Symposium: Mitochondria and human conception

Degradation of paternal mitochondria after fertilization: implications for heteroplasmy, assisted reproductive technologies and mtDNA inheritance

Peter Sutovsky gained his Master's degree from Slovak Agricultural University, Nitra, Slovakia, in 1989, and his PhD in Physiology from the Czech Academy of Sciences, Libechov/Prague, Czech Republic, in 1994. He was a post-doctoral fellow at the University of Wisconsin, Madison, USA from 1994 to 1997, and a staff scientist at the Oregon Health and Science University, Portland, Oregon, USA from 1997 to 2001. He is now jointly appointed as Assistant Professor in the Departments of Animal Science, and Obstetrics and Gynecology at the University of Missouri, Columbia, USA. Research in his laboratory includes the role of the ubiquitin system in mammalian spermatogenesis, epididymal sperm maturation, fertilization and zygotic development, as implicated in assisted reproduction, organelle inheritance, and the development of novel diagnostic tools for infertility treatment.

Dr Peter Sutovsky

Peter Sutovsky[1,2,6], Klaus Van Leyen[3], Tod McCauley[4,5], Billy N Day[1], Miriam Sutovsky[1]
[1]Department of Animal Science; [2]Department Obstetrics and Gynecology, University of Missouri-Columbia, MO, USA; [3]Neuroprotection Research Laboratory, Department of Radiology, Massachusetts General Hospital, 149 13th Street, R 2401, Charlestown, MA 02129, USA; [4]TMI Laboratories, The Marks Institute, 850 N. Kolb Road, Tucson, AZ 85710, USA; [5]Department of Animal Sciences, University of Arizona, Tucson, AZ 85721, USA
[6]Correspondence: University of Missouri-Columbia, S141 ASRC, 920 East Campus Drive, Columbia, MO 65211-5300, USA. Tel: +1 573 8823329; Fax: +1 573 8845540; e-mail: SutovskyP@missouri.edu

Abstract

Maternal inheritance of mitochondrial DNA has long been regarded as a major paradox in developmental biology. While some confusion may still persist in popular science, research data clearly document that the paternal sperm-borne mitochondria of most mammalian species enter the ooplasm at fertilization and are specifically targeted for degradation by the resident ubiquitin system. Ubiquitin is a proteolytic chaperone that forms covalently linked polyubiquitin chains on the targeted proteinaceous substrates. The polyubiquitin tag redirects the substrate proteins to a 26-S proteasome, a multi-subunit proteolytic organelle. Thus, specific proteasomal inhibitors reversibly block sperm mitochondrial degradation in ooplasm. Lysosomal degradation and the activity of membrane-lipoperoxidating enzyme 15-lipoxygenase (15-LOX) may also contribute to sperm mitochondrial degradation in the ooplasm, but probably is not crucial. Prohibitin, the major protein of the inner mitochondrial membrane, appears to be ubiquitinated in the sperm mitochondria. Occasional occurrence of paternal inheritance of mtDNA has been suggested in mammals including humans. While most such evidence has been widely disputed, it warrants further examination. Of particular concern is the documented heteroplasmy, i.e. mixed mtDNA inheritance after ooplasmic transplantation. Intracytoplasmic sperm injection (ICSI) has inherent potential for delaying the degradation of sperm mitochondria. However, paternal mtDNA inheritance after ICSI has not been documented so far.

Keywords: *assisted reproductive technologies, heteroplasmy, mitochondria, mtDNA, spermatozoa, ubiquitin*

How is the maternal inheritance of mitochondria and mtDNA in mammals ensured, and what is it good for?

Animal mitochondria are the only extranuclear organelles that harbour their own functional genome. In contrast to the nuclear genome, the mitochondrial genome encodes only a limited number of genes and even most of structural proteins of the mitochondria are the products of nuclear gene expression. Furthermore, mtDNA is not afforded protection by histones, or a highly efficient DNA repair mechanism seen in the nucleus (reviewed by Zeviani and Antozzi, 1997; St John, 2002). This probably contributes to the high mutation rate of the mitochondrial genome and, as will be discussed later, may be one of the reasons why paternal mitochondria, exposed to potential mutagens en route to the ovum, should not contribute

to the mitochondrial gene pool of a fertilized egg or embryo. Moreover, it seems that some human spermatozoa may have lost all their mtDNA through degradation (Diez-Sanchez *et al.*, 2003). It follows that the mode of uniparental, maternal inheritance of mitochondrial genes prevails in eukaryotic animals, and even in many plants (Birky, 1995; Runions and Owens, 1999). Notable exceptions among animals (reviewed in detail by Birky, 2001) include certain families of mussels (*Mytilidae*) in which the recombination of maternal and paternal mtDNA has also been proposed (Ladoukakis and Zouros, 2001), trematodes (*Schistosoma mansoni*; Jannotti-Passos *et al.*, 2001), bees (Meusel and Moritz, 1993), birds (Kvist *et al.*, 2003) and murid interspecific crosses (Gyllensten *et al.*, 1991; Kaneda *et al.*, 1995; Shitara *et al.*, 1998).

Maternal inheritance of mitochondrial DNA in mammals, which made it possible to develop standard tools for tracking evolutionary clocks in taxonomy and evolution, has long been regarded as a major paradox in developmental biology (see Hagelberg, 2003). Confusion concerning the fate of the sperm mitochondria during and after fertilization is still being propelled by popular science and by some general biology textbooks (see the critical review of such misconceptions by Ankel-Simons and Cummins, 1996). However, research data dating as far back as the 1960s clearly document that in most mammals, the paternal, sperm-borne mitochondria enter the oocyte cytoplasm at fertilization (reviewed by Sutovsky and Schatten, 2000).

There are several possible explanations for why it would be an evolutionary and/or developmental advantage for an embryo to eliminate all paternal, sperm-derived mtDNA. First of all, the level and stringency of gamete quality control differs between the ovary and the testis. In the testis, apoptotic mechanisms operate to reduce the number of germ cells, and could possibly eliminate some of the defective gametes (Sinha Hikkim and Sverdloff, 1999). This could further be aided by quality control in the epididymis, where some of the defective spermatozoa appear to be removed (e.g. Axnér *et al.*, 1999; Sutovsky *et al.*, 2001). However, most of the defective spermatozoa pass on to the ejaculate and some may still escape natural hurdles/filters encountered in the cervix, uterus and oviducts (reviewed by Suarez, 2002). Several recent studies demonstrated a high frequency of mtDNA mutations and deletions, and an overall increase in mtDNA contents of defective, morphologically abnormal human spermatozoa (e.g. Kao *et al.*, 1995; Lestienne *et al.*, 1997; May-Panloup *et al.*, 2002; O'Connell *et al.*, 2002). Contrary to such fairly relaxed sperm quality control and pre-selection mechanisms, only a handful of cells from the perinatal ovarian cohort of oogonia make the final cut and become ovulated (reviewed by Matzuk *et al.*, 2002). The majority of female germ cells undergo programmed cell death (Tilly, 2001), which among other factors could be the result of a genetic bottleneck, strictly preselecting only the fittest ova with least mutated mtDNA to be ovulated (Perez *et al.*, 2000; Shoubridge, 2000). While the existence of genetic bottlenecks has been disputed, it is true that the thousands of mitochondria found in a fully-grown oocyte originate from fewer than 10 mitochondria found in primordial germ cells (Birky, 2001). An alternative to the oocyte bottleneck hypothesis is the possibility that mtDNA segregation is a multi-step process, not restricted to a single moment during germ cell life (Smith *et al.*, 2002).

An additional reason for the complete eradication of paternal mitochondrial genes in the fertilized egg is the aggressive, leukocyte-infiltrated environment spermatozoa encounter during maturation in the epididymis, after discharge into the female reproductive tract and during transport through it. This environment is conducive to production of reactive oxygen species, thought to contribute to the high rate of mtDNA mutations in spermatozoa (reviewed by Aitken *et al.*, 1998; Agarwal *et al.*, 2003). It has been shown recently that the mammalian ovum does not have the capability to discriminate against foreign, mutated mitochondrial genomes derived from somatic cells (Rinaudo *et al.*, 1999; Inoue *et al.*, 2000). An additional reason for rapidly degrading sperm mitochondria may be the presence of a potentially anti-proliferative cargo of mitochondrial proteins. As will be discussed below, some mitochondrial membrane proteins, notably prohibitin (Nuell *et al.*, 1991), have distinct anti-proliferative properties that would probably be incompatible with rapid succession of embryonic mitosis after fertilization. It is thus not surprising that prohibitin appears to be modified by ubiquitin even before it enters the ooplasm (Thompson *et al.*, 2003), and was also implicated, together with ubiquitin, in the control of mitochondrial inheritance in budding yeast (Berger and Yaffe, 1998; Fisk and Yaffe, 1999).

Targeted degradation of sperm mitochondria after fertilization

Before discussing targeted destruction of the sperm mitochondria, consideration should be given to other factors that could cause the disappearance of paternal mitochondrial genes from the preimplantation mammalian embryo. These include a dilution effect (there would be about 1000 copies of oocyte-derived mtDNA for each sperm-derived one in a newly fertilized ovum; Smith and Alcivar, 1993; Shoubridge, 2000), and the possibility that the ovum could exclude or expel the sperm tail with mitochondria during fertilization, as documented in Chinese hamsters (Pickworth *et al.*, 1968). The dilution theory could account for the lack of paternal mtDNA detection observed in some earlier studies, before polymerase chain reaction (PCR) techniques were perfected. With the advent of long and nested PCR techniques, the sensitivity of mtDNA detection now appears sufficient to identify foreign mtDNA at concentrations less-than the proposed 10^3 dilution factor (St John, 2002). It was also proposed that some sperm mitochondria and mtDNA could be diverted into extra-embryonic tissues and participate in embryonic cell fate determination (St John, 2002).

Numerous ultrastructural studies documented in detail the fate of the sperm mitochondria after fertilization, showing the loss of their cristae, swelling and progressive disintegration within the first two cell cycles in rodent, primate and ungulate zygotes. Such active elimination of paternal mitochondria has not only been documented in mammals (Szöllösi, 1965; Hiraoka and Hirao, 1988; Sutovsky *et al.*, 1996a,b), but also in invertebrates (Longo and Anderson, 1968; Hinkley and Newman, 1989; Longo, 1991). The selective elimination of the sperm mitochondria after mammalian fertilization was observed using fluorescent mitochondrial probes in ungulates (Sutovsky *et al.*, 1996b, 2003), primates (Sutovsky *et al.*, 1996a, 1999) and rodents (Shalgi *et al.*, 1994; Kaneda *et al.*, 1995; Cummins *et al.*, 1997). Further confirmation was

provided by studies in mice expressing the green fluorescent protein exclusively in the mitochondria (mtGFP-tg mice; Shitara *et al.*, 2001).

Ubiquitin-dependent proteolysis and organelle degradation

Ultrastructural data provide strong indication that the sperm mitochondria are selectively targeted for degradation once they enter the oocyte cytoplasm. This evidence is fortified by studies showing the association of the universal proteolytic factor, ubiquitin, with the sperm mitochondrial sheath in the cytoplasm of fertilized mammalian ova (Sutovsky *et al.*, 1999, 2000). Ubiquitin binds covalently to Lys residues of substrate proteins and marks them for degradation by the formation of long polyubiquitin chains. This process of protein ubiquitination requires ATP hydrolysis and a set of co-factors, including ubiquitin ligases E1, E2 and E3. In turn, the deubiquitinating enzymes, or ubiquitin C-terminal hydrolases are necessary for the removal of polyubiquitin chains from the substrate prior to its degradation (reviewed by Hershko, 1998; Glickman and Ciechanover, 2002).

In the studies described here, two alternative pathways for the degradation of sperm mitochondria have been considered, both involving ubiquitin-dependent proteolysis. In general, the degradation of ubiquitinated substrates can occur by two different pathways, depending on the length of polyubiquitin chains ligated to the ubiquitinated substrates (reviewed by Glickman and Ciechanover, 2002). The most commonly described proteasomal pathway requires a chain of at least four ubiquitin molecules (tetra-ubiquitin), and leads to the docking of the ubiquitinated substrate to the 26-S proteasome. The proteasome is a multi-subunit, barrel-shaped holoenzyme with multiple protease activities. After docking to the 26-S proteasome, the polyubiquitin chains are cleaved off and the substrate protein is unravelled to make the enzymatic cleavage sites accessible to proteasomal core subunits. The substrate is then cleaved into small peptides of 3–23 amino acids, which are released from the proteasome and may be dismantled into individual amino acids by the activity of cytosolic endopeptidases (reviewed by Glickman and Ciechanover, 2002; Hochstrasser, 2002). Multiubiquitin chains of fewer than four molecules, as well as simple monoubiquitination may also target proteinaceous substrates for degradation, albeit not via proteasomal activity. The most notable case of such mono-/di-ubiquitination mediated proteolysis is the endocytosis of selected plasma membrane receptors (Strous and Govers, 1999). During such an endocytotic event, the endocytotic motif of the targeted receptor on the inner face of plasma membrane is monoubiquitinated and internalized by a lysosome. Lysosomal hydrolases then complete the task of substrate degradation.

In a special case of lysosomal proteolysis, not involving plasma membrane receptors, the whole organelles in the cell cytoplasm are degraded by the formation of an autophagosome. This event, termed macroautophagy has been divided into distinct steps, including induction, formation of autophagosome or autophagic vacuoles, docking/fusion with lysosome and the actual degradation/recycling (Klionsky and Emr, 2000). The ubiquitin system plays an important, yet not fully understood role in this process, possibly by degrading the intermediates of organelle breakdown. The ultrastructural appearance of macroautophaged erythrocyte mitochondria is somewhat reminiscent of the patterns seen during the degeneration and degradation of the sperm mitochondria in the ooplasm (see **Figure 1**). It appears, though, that during sperm mitochondrial degradation, the paternal mitochondria themselves could actually be transformed into autophagic vacuole-like vesicles (Sutovsky, 2003), rather than being engulfed by the preformed vacuoles or lysosomes. As discussed below, 15-lipoxygenase (15-LOX), a major component of the reticulocyte's organelle degradation system, is present in the mammalian ooplasm.

Contribution of 15-lipoxygenase (15-LOX) to mitochondrion degradation during reticulocyte maturation and after fertilization

Macroautophagy has been described in detail in yeast (reviewed by Klionsky and Emr, 2000), wherein 15-LOX activity has not been documented. In contrast, both the 15-LOX and the macroautophagic pathways are present in mammalian reticulocytes, the differentiating red blood cells (Grullich *et al.*, 2001). The reticulocyte model system is also a favourite for studying the ubiquitin system, and the existence of ubiquitin as a universal protein degradation factor was first proposed and subsequently described in reticulocyte lysates (Goldstein *et al.*, 1975; Etlinger and Goldberg, 1977). Subsequently, the ubiquitin dependence of mitochondrial degradation within the differentiating reticulocyte has been studied in detail (Rapoport *et al.*, 1985; Dubiel *et al.*, 1987). In addition to their protein components, the mitochondria and other reticulocyte organelles that need to be eliminated also contain a large proportion of lipid-rich membranes. This realization and further research led to the proposition that arachidonate-15-lipoxygenase (15-LOX) is involved in the degradation of mitochondria and other organelles in the differentiating reticulocyte (Van Leyen *et al.*, 1998). 15-LOX is the enzyme responsible for peroxidating the lipid component of the organelle membranes, effectively causing their collapse (reviewed by Kuhn *et al.*, 2002). The uniqueness of 15-LOX is in that it can target organelle membranes in the cytoplasm without damaging the integrity of the plasma membrane. It has been proposed that 15-LOX in the reticulocytes acts in synergy and/or in parallel with the resident ubiquitin system (Grulich *et al.*, 2001), yet it is still not clear how this relationship functions. Due to its preference for mitochondrial membranes, its compatibility with the ubiquitin system and its presumed inertness towards the plasma membrane, it is possible that 15-LOX may be a co-factor in the removal of sperm mitochondria after fertilization. Indeed, high expression of 15-LOX has been observed in bovine and porcine ova and zygotes (**Figure 2A–L**). Related LOX activities have also been detected in sea urchin (Hawkins and Brash, 1987) and surf clam ova (Hada *et al.*, 1997), and may be important for membrane remodelling after fertilization (Perry and Epel, 1985). Except for a few observations (see **Figure 2C**), it was not possible to document a consistent co-localization of 15-LOX with the sperm mitochondria incorporated in the cytoplasm of fertilized mammalian ova. Similarly, the block of 15-LOX activity by a competitive substrate, 15-eicosanotrienic acid (ETYA; arachidonic acid) did not prevent the degradation of sperm

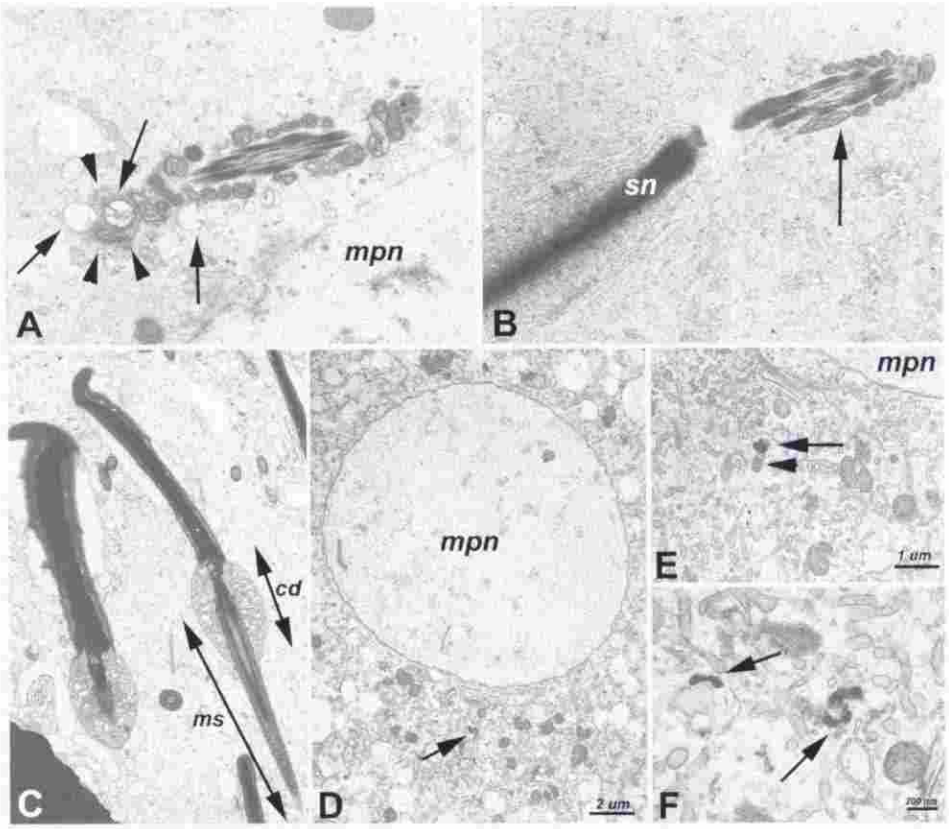

Figure 1. Ultrastructure of sperm mitochondrion degradation after IVF of bovine (**A**, **B**) and porcine (**C**–**F**) ova. (**A**) Loose association of the mitochondria within the sperm mitochondrial sheath, adjacent to male pronucleus (*mpn*) at 16 h after fertilization. Note the swollen sperm mitochondria showing partial or complete loss of mitochondrial cristae and matrix (arrows), surrounded by small particles (arrowheads; possibly proteasomes) and vesicles. (**B**) Most mitochondria remain tightly associated within the sperm mitochondrial sheath (arrow) inside a fertilized bovine ovum that was depleted of intrinsic glutathione by in-vitro maturation in medium containing glutathione synthesis inhibitor, L-buthioneine-[S,R]-sulphoximine (BSO; Sutovsky and Schatten, 1997). Note that glutathione depletion also prevented the decondensation of the sperm nucleus (*sn*), which remained mostly condensed at 20 h after fertilization. (**C**) Cauda epididymal porcine spermatozoa with characteriztic mitochondrial sheath (*ms*) and cytoplasmic droplets (*cd*). (**D**, **E**, **F**) Serial sections showing the remnants of the sperm tail midpiece (arrows) and sperm centriole (arrowhead in **E**) inside a porcine zygote at 20 h post-fertilization. Sperm mitochondria were not identified in these and other examined serial sections.

mitochondria inside a porcine zygote (**Table 1**), which has lately become our model of choice due to rapid degradation of sperm mitochondria within the first zygotic cell cycle (Sutovsky *et al.*, 2003). In the course of these studies, the accumulation of 15-LOX has also been observed in the boar sperm cytoplasmic droplet. Further observations suggest that LOX activity may be important for its removal during sperm maturation (Fischer *et al.*, 2002).

Proteasomal or lysosomal degradation of sperm mitochondrial proteins after fertilization

As has been shown previously, sperm mitochondria are already tagged by ubiquitin during spermatogenesis (Sutovsky *et al.*, 1999, 2000). In particular, prohibitin, an inner mitochondrial membrane protein expressed during spermiogenesis (Choongkittaworn *et al.*, 1993), is polyubiquitinated in the mammalian spermatozoa (Thompson *et al.*, 2003). Such ubiquitinated prohibitin species could be either directly docked to the proteasome/lysosome after fertilization, or they could be further ubiquitinated prior to being recognized by the ooplasmic proteolytic system. One way or another, the tagging of sperm mitochondria prior to fertilization, a death sentence which the sperm mitochondria carry with them into the ooplasm, is consistent with the observation that the paternal mitochondria are selectively degraded by the murine embryo even if they come from immature spermatogenic cells, spermatids or spermatocytes (Cummins *et al.*, 1998). However, rapid mitochondrial degradation is not observed when somatic cell mitochondria are injected into ooplasm (Vasilieva *et al.*, 1999; Shitara *et al.*, 2000). The degree of prohibitin ubiquitination suggests that this and other sperm mitochondrial membrane proteins should be mainly degraded by the 26-S proteasome. In earlier studies (Sutovsky *et al.*, 2000), it has been shown that the neutralization of the zygotic lysosomes with ammonium chloride delayed the degradation of sperm mitochondria in bovine embryos until the 8-cell stage. As discussed above, ultrastructural studies indicate that lysosomes or autophagic vacuoles could contribute to sperm mitochondrion degradation after fertilization. On the other hand, inhibitors such as

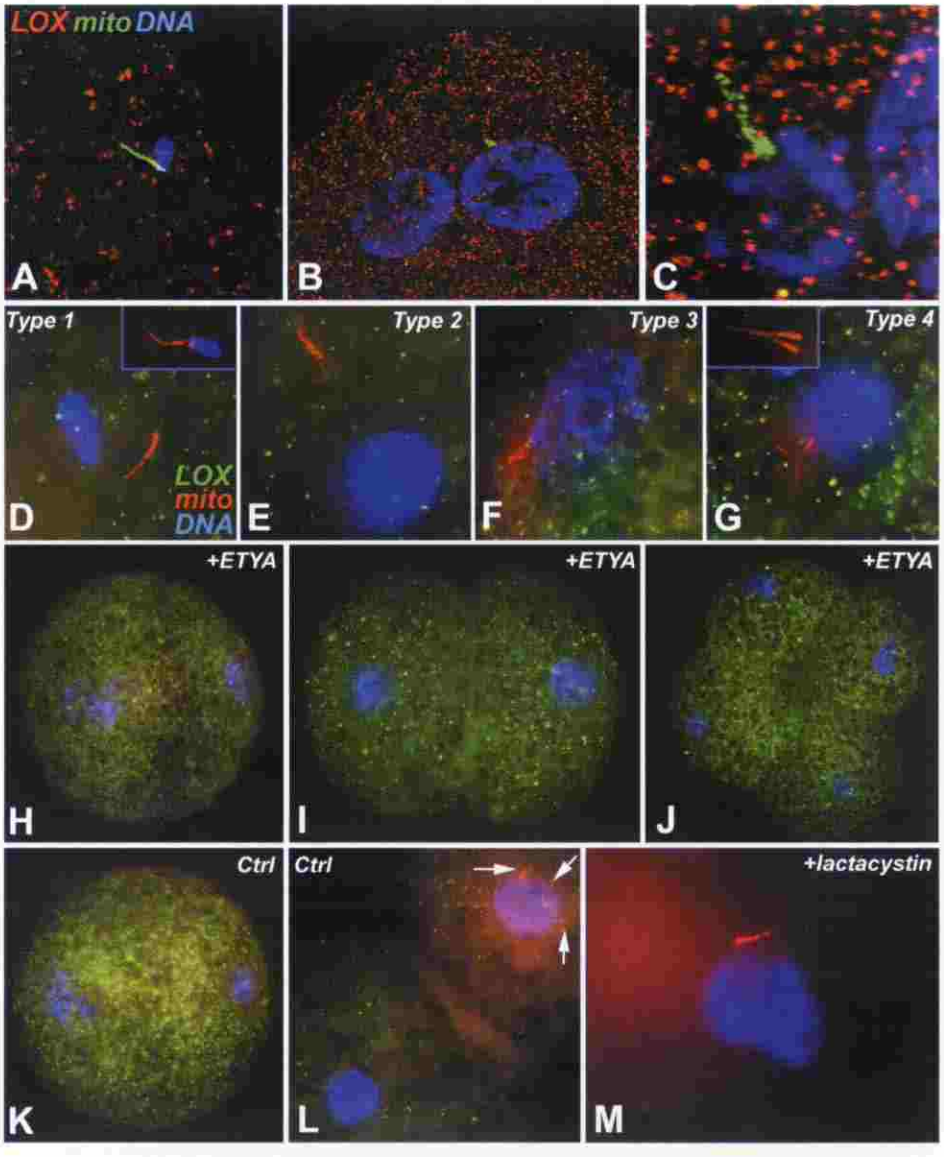

Figure 2. Expression of 15-lipoxygenase (15-LOX) in mammalian zygotes. (**A**, **B**, **C**) Confocal images of 15-LOX (red) and sperm mitochondria (green) in bovine zygotes at 16 h after IVF. Early pronuclear stage shortly after sperm incorporation (**A**) and pronuclear apposition stages (**B**, **C**) are shown. Note that some of the 15-LOX-immunoreactive structures appear adjacent to the sperm mitochondrial sheath and even inserted in the gaps left by missing, presumably degraded mitochondria (**C**). (**D**–**G**) Four types of sperm mitochondria (red), found inside porcine zygotes at 30 h after insemination. Insert (**D**) shows an intact spermatozoon. *Type 1*: an intact mitochondrial sheath, showing slight undulations, probably caused by the reduction of disulphide bond cross-linking in the membranes of mitochondrial capsule. *Type 2*: Partial degradation is observed, mitochondria are missing from the proximal part of outer dense fibres, which also seem to take up MitoTracker dye. *Type 3*: Mitochondria are mostly clustered and many are missing from the mitochondrial sheath. *Type 4*: Mitochondria are completely missing; only the outer dense fibres remain, forming a typical 'snake tongue' pattern. Insert (**G**) shows a detail of such 'snake tongue'. 15-LOX is labelled in green, DNA is counterstained blue with DAPI. (**H**–**J**). Detection of 15-LOX (green) and sperm mitochondria (red) in porcine pronuclear zygotes (**H**), 2-cell embryos (**I**) and 4-cell embryos (**J**) cultured for 30 h in presence of 50 µmol/l eicosanotrienic acid (ETYA; a potent inhibitor of 15-LOX activity). ETYA treatment did not prevent sperm mitochondrion degradation, or embryo development, while the polyspermy rates were increased, probably due to the effect of ETYA on acrosomal exocytosis. (**K**, **L**) Control porcine ova at 30 h after IVF, labelled with anti-15-LOX serum (green). Figure (**L**) shows seldom observed remnants of sperm mitochondrial sheath (arrows) in one of the blastomeres of a two cell embryo. (**M**) Persistence of sperm mitochondrial sheath (type 1–2) in a cytoplasm of oocyte fertilized and cultured for 30 h in presence of proteasomal inhibitor, lactacystin (10 µmol/l). IVF with MitoTracker-tagged spermatozoa was performed as described previously for bovine (Sutovsky *et al.*, 1996a) and porcine (Abeydeera and Day, 1997; Sutovsky *et al.*, 2003) IVF systems. The above studies also provide details on immunofluorescence processing. 15-LOX was detected using a rabbit serum against synthetic peptide corresponding to C-terminal amino acid sequence (YLRPSIVENSVAI) of rabbit reticulocyte 15-LOX. Confocal microscopy was performed using Leica NT2 microscope, conventional microscopy was performed using Nikon Eclipse 800 microscope equipped with QuickSnap CCD camera. Images were processed by MetaMorph 4.6.5 and Adobe Photoshop 5.5 software.

Table 1. Effects of the inhibition of 15-lipoxygenase and proteasome pathways on the degradation of paternal mitochondria after porcine fertilization *in vitro*.

Treatment[a]*/mito type*[b]	*Type 1*	*Type 2*	*Type 3*	*Type 4*	*Total*
Control	5 (10.9)	4 (8.7)	17 (37)	20 (43.5)	46 (100)
50 µmol/l ETYA at fertilization	2 (5.7)	7 (20)	9 (25.7)	17 (48.6)	35 (100)
100 µmol/l ETYA at fertilization	4 (19)	0	6 (28.6)	11 (52.4)	21 (100)
200 µmol/l ETYA at fertilization	1 (6.7)	1 (6.7)	5 (33.3)	8 (53.3)	15 (100)
10 µmol/l lactacystin at fertilization	11 (57.9)	3 (15.8)	2 (10.5)	3 (15.8)	19 (100)
10 µmol/l MG132 at fertilization[c]	0	0	0	0	19
10 µmol/l MG132 at 6 h PI	7 (31.8)	13 (59.1)	2 (9.1)	0	22
Total ova					177

Values in parentheses are percentages.
[a]All ova were fixed and processed 30 h post-insemination (PI).
[b]Type 1 represents intact sperm mitochondria, type 4 represent complete degradation of the sperm mitochondria (see **Figure 2** for details).
[c]No fertilization was observed in this group of 19 ova due to the MG132-induced block of ZP-penetration.

ammonium chloride are not specific for lysosomal activity alone and could affect the viability or developmental potential of the treated embryos. For these reasons, experiments were initiated with specific proteasomal inhibitors, including lactacystin and MG-132. These inhibitors are specific for proteasomal activity and do not affect non-proteasomal serine proteases such as chymotrypsin, trypsin or papain (Fenteany *et al.*, 1995; Goldberg *et al.*, 1995). Initially, the inclusion of these inhibitors into fertilization medium at insemination led to a partial (lactacystin at low concentrations) or complete (lactacystin at higher concentrations; MG132) block of porcine fertilization, most likely due to the inhibition of proteasomes present in the boar sperm acrosome, and necessary for sperm–zona penetration (Sawada *et al.*, 2002; Sutovsky *et al.*, 2003). This inhibitory effect forced the change of protocol in the experiments, incorporating the addition proteasomal inhibitors at 6 h after insemination. Such delayed anti-proteasomal treatment allows a time window sufficient for sperm incorporation into ooplasm. A complete and fully reversible block of sperm mitochondrial degradation was observed (Sutovsky *et al.*, 2003; see also **Table 1**), providing evidence that the ooplasmic 26-S proteasome is indeed crucial for enforcing maternal inheritance of mitochondria after fertilization.

If it is agreed that sperm mitochondria do enter oocyte cytoplasm to be degraded by 26-S proteasome, does this mean that their cargo of paternal mtDNA is hydrolysed at the same time? Most likely it is the case wherever the principle of uniparental mtDNA inheritance applies. However, conclusive evidence has not yet been established. If proteasomal degradation is the main route of sperm mitochondrial proteolysis, it is possible that nucleic acids in the mitochondrial matrix could be degraded concomitantly. Nuclease activity has been shown in proteasomes (Pouch *et al.*, 1995; Horikoshi *et al.*, 1998). Similarly, the lysosomes or autophagic vacuoles, which may also contribute to ubiquitin-dependent sperm mitochondrion degradation, contain acidic hydrolases capable of degrading DNA and various RNAs (Irie, 1999). Further insight into this mechanism could be brought by studies of mitochondrial inheritance in isogamous protist *Physarum polycephalum*. *Physarum* is an organism with hierarchical order of mitochondrial inheritance in which the fate of mtDNA from individual isogametes is determined by the mating-type locus matA (Kawano *et al.*, 1987). After mating, the mitochondria from one parent are first deprived of mtDNA and only after mtDNA digestion does the mitochondrial membrane collapse and the whole mitochondrial sheath is eliminated (Moriyama and Kawano, 2003). Even though such early uniparental degradation of mtDNA in *Physarum* preceded the complete degradation of the mitochondrial sheath, it appeared to be paralleled by a collapse of the inner mitochondrial membrane in individual mitochondria. It would be useful to determine whether the degradation of both mitochondria and mtDNA in this intriguing model depends upon proteasomal activity, or whether it relies on an independent, nuclease-type activity.

Why is sperm mitochondrion degradation species specific and restricted to ooplasm?

Sperm mitochondria are metabolically functional and under certain conditions can be propagated when microinjected into somatic, mitochondrion-free rho0 cells (Manfredi *et al.*, 1997). However, such repopulation with sperm mitochondria occurs only in a small portion of injected rho0 cells. This could argue for the hypothesis of ubiquitin-dependent degradation after fertilization, since the somatic cells, similar to oocytes, posses an active ubiquitin–proteasome system. As for the lesser efficiency of sperm mitochondrion elimination in rho0 cells, these somatic cells may not possess the capability to unmask the ubiquitinated mitochondrial membranes in the same way that oocyte cytoplasm does. In particular, the priming of sperm mitochondria for degradation could be accelerated by disulphide-bond reduction of the mitochondrial capsule proteins rich in both lysine (ubiquitination epitopes) and cysteine (disulphide bond-formation epitope). This is achieved by the reducing action of the oocyte-produced intrinsic peptide, glutathione (Perreault *et al.*, 1984) and possibly other ooplasmic co-factors. Earlier studies indeed demonstrated that the suppression of glutathione synthesis during oocyte maturation not only hinders the transformation of the sperm nucleus into male pronucleus, but also delays the disassembly of sperm flagellum and swelling of the sperm mitochondria (Sutovsky and Schatten, 1997; see also **Figure 1B**). In addition, a family of sperm-specific thioredoxins, proteins

capable of managing the balance of disulphide bond reduction and formation, has been recently described (Miranda-Vizuete *et al.*, 2001; Sadek *et al.*, 2001). Such co-factors may not be present in somatic cells cytoplasm in quantities comparable to ooplasm. Alternatively, the gamete-specific ubiquitin ligases may participate in the ubiquitination and proteolysis of the sperm mitochondria after fertilization. It is now well established that despite extreme conservation of ubiquitin sequence (Özkaynak *et al.*, 1984), ubiquitination is a highly substrate-specific event, mainly due to a wide array of tissue-specific ubiquitin ligases and a variety of narrowly specific ubiquitination/degradation domains or degrons, within the amino acid sequences of the ubiquitination-prone substrates (Laney and Hochstrasser, 1999). One such oocyte-specific ubiquitin ligase, the E3-type ligase RFPL4 (Ret Finger Protein-Like 4 gene product), has been described recently in mice (Suzumori *et al.*, 2003). Similarly, the acrosome of mouse sperm head contains a testis/germ cell-specific deubiquitinating enzyme mUBPy (Berruti and Martegani, 2002).

The existence of (ovarian and testicular) tissue specific ubiquitin ligases and C-terminal hydrolases could also account for species specificity of maternal mtDNA inheritance. Interspecific hybrids between domestic mouse and Spanish wild mouse (*Mus musculus* × *M. spretus*) provide a good model to study the species specificity of sperm mitochondrion degradation. In such crosses, paternal mtDNA can be detected in F1 offspring (Gyllensten *et al.*, 1991; Kaneda *et al.*, 1995), but not in the F2 generation (Shitara *et al.*, 2000). The main lesson learned from these studies is that the sperm mitochondrial membrane proteins, rather than mtDNA itself, are recognized by the oocyte and are conducive to sperm mitochondrion recognition and degradation after fertilization. Second to that, the observation of lacking paternal mtDNA transmission in backcrosses is of interest (Shitara *et al.*, 2000). This is not surprising when F1 hybrid males are backcrossed with wild type females of either species: mitochondrial membranes of such F1 males should contain proteins encoded by nuclear genes of both parental species, and thus should be vulnerable to recognition and proteolysis in the ooplasm of either of the two parental species. More surprising is the lack of paternal mtDNA transmission when F1 females are backcrossed with wt males. This seeming paradox can be reconciled by the observation of genetic drift of paternal mtDNA in the F1 hybrid females, wherein only certain tissues, not including germ line, appear to receive paternal mtDNA (Shitara *et al.*, 2000).

Heteroplasmy after assisted reproduction

Heteroplasmy or the coexistence of two distinct mitochondrial genomes within the same cytoplast may arise from mtDNA mutations resulting in a mixture of normal and mutated mtDNA, derived from the common progenitor mitochondrial genome (Graff *et al.*, 2002). By a stricter definition, true heteroplasmy arises from the introduction and propagation of foreign mitochondria into a recipient cytoplast (Cummins, 2001). Perhaps the most common occurrence of heteroplasmy is seen after nuclear transfer (NT) in animals. Heteroplasmy due to the presence of donor cell mtDNA has been documented in NT embryos cloned from both an embryonic blastomere (Hiedlander *et al.*, 1999) and a somatic cell (e.g. Do *et al.*, 2002; Steinborn *et al.*, 2002; Hiedlander *et al.*, 2003; Takeda *et al.*, 2003). Thus far, the abnormalities commonly seen in cloned embryos have not been attributed to heteroplasmy. In this regard, it should also be considered that the recipient, ooplast-derived mitochondria arise from a pooled, heterologous population of oocytes, thus increasing the variability of mtDNA inheritance seen in offspring.

With regard to human assisted reproduction, the oocyte cytoplasm donation, or ooplasmic transplantation procedure has been used successfully to rejuvenate the mitochondrial pools of aged ova from premenopausal women in some IVF programmes. The major concern is that the ooplasmic transplantation procedure inevitably propagates heteroplasmy, i.e. the perpetuation of mitochondria and mitochondrial genomes from both the donor and the recipient cytoplasm. It has been documented that children born after cytoplasm donation are heteroplasmic (Brenner *et al.*, 2000; Barritt *et al.*, 2001a). The major concern in this treatment is that the persistence and replication of the recipient's mtDNA, which is derived from an aged mitochondrial pool and may bear more mutations than that of the donor. This could affect the fitness of children born after such procedure. The initial survey of such heteroplasmic babies found all of them healthy (Barritt *et al.*, 2001a). However, a follow-up report revealed two occurrences of 45,XO syndrome resulting in one spontaneous miscarriage and one selective abortion, and one case of pervasive developmental disorder, a spectrum of autism-related symptoms, diagnosed at 18 months of age (Barritt *et al.*, 2001b). It is difficult to determine whether the above anomalies were inherent to IVF procedure or they were a direct result of mitochondrial transplantation. The critics of this technique urge clinicians to consider that a mitochondrial deficiency stemming from either recipient or donor mtDNA could be manifested only later during post-natal life, rather than during early childhood.

Similar concerns exist with regard to possible transmission of paternal mitochondria by ICSI, mainly due to a possibility that the intact plasma membrane overlaying the sperm mitochondrial sheath could hinder the access of ooplasmic factors to the sperm mitochondrial membranes (Sutovsky, 2003). Thus far, none of the follow-up studies conducted to date in ICSI babies has substantiated such fears (Houshmand *et al.*, 1997; Danan *et al.*, 1999; Marchington *et al.*, 2002). Furthermore, experiments in mice showed that sperm mitochondria are degraded after ICSI with similar dynamics to what is seen after natural fertilization (Cummins *et al.*, 1997). The presence of paternal mtDNA has been described in defective preimplantation human embryos (St John *et al.*, 2000), an indication that warrants further research. At the same time, it is difficult to rule out that the screened embryos could carry accessory spermatozoa on their surface even after the mitochondria of the fertilizing, incorporated spermatozoon were degraded. Such accessory spermatozoa, bound to the oolemma, yet prevented from incorporation into ooplasm by anti-polyspermy defence, are common in both in vivo- and in vitro-derived zygotes and early embryos of other mammals (P. Sutovsky, unpublished observations).

Challenges to the hypothesis

As mentioned previously, some animals such as shellfish may avoid the degradation of paternal mitochondria, and occasional paternal inheritance of mtDNA has been suggested to occur in mammals, including humans. These reports include molecular studies in ruminants (Zhao *et al.*, 2000; Slate and Phua, 2003), and population studies in chimpanzees (Awadalla *et al.*, 1999) and the isolated Pacific Islander populations (Eyre-Walker *et al.*, 1999; Hagelberg *et al.*, 1999), based on a survey of the hypervariable region mtDNA, the D-loop. Such evidence has been disputed (e.g. Inan and Nordborg, 2002) and in certain extent contradicted by studies exploring the whole mitochondrial genome (Ingman *et al.*, 2000; Herrnstadt *et al.*, 2002), yet warrants further examination (Hagelberg, 2003). The purpose of this review is not to discuss the possibility of recombination between paternal and maternal, human genomes after fertilization. To date, the only reliably documented, naturally occurring deviation from this pattern is the instances of severe mitochondrial pathology in humans (Schwartz and Vissing, 2002), wherein the transmission of paternal mtDNA, but no paternal–maternal mtDNA recombination has been found in a man carrying a de-novo deletion of mitochondrial NADH dehydrogenase subunit-2 (*ND2*) gene. While the degradation of the sperm mitochondria may not be synonymous with the degradation of paternal mtDNA, the data from fertilization studies show unambiguous evidence for the former. It remains to determine whether paternal mtDNA is degraded concomitantly with the degradation of sperm mitochondrial membranes and mitochondrial matrix.

Acknowledgements

The authors thank Randy Tindall and Cheryl Jensen for the preparation of TEM samples, and Angela George, Beth Azdell and Heinz Leigh for technical and clerical assistance. This work was supported by grants from USDA (99-35203 and 2002-02069) and NIH/NIOSH (OH 07324–01) to P.S., who is also supported by the F21C Program of the University of Missouri-Columbia.

References

Agarwal A, Saleh RA, Bedaiwy MA 2003 Role of reactive oxygen species in the pathophysiology of human reproduction. *Fertility and Sterility* **79**, 829–843.

Aitken R J, Gordon E, Harkiss D *et al.* 1998 Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biology of Reproduction* **59**, 1037–1046.

Ankel-Simons F, Cummins JM 1996 Misconceptions about mitochondria and mammalian fertilization: implications for theories on human evolution. *Proceedings of the National Academy of Sciences of the USA* **93**, 13859–13863.

Awadalla P, Eyre-Walker A, Smith JM 1999 Linkage disequilibrium and recombination in hominid mitochondrial DNA. *Science* **286**, 2524–2525.

Axnér E, Ström B, Linde-Forsberg C 1999 Morphology and motility of spermatozoa from different regions of the epididymal duct in the domestic cat. *Theriogenology* **52**, 767–778.

Barritt JA, Brenner CA, Malter HE, Cohen J 2001a Mitochondria in human offspring derived from ooplasmic transplantation. *Human Reproduction* **16**, 513–516.

Barritt JA, Brenner CA, Malter HE, Cohen J 2001b Rebuttal: interooplasmic transfers in humans. *Reproductive BioMedicine Online* **3**, 47–48.

Berger KH, Yaffe MP 1998 Prohibitin family members interact genetically with mitochondrial inheritance components in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **18**, 4043–4052.

Berruti G, Martegani E 2002 mUBPy and MSJ-1, a deubiquitinating enzyme and a molecular chaperone specifically expressed in testis, associate with the acrosome and centrosome in mouse germ cells. *Annals of the New York Academy of Sciences* **973**, 5–7.

Birky Jr CW 1995 Uniparental inheritance of mitochondrial and chloroplast genes: mechanisms and evolution. *Proceedings of the National Academy of Sciences of the USA* **92**, 11331–11338.

Birky WC Jr 2001 The inheritance of genes in mitochondria and chloroplasts: laws, mechanisms, and models. *Annual Review of Genetics* **35**, 125–148.

Brenner CA, Barritt JA, Willadsen S, Cohen J 2000 Mitochondrial DNA heteroplasmy after human ooplasmic transplantation. *Fertility and Sterility* **74**, 573–578.

Choongkittaworn NM, Kim KH, Danner DB, Griswold MD 1993 Expression of prohibitin in rat seminiferous epithelium. *Biology of Reproduction* **49**, 300–310.

Cummins JM 2001 Cytoplasmic inheritance and its implications for animal biotechnology. *Theriogenology* **55**, 1381–1399.

Cummins JM, Wakayma T, Yanagimachi R 1997 Fate of microinjected sperm components in the mouse oocyte and embryo. *Zygote* **5**, 301–308.

Cummins J M, Wakayma T, Yanagimachi R 1998 Fate of microinjected spermatid mitochondria in the mouse oocyte and embryo. *Zygote* **6**, 213–222.

Danan C, Sternberg D, Van Steirteghem A *et al.* 1999 Evaluation of parental mitochondrial inheritance in neonates born after intracytoplasmic sperm injection. *American Journal of Human Genetics* **65**, 463–473.

Diez-Sanchez C, Ruiz-Pesini E, Lapena AC *et al.* 2003 Mitochondrial DNA content of human spermatozoa. *Biology of Reproduction* **68**, 180–185.

Do JT, Lee JW, Lee BY *et al.* 2002 Fate of donor mitochondrial DNA in cloned bovine embryos produced by microinjection of cumulus cells. *Biology of Reproduction* **67**, 555–560.

Dubiel W, Drung I, Muller M, Rapoport SM 1987 Kinetic studies on the ATP- and ubiquitin-dependent proteolytic system of reticulocytes. *Biochimica et Biophysica Acta* **46**, 565–570.

Etlinger JD, Goldberg AL 1977 A soluble ATP-dependent proteolytic system responsible for the degradation of abnormal proteins in reticulocytes. *Proceedings of the National Academy of Sciences of the USA* **74**, 54–58.

Eyre-Walker A, Smith NH, Smith JM 1999 How clonal are human mitochondria? *Proceedings of the Royal Society of London B Biological Sciences* **266**, 477–483.

Fenteany G, Standaert RF, Lane WS *et al.* 1995 Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science* **268**, 726–731.

Fischer KA, Safranski TJ, Van Leyen K *et al.* 2002 Components of lipoxygenase and ubiquitin-dependent pathways for organelle degradation colocalize in the cytoplasmic droplets of mammalian spermatozoa. *Biology of Reproduction* **67**, 114.

Fisk HA, Yaffe MP 1999 A role for ubiquitination in mitochondrial inheritance in *Saccharomyces cerevisiae*. *Journal of Cell Biology* **145**, 1199–1208.

Glickman MH, Ciechanover A 2002 The ubiquitin–proteasome proteolytic pathway: destruction for the sake of construction. *Physiological Reviews* **82**, 373–428.

Goldberg AL, Stein R, Adams J 1995 New insights into proteasome function: from archaebacteria to drug development. *Chemical Biology* **2**, 503–508.

Goldstein G, Scheid M, Hammerling U *et al.* 1975 Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells. *Proceedings of the National Academy of Sciences of the USA* **72**, 11–15.

Graff C, Bui TH, Larsson NG 2002 Mitochondrial diseases. *Best*

Practice Research in Clinical Obstetrics and Gynaecology **16**, 715–728.

Grullich C, Duvoisin RM, Wiedmann M, van Leyen K 2001 Inhibition of 15-lipoxygenase leads to delayed organelle degradation in the reticulocyte. *FEBS Letters* **489**, 51–54.

Gyllensten U, Wharton D, Josefsson A, Wilson AC 1991 Paternal inheritance of mitochondrial DNA in mice. *Nature* **352**, 255–257.

Hada T, Swift LL, Brash AR 1997 Discovery of 5R-lipoxygenase activity in oocytes of the surf clam, *Spisula solidissima*. *Biochimica et Biophysica Acta* **1346**, 109–119.

Hagelberg E, Goldman N, Lio P *et al.* 1999 Evidence for mitochondrial DNA recombination in a human population of island Melanesia. *Proceedings of the Royal Society of London B Biological Sciences* **266**, 485–492.

Hawkins DJ, Brash AR 1987 Eggs of the sea urchin, *Strongylocentrotus purpuratus*, contain a prominent (11R) and (12R) lipoxygenase activity. *Journal of Biological Chemistry* **262**, 7629–7634.

Herrnstadt C, Elson JL, Fahy E *et al.* 2002 Reduced-median-network analysis of complete mitochondrial DNA coding-region sequences for the major African, Asian, and European haplogroups. *American Journal of Human Genetics* **70**, 1152–1171.

Hershko A 1998 The ubiquitin system: past, present and future perspectives. In: Peters J-M, Harris JR, Finley D (eds) *Ubiquitin and the Biology of the Cell*. Plenum Press, New York, pp. 1–17.

Hiendleder S, Schmutz SM, Erhardt G *et al.* 1999 Transmitochondrial differences and varying levels of heteroplasmy in nuclear transfer cloned cattle. *Molecular Reproduction and Development* **54**, 24–31.

Hiendleder S, Zakhartchenko V, Wenigerkind H *et al.* 2003 Heteroplasmy in bovine fetuses produced by intra- and inter-subspecific somatic cell nuclear transfer: neutral segregation of nuclear donor mitochondrial DNA in various tissues and evidence for recipient cow mitochondria in fetal blood. *Biology of Reproduction* **68**, 159–166.

Hinkley RE Jr, Newman AN 1989 Selective identification of the paternal mitochondrion in living sea urchin eggs and embryos by chlorotetracycline. *Journal of Experimental Zoology* **249**, 111–114.

Hiraoka J, Hirao Y 1988 Fate of sperm tail components after incorporation into the hamster egg. *Gamete Research* **19**, 369–380.

Hochstrasser M 2002 Molecular biology. New proteases in a ubiquitin stew. *Science* **298**, 549–552.

Horikoshi T, Page J, Lei G *et al.* 1998 Proteasomal RNase activity in human epidermis. *In Vivo* **12**, 155–158.

Houshmand M, Holme E, Hanson C *et al.* 1997 Is paternal mitochondrial DNA transferred to the offspring following intracytoplasmic sperm injection? *Journal of Assisted Reproduction and Genetics* **14**, 223–227.

Ingman M, Kaessmann H, Paabo S, Gyllensten U 2000 Mitochondrial genome variation and the origin of modern humans. *Nature* **408**, 708–713.

Innan H, Nordborg M 2000 Recombination or mutational hot spots in human mtDNA? *Molecular Biology and Evolution* **19**, 1122–1127.

Inoue K, Nakada K, Ogura A *et al.* 2000 Generation of mice with mitochondrial dysfunction by introducing mouse mtDNA carrying a deletion into zygotes. *Nature Genetics* **26**, 176–181.

Irie M 1999 Structure–function relationships of acid ribonucleases: lysosomal, vacuolar, and periplasmic enzymes. *Pharmacology and Therapeutics* **81**, 77–89.

Jannotti-Passos LK, Souza CP, Parra JC, Simpson AJ 2001 Biparental mitochondrial DNA inheritance in the parasitic trematode *Schistosoma mansoni*. *Journal of Parasitology* **87**, 79–82.

Kaneda H, Hayashi J-I, Takahama S *et al.* 1995 Elimination of paternal mitochondrial DNA in intraspecific crosses during early mouse embryogenesis. *Proceedings of the National Academy of Sciences of the USA* **92**, 4542–4546.

Kao S-H, Chao H-T, Wei Y-H 1995 Mitochondrial deoxyribonucleic acid 4977-bp deletion is associated with diminished fertility and motility of human sperm. *Biology of Reproduction* **52**, 729–736.

Kawano S, Anderson RW, Nanba T, Kuroiwa T 1987 Polymorphism and uniparental inheritance of mitochondrial DNA in *Physarum polycephalum*. *Journal of General Microbiology* **133**, 3175–3182.

Klionsky DJ, Emr SD 2000 Autophagy as a regulated pathway of cellular degradation. *Science* **290**, 1717–1721.

Kuhn H, Walther M, Kuban RJ 2002 Mammalian arachidonate 15-lipoxygenases structure, function, and biological implications. *Prostaglandins and Other Lipid Mediators* **68–69**, 263–290.

Kvist L, Martens J, Nazarenko AA, Orell M 2003 Paternal leakage of mitochondrial DNA in the great tit (*Parus major*). *Molecular Biology and Evolution* **20**, 243–247.

Ladoukakis ED, Zouros E 2001 Direct evidence for homologous recombination in mussel (*Mytilus galloprovincialis*) mitochondrial DNA. *Molecular Biology and Evolution* **18**, 1168–1175.

Laney JD, Hochstrasser M 1999 Substrate targeting in the ubiquitin system. *Cell* **97**, 427–430.

Lestienne P, Reynier P, Chretien M-F *et al.* 1997 Oligoasthenospermia associated with multiple mitochondrial DNA rearrangements. *Molecular Human Reproduction* **3**, 811–814.

Longo FJ 1991 Gamete interactions and the fate of sperm organelles in fertilized echinoderm eggs. *Journal of Electron Microscopy Technology* **17**, 246–265.

Longo FJ, Anderson E 1968 The fine structure of pronuclear development and fusion in the sea urchin, *Arbacia punctulata*. *Journal of Cell Biology* **39**, 339–368.

Manfredi G, Thyagarajan D, Papadopoulou LC *et al.* 1997 The fate of human sperm-derived mtDNA in somatic cells. *American Journal of Human Genetics* **61**, 953–960.

Marchington DR, Scott Brown MS, Lamb VK *et al.* 2002 No evidence for paternal mtDNA transmission to offspring or extra-embryonic tissues after ICSI. *Molecular Human Reproduction* **8**, 1046–1049.

Matzuk MM, Burns KH, Viveiros MM, Eppig JJ 2002 Intercellular communication in the mammalian ovary: oocytes carry the conversation. *Science* **296**, 2178–2180.

May-Panloup P, Chretien MF, Savagner F *et al.* 2003 Increased sperm mitochondrial DNA content in male infertility.*Human Reproduction* **18**, 550–556.

Meusel MS, Moritz RF 1993 Transfer of paternal mitochondrial DNA during fertilization of honeybee (*Apis mellifera* L.) eggs. *Current Genetics* **24**, 539–543.

Miranda-Vizuete A, Ljung J, Damdimopoulos AE *et al.* 2001 Characterization of Sptrx, a novel member of the thioredoxin family specifically expressed in human spermatozoa. *Journal of Biological Chemistry* **276**, 31567–31574.

Moriyama Y, Kawano S 2003 Rapid, selective digestion of mitochondrial DNA in accordance with the matA hierarchy of multiallelic mating types in the mitochondrial inheritance of *Physarum polycephalum*. *Genetics* **164**, 963–975.

Nuell MJ, Stewart DA, Walker L *et al.* 1991 Prohibitin, an evolutionarily conserved intracellular protein that blocks DNA synthesis in normal fibroblasts and HeLa cells. *Molecular and Cellular Biology* **11**, 1372–1381.

O'Connell M, McClure N, Lewis SE 2002 A comparison of mitochondrial and nuclear DNA status in testicular sperm from fertile men and those with obstructive azoospermia. *Human Reproduction* **17**, 1571–1577.

Özkaynak E, Finley D, Varshavsky A 1984 The yeast ubiquitin gene: head-to-tail repeats encoding a polyubiquitin precursor protein. *Nature* **312**, 663–666.

Perez GI, Trbovich AM, Gosden RG, Tilly JL 2000 Mitochondria and the death of oocytes. *Nature* **403**, 500–501.

Perreault SD, Wolff RA, Zirkin BR 1984 The role of disulfide bond reduction during mammalian sperm nuclear decondensation *in vivo*. *Developmental Biology* **101**, 160–167.

Perry G, Epel D 1985 Characterization of a Ca^{2+}-stimulated lipid

peroxidizing system in the sea urchin egg. *Developmental Biology* **107**, 47–57.

Pickworth S, Yerganian G, Chang MC 1968 Fertilization and early development in the Chinese hamster, *Cricetulus griseus*. *Anatomical Record* **162**, 197–208.

Pouch MN, Petit F, Buri J *et al.* 1995 Identification and initial characterization of a specific proteasome (prosome) associated RNase activity. *Journal of Biological Chemistry* **270**, 22023–22028.

Rapoport S, Dubiel W, Muller M 1985 Proteolysis of mitochondria in reticulocytes during maturation is ubiquitin-dependent and is accompanied by a high rate of ATP hydrolysis. *FEBS Letters* **180**, 249–252.

Rinaudo P, Niven-Fairchild T, Buradagunta S *et al.* 1999 Microinjection of mitochondria into zygotes creates a model for studying the inheritance of mitochondrial DNA during reimplantation development. *Fertility and Sterility* **71**, 912–918.

Runions CJ, Owens JN 1999 Sexual reproduction of interior spruce (Pinaceae). II. Fertilization to early embryo formation. *International Journal of Plant Science* **160**, 641–652.

Sadek CM, Damdimopoulos AE, Pelto-Huikko M *et al.* 2001 Sptrx-2, a fusion protein composed of one thioredoxin and three tandemly repeated NDP-kinase domains is expressed in human testis germ cells. *Genes to Cells* **6**, 1077–1090.

Sawada H, Sakai N, Abe Y *et al.* 2002 Extracellular ubiquitination and proteasome-mediated degradation of the ascidian sperm receptor. *Proceedings of the National Academy of Sciences of the USA* **99**, 1223–1228.

Schwartz M, Vissing J 2002 Paternal inheritance of mitochondrial DNA. *New England Journal of Medicine* **22**, 576–580.

Shalgi R, Magnus A, Jones R, Phillips DM 1994 Fate of sperm organelles during early embryogenesis in the rat. *Molecular Reproduction and Development* **37**, 264–271.

Shitara H, Hayashi JI, Takahama S *et al.* 1998 Maternal inheritance of mouse mtDNA in interspecific hybrids: segregation of the leaked paternal mtDNA followed by the prevention of subsequent paternal leakage. *Genetics* **148**, 851–857.

Shitara H, Kaneda H, Sato A *et al.* 2000 Selective and continuous elimination of mitochondria microinjected into mouse eggs from spermatids, but not from liver cells, occurs throughout embryogenesis. *Genetics* **156**, 1277–1284.

Shitara H, Kaneda H, Sato A *et al.* 2001 Non-invasive visualization of sperm mitochondria behavior in transgenic mice with introduced green fluorescent protein (GFP). *FEBS Letters* **500**, 7–11.

Shoubridge EA 2000 Mitochondrial DNA segregation in the developing embryo. *Human Reproduction* **15**, 229–234.

Sinha Hikim AP, Swerdloff RS 1999 Hormonal and genetic control of germ cell apoptosis in the testis. *Reviews in Reproduction* **4**, 38–47.

Slate J, Phua SH 2003 Patterns of linkage disequilibrium in mitochondrial DNA of 16 ruminant populations. *Molecular Ecology* **12**, 597–608.

Smith LC, Alcivar AA 1993 Cytoplasmic inheritance and its effects on development and performance. *Journal of Reproduction and Fertility* (Suppl.) **48**, 31–43.

Smith LC, Bordignon V, Couto MM *et al.* 2002 Mitochondrial genotype segregation and the bottleneck. *Reproductive BioMedicine Online* **4**, 248–255.

Steinborn R, Schinogl P, Wells DN *et al.* 2002 Coexistence of *Bos taurus* and *B. indicus* mitochondrial DNAs in nuclear transfer-derived somatic cattle clones. *Genetics* **162**, 823–829.

St John JC 2002 The transmission of mitochondrial DNA following assisted reproductive techniques. *Theriogenology* **57**, 109–123.

St John JC, Sakkas D, Dimitriadi K *et al.* 2000 Failure of elimination of paternal mitochondrial DNA in abnormal embryos. *Lancet* **355**, 200.

Strous GJ, Govers R 1999 The ubiquitin–proteasome system and endocytosis. *Journal of Cell Science* **112**, 1417–1423.

Suarez SS 2002 Gamete transport. In: Hardy D (ed.) *Fertilization*. Academic Press, San Diego, pp. 3–28.

Sutovsky P 2003 Ubiquitin-dependent proteolysis in mammalian spermatogenesis, fertilization, and sperm quality control: killing three birds with one stone. *Microscopy Research and Technology* **61**, 88–102.

Sutovsky P, Schatten G 1997 Depletion of glutathione during oocyte maturation reversibly blocks the decondensation of the male pronucleus and pronuclear apposition during fertilization. *Biology of Reproduction* **56**, 1503–1512.

Sutovsky P, Schatten G 2000 Paternal contributions to the mammalian zygote: fertilization after sperm–egg fusion. *International Reviews in Cytology* **195**, 1–65.

Sutovsky P, Hewitson L, Simerly CR *et al.* 1996a Intracytoplasmic sperm injection (ICSI) for Rhesus monkey fertilization results in unusual chromatin, cytoskeletal, and membrane events, but eventually leads to pronuclear development and sperm aster assembly. *Human Reproduction* **11**, 1703–1712.

Sutovsky P, Navara CS, Schatten G 1996b The fate of the sperm mitochondria, and the incorporation, conversion and disassembly of the sperm tail structures during bovine fertilization *in vitro*. *Biology of Reproduction* **55**, 1195–1205.

Sutovsky P, Moreno R, Ramalho-Santos J *et al.* 1999 Ubiquitin tag for sperm mitochondria. *Nature* **402**, 371–372.

Sutovsky P, Moreno R, Ramalho-Santos J *et al.* 2000 Ubiquitinated sperm mitochondria, selective proteolysis and the regulation of mitochondrial inheritance in mammalian embryos. *Biology of Reproduction* **63**, 582–590.

Sutovsky P, Moreno R, Ramalho-Santos J *et al.* 2001 A putative, ubiquitin-dependent mechanism for the recognition and elimination of defective spermatozoa in the mammalian epididymis. *Journal of Cell Science* **114**, 1665–1675.

Sutovsky P, McCauley TC, Sutovsky M, Day BN 2003 Early degradation of paternal mitochondria in domestic pig *(Sus scrofa)* is prevented by selective proteasomal inhibitors lactacystin and MG 132. *Biology of Reproduction* **68**, 1793–1800.

Suzumori N, Burns KH, Yan W, Matzuk MM 2003 RFPL4 interacts with oocyte proteins of the ubiquitin–proteasome degradation pathway. *Proceedings of the National Academy of Sciences of the USA* **100**, 550–555.

Szöllösi D 1965 The fate of the sperm middle-piece mitochondria in the rat egg. *Journal of Experimental Zoology* **159**, 367–378.

Takeda K, Akagi S, Kaneyama K *et al.* 2003 Proliferation of donor mitochondrial DNA in nuclear transfer calves (*Bos taurus*) derived from cumulus cells. *Molecular Reproduction and Development* **64**, 429–437.

Thompson WE, Ramalho-Santo J, Sutovsky P 2003 Ubiquitination of prohibitin in mammalian sperm mitochondria: possible roles in the regulation of mitochondrial inheritance and sperm quality control. *Biology of Reproduction* **69**, 254–260.

Tilly JL 2001 Commuting the death sentence: how oocytes strive to survive. *Nature Reviews. Molecular Cell Biology* **2**, 838–848.

van Leyen K, Duvoisin RM, Engelhardt H, Wiedmann M 1998 A function for lipoxygenase in programmed organelle degradation. *Nature* **395**, 392–395.

Vasilyev VB, Sokolova VA, Sorokin AV *et al.* 1999 Persistence of human mitochondrial DNA throughout the development to the blastocyst of mouse zygotes microinjected with human mitochondria. *Zygote* **7**, 279–283.

Zeviani M, Antozzi C 1997 Mitochondrial disorders. *Molecular Human Reproduction* **3**, 133–148.

Zhao XB, Chu MX, Li N, Wu CX 2001 Paternal inheritance of mitochondrial DNA in the sheep (*Ovis aries*). *Science in China Life Sciences* **44**, 321–326.

Received 28 May 2003; refereed 11 July 2003; accepted 2 September 2003.