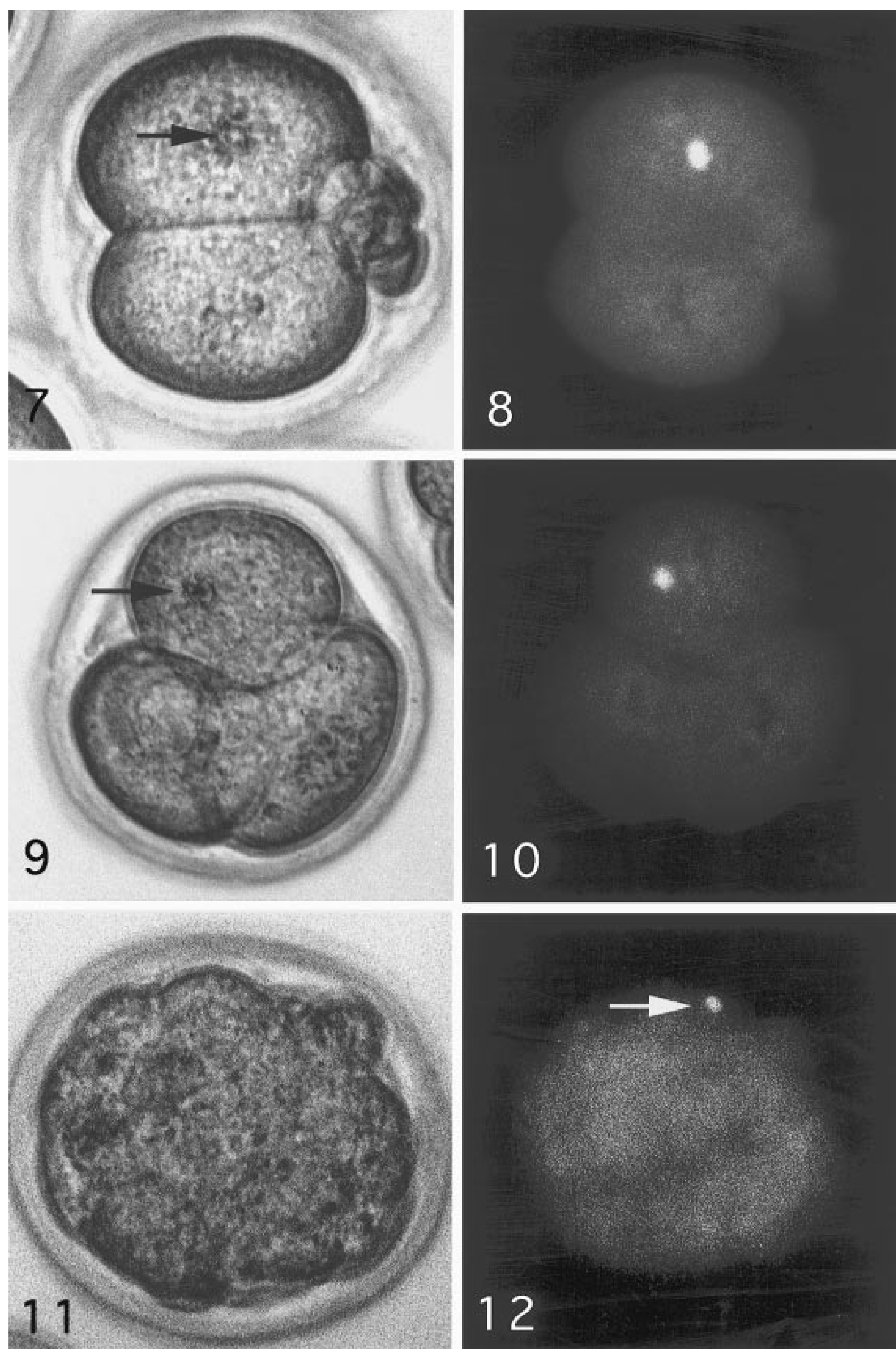

Figures 7–12 All figures are the same magnification as Figs 1–6. **Figure 7** Two-cell embryo by phase contrast microscopy. The small patch of agglutinated spermatid mitochondria close to the interphase nucleus is just visible (arrow). **Figure 8** The same embryo as in Fig. 7 by fluorescence microscopy to demonstrate MitoTracker staining. **Figure 9** Four-cell embryo by phase contrast microscopy. A small group of spermatid mitochondria is visible in the upper cell (arrow). **Figure 10** The same embryo as in Fig. 9 by fluorescence microscopy to demonstrate a small patch of MitoTracker staining. **Figure 11** Day 4 morula by phase contrast microscopy. **Figure 12** The same morula as in Fig. 11 demonstrating that very occasionally some spermatid mitochondria can survive (arrow). Only one such embryo was observed. In the majority of cases no spermatid mitochondria persisted past the 4-cell stage (Table 2, Fig. 13).

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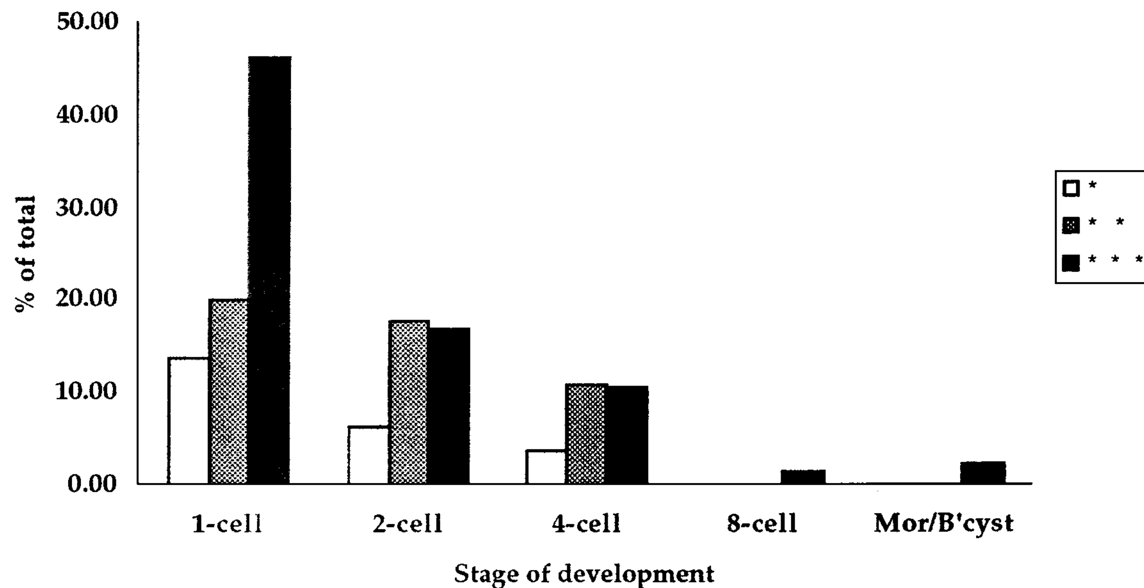

Figure 13 Survival of MitoTracker-stained spermatid mitochondria in normal embryos. The intensity of fluorescence is indicated by the number of asterisks.

could substitute for human mtDNA. It has been known for some years that mitochondrial replication in hybrid cell lines is strongly influenced by the nuclear genome (De Francesco *et al.*, 1980), and mitochondrial genomes in such heteroplasmic situations can behave in either a co-dominant or a dominant/recessive mode (Hayashi *et al.*, 1987). Jenuth *et al.* (1977) found strong tissue-specific and age-related selection for different mitochondrial genotypes in heteroplasmic mice created by embryo–cytoplast fusion. This again highlights the complex nature of nucleo-cytoplasmic interactions for mitochondrial segregation and tissue energetics.

What of the sperm's mtDNA? What estimates can be place on the remote possibility of survival and proliferation? Gyllensten *et al.* (1991, 1985) detected a 'leakage' of paternal mtDNA at around 1 in 1000 in back-crossed mice. This is close to the calculated dilution ratio of sperm to oocyte mitochondrial numbers at fertilisation (Ankel-Simons & Cummins, 1996). We know that single mitochondria have the potential to colonise depleted cell lines under experimental conditions *in vitro*, albeit at low efficiency (King & Attardi, 1988, 1989). Manfredi *et al.* (1977) found even lower long-term persistence rates when sperm were fused with mitochondrially depleted somatic cell lines *in vitro*. Only 0.01% of cells showed repopulation with sperm mitochondria even though a high proportion (10–20%) of cells had mitochondria demonstrating normal transmembrane potential when examined at 4 h. Mitochondria probably have the potential to fuse and to form stable heteroplasmic lineages under certain conditions (Meirelles & Smith, 1997). While this latter possibility is unlikely at fertilisation as sperm mitochondria are highly differentiated and probably tightly sequestered within the midpiece capsular proteins (see below), we should perhaps keep our minds open to the sometimes bizarre tricks that competing genetic elements can play in natural systems (Haig, 1995; Hurst, 1992; Birky, 1995).

We can still only speculate on the factors of the sperm midpiece that may act as recognition for selective destruction in the embryo. From the observations above spermatid mitochondria clearly behave very differently from those of spermatocytes or somatic cells. Instead of dispersing they remain tightly agglutinated even up to the morula stage (Table 2), but they are probably held together by precursors of capsular proteins. Sperm mitochondria show a complex set of changes during spermiogenesis, as reviewed by Oko & Clermont (1990). An early burst of mtDNA synthesis may indicate proliferation (Hecht & Liem, 1984). During formation of the midpiece by caudal movement of the annulus, the mitochondria rapidly migrate along the axoneme in a controlled stepwise fashion to form a tight array (Otani *et al.*, 1988). It therefore seems likely that early spermatid mitochondria may be already be tagged with cytoskeletal elements preparing for this move. The mitochondria differentiate structurally by elongating and becoming denser and crescent-shaped to surround the axoneme under the forming coarse fibres. This differentiation appears pre-programmed, as it also occurs in the superfluous mitochondria of the excess cytoplasm that later degenerates to form the residual body and is resorbed by the Sertoli cell (Russell, 1993).

Formation of the midpiece is accompanied by the

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secretion of a keratinous and selenium-rich capsule on the outside of the mitochondrial sheath, and a submitochondrial reticulum that apparently anchors the sheath to the axoneme (Olson & Winfrey, 1992). This capsule consists of at least four proteins of 17, 20, 29 and 31 kDa (Cataldo *et al.*, 1996; Pallini *et al.*, 1979). The 20 kDa protein is cysteine- and proline-rich (Cataldo *et al.*, 1996). Spermatozoa contain the highest relative concentration of selenium of any cell line in the body, and animals fed a selenium-deficient diet show midpiece defects (Cataldo *et al.*, 1996). The selenoprotein is most likely the antioxidant phospholipid hyperperoxide glutathione peroxidase (Roveri *et al.*, 1992, 1994), which is intriguing given the susceptibility of spermatozoa to oxidative damage and lipid peroxidation (Aitken, 1995, 1997). There is thus a series of unique haploid-encoded protein components of the mitochondrial capsule that potentially could act as targets for the embryo's proteolytic apparatus. It would be interesting to use immunocytochemistry based on antibodies to capsule elements to see how these components may be involved in the necrosis of the sperm mitochondria described by Szöllösi (1965).

Other internal features of the mitochondria also differentiate during spermiogenesis. A sperm-specific lactic dehydrogenase isozyme appears early (Machado de Domenech *et al.*, 1972) and there is a unique isoform of cytochrome *c* that appears first in spermatocytes and increases in post-meiotic cells (Goldberg *et al.*, 1977; Morales *et al.*, 1993). Moreover there is – perhaps terminal – differentiation of the mtDNA with loss of the mitochondrial targeting sequence of a testis-specific isoform of nuclear mitochondrial transcription factor A (mtTFA) (Larsson *et al.*, 1996, 19097). However, the sperm's mitochondrial transcriptional potential is apparently unchanged during this process (Hecht *et al.*, 1984; Alcivar *et al.*, 1989). Larsson *et al.* (1996) suggested that the nuclear isoform of mtTFA, which is also present in spermatocytes, may play a secondary structural role in compacting nuclear DNA during spermiogenesis. This would be consistent with its known capacity to induce conformational changes in DNA (Fisher *et al.*, 1992).

It is perhaps significant that the sperm mitochondria disappear at about the same time that embryonic maternally derived mitochondria start to become active (Pikó & Taylor, 1987; Taylor & Pikó, 1995). Mature oocyte mitochondria appear to be in a relatively quiescent state with high cytosolic levels of ATP maintaining meiotic arrest (Downs *et al.*, 1989; Eppig, 1991) and with inactive mitochondrial DNA (Pikó & Taylor, 1987; Taylor & Pikó, 1995). The embryo's mitochondrial transcription commences at the 4-cell stage, with the start of respiration and changes in ultrastructural appearance including increased numbers of cristae, but mitochondrial mtDNA replication does not commence until the egg-cylinder stage at day 7–8 (Ebert *et al.*, 1988). This delay in mtDNA replication before differentiation of the germ cell lineage could explain the 'bottleneck' in the mitochondrial genome copy number that occurs during oogenesis and that generally ensures homoplasmy (Jenuth *et al.*, 1996; Blok *et al.*, 1997; Marchington *et al.*, 1997).

Understanding the onset of mitochondrial replication in mammalian embryogenesis would therefore seem to be a key question in biology, as it is possible that the majority of sperm mitochondria simply lose membrane potential and die in the absence of appropriate signals from the nucleus (Poyton & McEwen, 1996). Possibly leakage of cytochrome *c* from the outer mitochondrial space may act as an apoptotic-type trigger to the endogenous proteolytic system of the embryo (Ozawa *et al.*, 1997). Against this scenario must be weighed our observations that sperm mitochondria in non-activated oocytes disperse and can persist for up to 5 days (Cummins *et al.*, 1997); however, this is a highly abnormal situation in which the normal regulatory cell mechanisms of the differentiating embryo are obviously not functioning. One central puzzle to this whole question is the observation of Kaneda *et al.* (1995) that paternal mtDNA can be detected in interspecific but not intraspecific *Mus* hybrids. As there appears to be consensus that evolutionary processes have mediated against such cross-species mitochondrial transcription (Fisher *et al.*, 1992) it is far from clear how in this case the paternally derived 'foreign' mitochondria can survive.

Note added in proof

Since submitting this manuscript Shitara *et al.* (*Genetics* 1998; **148:** 851–7) have shown that paternal mtDNA survives in F1 inter-strain hybrid mice offspring but not in subsequent generations. This is at variance with Gyllensten *et al.*'s (1991) findings of long-term low-level leakage.

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References

Aitken, R.J. (1995). Free radicals, lipid peroxidation and sperm function. *Reprod. Fertil. Dev.* 7, 659–68.

https://doi.org/10.1017/S0967199498000148 Published online by Cambridge University Press

Aitken, R.J. (1997). Molecular mechanisms regulating human sperm function. *Mol. Hum. Reprod.* **3**, 169–73.

Alcivar, A.A., Hake, L.E., Millette, C.F. *et al.* (1989). Mitochondrial gene expression in male germ cells of the mouse. *Dev. Biol.* **135**, 263–71.

Ankel-Simmons, F. & Cummins, J.M. (1996). Misconceptions about mitochondria and mammalian fertilization: implications for theories on human evolution. *Proc. Nat. Acad. Sci. USA* **93**, 13859–63.

Ashley, M.V., Laipis, P.J. & Hauswirth, W.W. (1989). Rapid segregation of heteroplasmic bovine mitochondria. *Nucleic Acids Res.* **17**, 7325–31.

Bedford, J.M. (1972). An electron microscopic study of sperm penetration into the rabbit egg after natural mating. *Am. J. Anat.* **133**, 213–54.

Birky, C.W. (1995). Uniparental inheritance of mitochondrial and chloroplast genes: mechanisms and evolution. *Proc. Natl. Acad. Sci. USA* **92**, 11331–8.

Blok, B.B., Gook, D.A., Thorburn, D.R. *et al.* (1997). Skewed segregation of the mtDNA nt 8993 (T–G) mutation in human oocytes. *Am. J. Hum. Genet.* **60**, 1495–501.

Bos-Mikich, A. & Whittingham, D.G. (1995). Analysis of the chromosome complement of frozen-thawed mouse oocytes after parthenogenetic activation. *Mol. Reprod. Dev.* **42**, 254–60.

Cataldo, L., Baig, K., Oko, R. *et al.* (1996). Developmental expression, intracellular localization, and selenium content of the cysteine-rich protein associated with the mitochondrial capsules of mouse sperm. *Mol. Reprod. Dev.* **45**, 320–31.

Chatot, C.L., Ziomek, C.A., Bavister, B.D. *et al.* (1989). An improved culture medium supports development of random-bred 1-cell mouse embryos *in vitro*. *J. Reprod. Fertil.* **86**, 679–88.

Cummins, J.M. (1977). Mitochondrial DNA: implications for the genetics of human male infertility. In *Genetics of Human Male Fertility*, ed. C. Barratt, C. De Jonge, D. Mortimer & J. Parinaud, pp. 287–307. Paris: Editions EDK.

Cummins, J.M. & Jequier, A.M. (1995). Concerns and recommendations for intracytoplasmic sperm injection (ICSI) treatment. *Hum. Reprod.* **10**, 138–43.

Cummins, J.M., Fleming, A.D., Crozet, N. *et al.* (1986). Labelling of living mammalian spermatozoa with the fluorescent thiol alkylating agent, monobromobimane (MB): fate of labelled sperm components in the embryo and developmental capacity. *J. Exp. Zool.* **237**, 383–90.

Cummins, J.M., Wakayama, T. & Yanagimachi, R. (1997). Fate of microinjected sperm tail components in the mouse embryo and oocyte. *Zygote* **5**, 301–8.

De Francesco, L., Attardi, G. & Groce, C.M. (1980). Uniparental propagation of mitochondrial DNA in mouse–human cell hybrids. *Proc. Natl. Acad. Sci. USA* **77**, 4079–83.

Downs, S., Daniel, S., Bornslaeger, E. *et al.* (1989). Maintenance of meiotic arrest in mouse oocytes by purines: modulation of cAMP levels and cAMP phosphodiesterase activity. *Gamete Res.* **23**, 323–35.

Ebert, K.M., Liem, H. & Hecht, N.B. (1988). Mitochondrial DNA in the mouse preimplantation embryo. *J. Reprod. Fertil.* **82**, 145–9.

Eppig, J.J. (1991). Maintenance of meiotic arrest and the induction of oocyte maturation in mouse oocyte–granulosa cell complexes developed *in vitro* from preantral follicles. *Biol. Reprod.* **45**, 824–30.

Fisher, R.P., Lisowsky, T., Parisi, M.A. *et al.* (1992). DNA wrapping and bending by a mitochondrial high mobility group-like transcriptional activator protein. *J. Biol. Chem.* **267**, 3358–67.

Goldberg, E., Sberna, D., Wheat, T.E. *et al.* (1977). Cytochrome *c*: immunofluorescent localization of the testis-specific form. *Science* **196**, 1010–2.

Gyllensten, U., Wharton, D. & Wilson, A.C. (1985). Maternal inheritance of mitochondrial DNA during backcrossing of two species of mice. *J. Hered.* **76**, 321–4.

Gyllensten, U., Wharton, D., Josefsson, A. *et al.* (1991). Paternal inheritance of mitochondrial DNA in mice. *Nature* **352**, 255–7.

Haig, D. (1996). Altercation of generations: genetic conflicts of pregnancy. *Am. J. Reprod. Immunol.* **35**, 226–32.

Hayashi, J., Yonekawa, H., Murakami, J. *et al.* (1987). Mitochondrial genomes in intraspecies mammalian cell hybrids display codominant or dominant/recessive behavior. *Exp. Cell. Res.* **172**, 218–27.

Hecht, N.B. & Liem, H. (1984). Mitochondrial DNA is synthesized during meiosis and spermiogenesis in the mouse. *Exp. Cell. Res.* **154**, 293–8.

Hecht, N.B., Liem, H., Kleene, K.C. *et al.* (1984). Maternal inheritance of the mouse mitochondrial genome is not mediated by a loss or gross alteration of the paternal mitochondrial DNA or by methylation of the oocyte mitochondrial DNA. *Dev. Biol.* **102**, 452–61.

Hiraoka, J. & Hirao, Y. (1988). Fate of sperm tail components after incorporation into the hamster egg. *Gamete Res.* **19**, 369–80.

Houshmand, M., Holme, E., Hanson, C. *et al.* (1997). Paternal mitochondrial DNA transferred to the offspring following intracytoplasmic sperm injection. *J. Assist. Reprod. Genet.* **14**, 223–7.

Hurst, L.D. (1992). Intragenomic conflict as an evolutionary force. *Proc. R. Soc. Lond., Ser. B* **248**, 135–40.

Jenuth, J.P., Peterson, A.C., Fu, K. *et al.* (1996). Random genetic drift in the female germline explains the rapid segregation of mammalian mitochondrial DNA. *Nature Genet.* **14**, 146–51.

Jenuth, J.P., Peterson, A.C. & Shoubridge, E.A. (1997). Tissue-specific selection for different mtDNA genotypes in heteroplasmic mice. *Nature Genet.* **16**, 93–5.

Johns, D.R. (1996). The other human genome: mitochondrial DNA and disease. *Nature Med.* **2**, 1065–8.

Kaneda, H., Hayashi, J.I., Takahama, S. *et al.* (1995). Elimination of paternal mitochondrial DNA in intraspecific crosses during early mouse embryogenesis. *Proc. Natl. Acad. Sci. USA* **92**, 4542–6.

Kenyon, K. & Moraes, C.T. (1997). Expanding the functional human mitochondrial DNA database by the establishment of primate xenomitochondrial cybrids. *Proc. Natl. Acad. Sci. USA* **94**, 9131–5.

Kimura, Y. & Yanagimachi, R. (1995*a*). Intracytoplasmic sperm injection in the mouse. *Biol. Reprod.* **52**, 709–20.

Kimura, Y. & Yanagimachi, R. (1995*b*). Mouse oocytes injected with testicular spermatozoa or round spermatids can develop into normal offspring. *Development* **121**, 2397–405.

https://doi.org/10.1017/S0967199498000148 Published online by Cambridge University Press

King, M.P. & Attardi, G. (1988). Injection of mitochondria into human cells leads to a rapid replacement of the endogenous mitochondrial DNA. *Cell* **52**, 811–19.

King, M.P. & Attardi, G. (1989). Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. *Science* **246**, 500–3.

Kuretake, S., Kimura, Y., Hoshi, K. *et al.* (1996). Fertilization and development of mouse oocytes injected with isolated sperm heads. *Biol. Reprod.* **55**, 789–95.

Larsson, N.G., Garman, J.D., Oldfors, A. *et al.* (1996). A single mouse gene encodes the mitochondrial transcription factor A and a testis-specific nuclear hmg-box protein. *Nature Genet.* **13**, 296–302.

Larsson, N.G., Oldfors, A., Garman, J.D. *et al.* (1997). Down-regulation of mitochondrial transcription factor A during spermatogenesis in humans. *Hum. Mol. Genet.* **6**, 185–91.

Linnane, A.W., Baumer, A., Boubolas, A. *et al.* (1992). Mitochondria mutations and the ageing process. In *Adenine Nucleotides in Cellular Energy Transfer and Signal Transduction*, ed. S. Papa, A. Azzi & J.M. Tager, pp. 137–49. Basel: Birkhäuser Verlag.

Machado de Domenech, E., Domenech, C.E., Akoi, A. *et al.* (1972). Association of the testicular lactate dehydrogenase isozyme with a special type of mitochondria. *Biol. Reprod.* **6**, 136–47.

Manfredi, G., Thyagarajan, D., Papadopoulou, L.C. *et al.* (1997). The fate of human sperm-derived mtDNA in somatic cells. *Am. J. Hum. Genet.* **61**, 953–60.

Marchington, D.R., Hartshorne, G.M., Barlow, D. *et al.* (1997). Homopolymeric tract heteroplasmy in mtDNA from tissues and single oocytes: support for a genetic bottleneck. *Am. J. Hum. Genet.* **60**, 408–16.

Meirelles, F.V. & Smith, L.C. (1997). Mitochondrial genotype segregation in a mouse heteroplasmic lineage produced by embryonic karyoplast transplantation. *Genetics* **145**, 445–51.

Morales, C.R., Hake, L.E. & Hecht, N.B. (1993). Cellular localization of the mRNAs of the somatic and testis-specific cytochromes *c* during spermatogenesis in the rat. *Mol. Reprod. Dev.* **34**, 196–205.

Ogura, A., Yanagimachi, R., Usui, N. (1993). Behaviour of hamster and mouse round spermatid nuclei incorporated into mature oocytes by electrofusion. *Zygote* **1**, 1–8.

Ogura, A., Matsuda, J. & Yanagimachi, R. (1994). Birth of normal young after electrofusion of mouse oocytes with round spermatids. *Proc. Natl. Acad. Sci. USA* **91**, 7460–2.

Oko, R. & Clermont, Y. (1990). Mammalian spermatozoa: structure and assembly of the tail. In *Controls of Sperm Motility: Biological and Clinical Aspects*, ed. C. Gagnon, pp. 3–27. Boca Raton: CRC Press.

Olson, G.E. & Winfrey, V.P. (1992). Structural organization of surface domains of sperm mitochondria. *Mol. Reprod. Dev.* **33**, 89–98.

Otani, H., Tanaka, O., Kasai, K. *et al.* (1988). Development of mitochondrial helical sheath in the middle piece of the mouse spermatid tail: regular dispositions and synchronized changes. *Anat. Res.* **222**, 26–33.

Ozawa, T. (1997). Genetic and functional changes in mitochondria associated with aging. *Physiol. Rev.* **77**, 425–64.

Ozawa, T., Hayakawa, M., Katsumata, K. *et al.* (1997). Fragile mitochondrial DNA: the missing link in the apoptotic neuronal cell death in Parkinson's disease. *Biochem. Biophys. Res. Commun.* **235**, 158–61.

Pallini, V., Baccetti, B. & Burini, A.G. (1979). A peculiar cysteine-rich polypeptide related to some unusual properties of mammalian sperm mitochondria. In *The Spermatozoon*, ed. D.W. Fawcett & J.M. Bedford, pp. 141–50. Baltimore: Urban and Schwarzenberg.

Pikó, L. & Taylor, KD. (1987). Amounts of mitochondrial DNA and abundance of some mitochondrial gene transcripts in early mouse embryos. *Dev. Biol.* **123**, 364–74.

Poyton, R.O. & McEwen, J.E. (1996). Crosstalk between nuclear and mitochondrial genomes. *Annu. Rev. Biochem.* **65**, 563–607.

Roveri, A., Casasco, A., Maiorino, M. *et al.* (1992). Phospholipid hydroperoxide glutathione peroxidase of rat testis: gonadotropin dependence and immunocytochemical identification. *J. Biol. Chem.* **267**, 6142–6.

Roveri, A., Maiorino, M., Nisii, C. *et al.* (1994). Purification and characterization of phospholipid hydroperoxide glutathione peroxidase from rat testis mitochondrial membranes. *Biochim. Biophys. Acta* **1208**, 211–21.

Russell, L.D. (1993). Role in spermiation. In *The Sertoli Cell*, ed. L.D. Russell & M.C. Griswold, pp. 269–303. Clearwater, FL: Cache River Press.

Sasagawa, I., Kuretake, S., Eppig, L.J. *et al.* (1988). Mouse primary spermatocytes can complete two meiotic divisions within the oocyte cytoplasm. *Biol. Reprod.* **58**, 248–54.

Shalgi, R., Magnus, A., Jones, R. *et al.* (1994). Fate of sperm organelles during early embryogenesis in the rat. *Mol. Reprod. Dev.* **37**, 264–71.

Shoffner, J.M. & Wallace, D.C. (1990). Oxidative phosphorylation diseases: disorders of two genomes. *Adv. Hum. Genet.* **19**, 267–330.

Sutherland, B., Stewart, D., Kenchington, E.R. *et al.* (1998). The fate of paternal mitochondrial DNA in developing female mussels, *Mytilus edulis*: implications for the mechanism of doubly uniparental inheritance of mitochondrial DNA. *Genetics* **148**, 341–7.

Sutovsky, P., Navara, C.S. & Schatten, G. (1996). Fate of the sperm mitochondria, and the incorporation, conversion, and disassembly of the sperm tail structures during bovine fertilisation. *Biol. Reprod.* **55**, 1195–205.

Szöllösi, D. (1965). The fate of sperm middle-piece mitochondria in the rat egg. *J. Exp. Zool.* **159**, 367–78.

Taylor, K.D. & Pikó, L. (1995). Mitocondrial biogenesis in early mouse embryos: expression of the mRNAs for subunits iIV, VB, and VIIC of cytochrome *c* oxidase and subunit 9 (p1) of H^+-ATP synthase. *Mol. Reprod. Dev.* **40**, 29–35.

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