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Generation of mice with mitochondrial dysfunction by introducing mouse mtDNA carrying a deletion into zygotes

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Mice carrying mitochondrial DNA (mtDNA) with pathogenic mutations would provide a system in which to study how mutant mtDNAs are transmitted and distributed in tissues, resulting in expression of mitochondrial diseases. However, no effective procedures are available for the generation of these mice. Isolation of mouse cells without mtDNA (ρ^0) enabled us to trap mutant mtDNA that had accumulated in somatic tissues into ρ^0 cells repopulated with mtDNA (cybrids). We isolated respiration-deficient cybrids with mtDNA carrying a deletion and introduced this mtDNA into fertilized eggs. The mutant mtDNA was transmitted maternally, and its accumulation induced mitochondrial dysfunction in various tissues. Moreover, most of these mice died because of renal failure, suggesting the involvement of mtDNA mutations in the pathogeneses of new diseases.

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Introduction

Human mtDNAs with point mutations in genes encoding tRNA and large-scale deletions (including several tRNA genes) have been associated with mitochondrial diseases[1,2]. Aside from the clinically distinct syndromes of mitochondrial diseases, these mtDNA mutations have been identified in association with human ageing and age-associated disorders including diabetes and neurodegenerative diseases[1,2]. Although the pathogenicities of these mtDNA mutations were proved by co-transmission of the mutant mtDNAs and respiration deficiency to ρ^0 human cells[3–6], there is as yet no convincing evidence to explain whether accumulation of these pathogenic mutant mtDNAs in tissues is responsible for the expressions of various clinical phenotypes.

Establishment of mice with pathogenic mutant mtDNA may provide a model system in which to study how pathogenic mutant mtDNA is transmitted and distributed in tissues. But no procedures are thus far available for introduction of mutagenized mammalian whole mtDNA into mitochondria in living cells, or even into isolated mitochondria. We have previously demonstrated that ρ^0 human cells can be used to trap mtDNAs with somatic mutations that have accumulated in somatic tissues of patients with Alzheimer disease[7]. Moreover, we have isolated ρ^0 mouse cells[8,9], which should be effective for trapping mutant mtDNAs accumulated in mouse somatic tissues or cells by their fusion with brain synaptosomes or enucleated cultured cells for isolation of mtDNA repopulated cybrids. Here we isolated respiration-deficient cybrids due to mutant mDNA carrying a somatic deletion, and generated mice modelling disease by electrofusion of fertilized mouse eggs with enucleated cybrids.

Results

Isolation of cybrids with a somatic deletion-mutant mtDNA

We carried out screening of cybrid clones with imported deletion mutant mtDNAs by PCR analysis using published primer sets[8]. Signals for deletion mutations were observed in 8 of 26 cybrid clones, and cybrids with these signals were further selected for quantitative estimation of deletion-mutant mtDNAs. Although most cybrid clones did not possess sufficient mutant mtDNAs to be detected by Southern-blot analysis, one clone (Cy4696) had 30% mutant mtDNA with a large-scale deletion derived from cultured mouse cells. Sequence analysis of the PCR products of Cy4696 cybrids revealed that the deletion was 4,696 bp (nt 7,759–12,454) and included 6 tRNA genes and 7 structural genes (Fig. 1*a*). This deletion-mutant mtDNA (ΔmtDNA4696) did not have a direct repeat surrounding the breakpoint (Fig. 1*b*), unlike other mouse ΔmtDNAs (refs 8,10,11). The amount of ΔmtDNA4696 in Cy4696 increased during prolonged cultivation from 30 to 83% and then remained stable. This behaviour was very similar to that in human cybrid clones with ΔmtDNA, which have been shown to be responsible for the pathogenesis of a mitochondrial encephalomyopathy, Kearns-Sayre syndrome[3]. Smaller ΔmtDNAs probably had a replication advantage over wild-type mtDNA during prolonged cultivation[3], although a smaller size might not be the only factor required for the propagational advantage[12]. We demonstrated the pathogenicity of the ΔmtDNA4696 by the simultaneous decreases in activities of cytochrome *c* oxidase (COX) and mitochondrial translation in cybrids with a predominant amount of ΔmtDNA4696 (Fig. 1*c*–*e*). As these cybrids share

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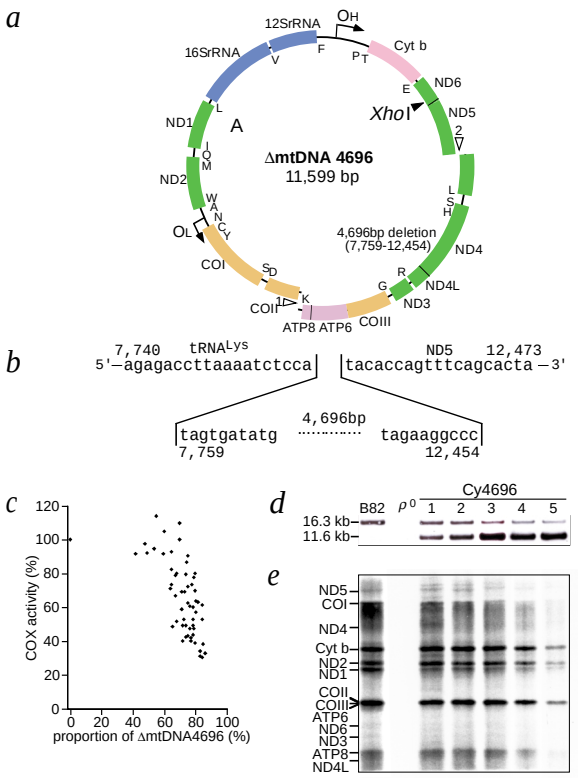

Fig. 1 Genetic characterization of ΔmtDNA4696 in somatic cells. ***a***, Gene map of ΔmtDNA4696. The arc indicates the deleted region expanded from $tRNA^{Lys}$ to ND5 genes. Primers used for sequencing or detection of ΔmtDNA4696 are indicated by open arrows (forward primer 1, 7,558–7,581; reverse primer 2, 12,678–12,658). Region A (1,751–3,803) indicates probe A used for detection of the wild-type and ΔmtDNA4696. *Xho*I, *Xho*I site. ***b***, Sequences of light strand mtDNA around the region of the deletion breakpoint of ΔmtDNA4696. The deletion was 4,696 bp with a breakpoint from nt 7,759 to 12,454 (brackets). ***c***, Effect of the amount of ΔmtDNA4696 on COX activity. Cy4696 cybrids possessing various proportions of ΔmtDNA4696 were used for biochemical analysis of COX activity. Southern blots are shown of *Xho*I fragments (***d***) and [^{35}S]methionine incorporation into mitochondria (***e***) of Cy4696 cybrids. B82, parental B82 cells of ρ^0 mouse cells; ρ^0, ρ^0 mouse cells; 1–5, Cy4696 cybrid clones. Probe A was used for Southern-blot analysis. The 16.3- and 11.6-kb fragments in (*d*) correspond to wild-type mtDNA and ΔmtDNA4696, respectively. ND5-ND4L in (*e*) are polypeptides assigned to mtDNA.

the same nuclear background as the parental ρ^0 cells, overall reduction of mitochondrial translation and resultant reduction of respiratory function is likely caused by accumulation of ΔmtDNA4696 (Fig. 1*a*).

Generation of mice with ΔmtDNA4696

We used respiration-deficient Cy4696 cybrids with a predominant amount of the pathogenic ΔmtDNA4696 as mtDNA donors for generation of mice with ΔmtDNA4696 and respiration defects. We introduced ΔmtDNA4696 into mouse zygotes by electrofusion of pronucleus-stage embryos with several enucleated cytoplasts of the Cy4696 cybrids (Fig. 2). To explore the fate of introduced ΔmtDNA4696, embryos fused with Cy4696 cytoplasts were cultured *in vitro* for 24–48 hours and transferred to the oviduct of pseudopregnant females. Of 111 newborn mice, 98 survived to adulthood. On PCR analysis, tail samples from 31 of these mice showed PCR signals for ΔmtDNA4696. Southern-blot analysis of muscle biopsy samples showed that 24 of these 31 mice had 5.7–41.8% ΔmtDNA4696 (Fig. 3*a*). Because mtDNA is inherited strictly maternally[13,14], we selected 5 founder (F0) females with 5.7–13.0% ΔmtDNA4696 as mothers for breeding, and obtained progeny with predominant ΔmtDNA4696, demonstrating transmission of ΔmtDNA4696 through female germ lines from F0 mothers to their progeny (Fig. 3*b*). Mice with more than 90% ΔmtDNA4696 in their muscle tissues were not obtained, probably due to lethality of eggs or embryos with a predominant amount of ΔmtDNA4696.

Partially duplicated mtDNA, but not ΔmtDNA, has been associated with maternally transmitted mitochondrial diseases[15–18]. We therefore examined the presence of a partially duplicated form consisting of one full-length mtDNA and one ΔmtDNA4696 (Fig. 4*a*), and its involvement in maternal transmission. A partially duplicated mtDNA, as well as ΔmtDNA4696 and wild-type mtDNA, was present in samples from tails of F0–F3 mice, but was not observed in any clones of the Cy4696 cybrids (Fig. 4*b*). We next determined the proportions of three mtDNA forms, ΔmtDNA4696, partially duplicated mtDNA and wild-type mtDNA, using tissues from F1 and F2 mice (Fig. 4*c*). Skeletal muscle, heart and blood contained 0–17% of the partially duplicated mtDNA, but it was not detected in other tissues. These observations suggest that the amount of the duplicated form increased to detectable levels after its introduction into embryos, or that it was newly created in the host embryos by recombination

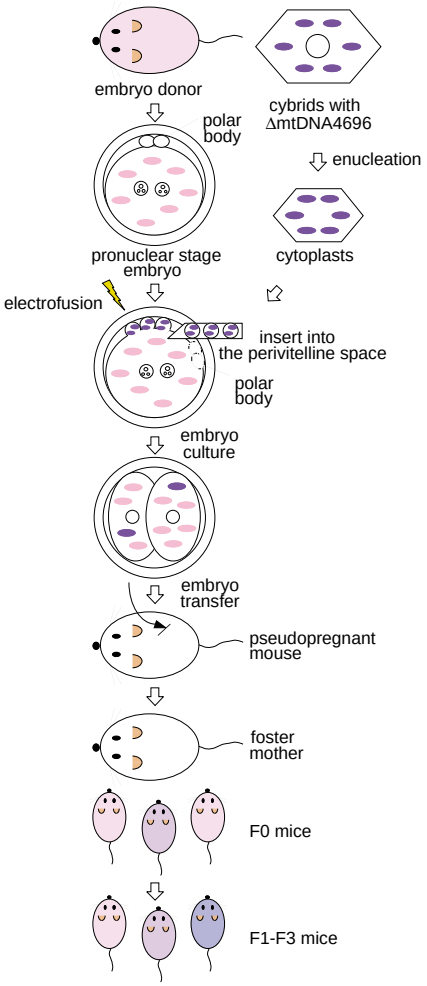

Fig. 2 Experimental scheme for producing heteroplasmic mice possessing wild-type mtDNA and exogenously introduced ΔmtDNA4696 from Cy4696 cybrids. Mice or mitochondria possessing wild-type mtDNA are shown in pink, and those with ΔmtDNA4696, in blue. Heteroplasmic mice with a small amount of ΔmtDNA4696 are shown in red, whereas those with a large amount are shown in violet.

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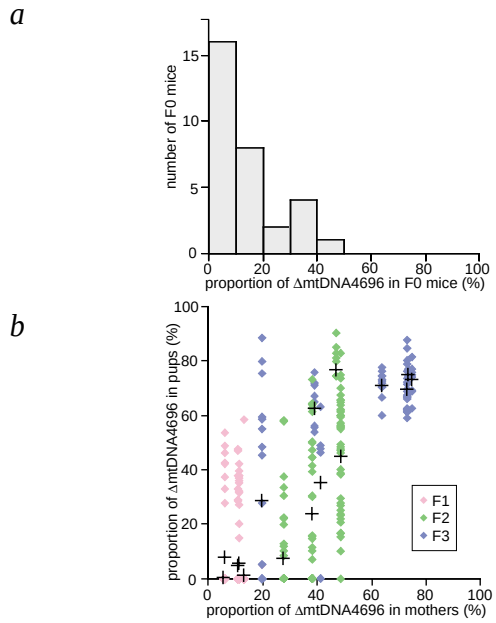

Fig. 3 Transmission and distribution of ΔmtDNA4696 in F0–F3 mice. ***a***, Histogram illustrating the proportion of ΔmtDNA4696 in muscle (*M. tibialis anterior*) biopsy samples from F0 mice and their numbers. F0 mice were obtained as shown in Fig. 2. Of 111 newborn mice, 98 grew to adults, and 31 showed PCR signals for ΔmtDNA4696 in their tails. Muscle biopsy samples were prepared from the 31 F0 mice with PCR signals and used for quantitative estimation of ΔmtDNA4696 by Southern-blot analysis. ***b***, Comparison of ΔmtDNA4696 proportions in mothers and their pups. Transmissions of ΔmtDNA4696 from F0 to F1, from F1 to F2, and from F2 to F3 generations were analysed using muscle biopsy samples. Five F0, four F1 and seven F2 females were used as mothers for production of F1 (pink), F2 (green) and F3 (blue diamonds) pups, respectively. Five F0 mothers had 5.7, 6.1, 10.8, 11.2 and 13.0% ΔmtDNA4696, four F1 mothers had 27.4, 38.0, 47.0 and 48.7% ΔmtDNA4696, and seven F2 mothers had 19.6, 39.1, 41.3, 63.9, 73.2, 73.5 and 74.8% ΔmtDNA4696. Crosses indicate the mean level of ΔmtDNA4696 within each litter. Note that the variance in the proportion of ΔmtDNA4696 in pups from mothers possessing a predominant amount of the deletion mtDNA was extremely small compared with the variance in pups from mothers possessing a lower proportion of ΔmtDNA4696. This observation could be explained by assuming that ΔmtDNA4696 possesses a replication advantage over wild-type mtDNA, whereas more than 90% deletion mtDNA in eggs or embryos is lethal due to induction of progressive respiration defects, resulting in reproduction of pups with 60–90% ΔmtDNA4696 from mothers with a predominant amount of ΔmtDNA4696.

of wild-type mtDNA and exogenous ΔmtDNA4696. Considering that partially duplicated mtDNAs, but not ΔmtDNAs, are transmitted maternally to the following generations in human subjects[16–18], apparent maternal transmission of mouse ΔmtDNA4696 to the following generations (Fig. 3*b*) may be explained by assuming maternal transmission of the partially duplicated form and its subsequent rearrangement to reproduce pathogenic ΔmtDNA4696 in tissues of the progeny (Fig. 4*b*,*c*).

To determine the pathogenicity of the mutant mtDNA, muscle biopsy samples from F2 males with various amounts of the mutant mtDNA were subjected to histochemical and quantitative single-fibre PCR analyses, using serial sections for comparison of COX activity and the proportion of the mutant mtDNA in single muscle fibres. Only mice with predominant amounts of ΔmtDNA4696 showed mosaic distribution of COX-positive and -negative fibres (Fig. 5*a*,*b*). We were not able to determine whether the PCR products were derived from ΔmtDNA4696 and/or the ΔmtDNA4696 region of partially duplicated mtDNA (Fig. 4*a*); however, we previously showed that mitochondria in

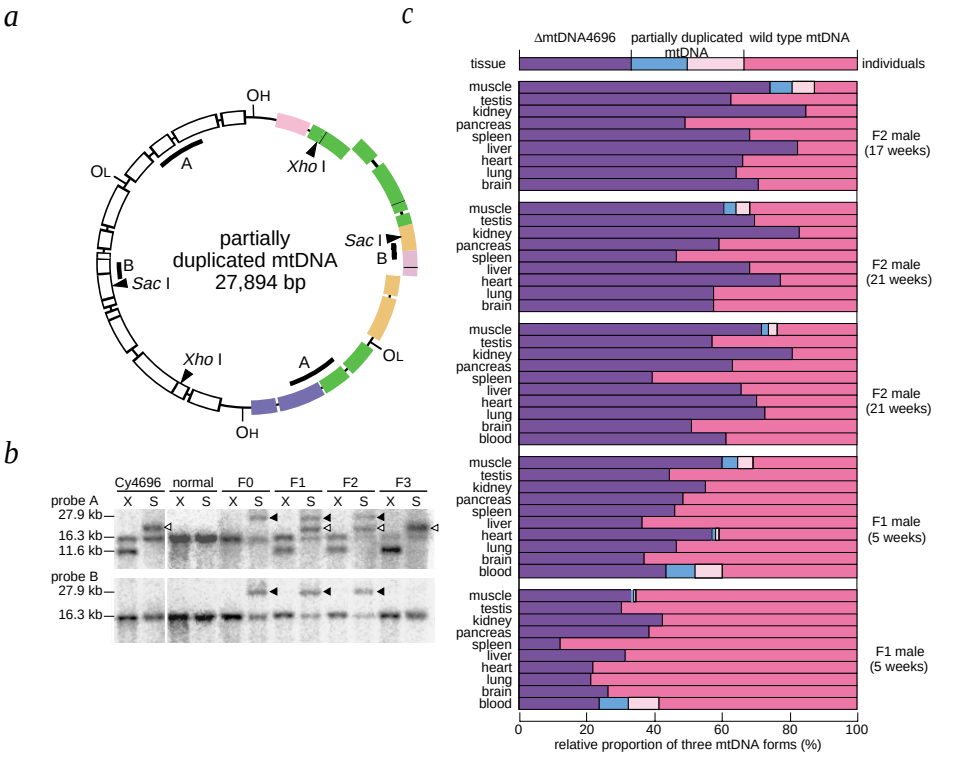

Fig. 4 Detection of a partially duplicated mtDNA and its distribution in tissues of F0–F3 generations. ***a***, Gene map of a partially duplicated mtDNA molecule consisting of one wild-type mtDNA (open boxes) and one ΔmtDNA4696 (coloured boxes). Wild-type mtDNA possesses one *Xho*I and one *Sac*I site, whereas ΔmtDNA4696 possesses one *Xho*I site but no *Sac*I site, which is located in the deleted region. Regions A (1,751–3,803) and B (8,410–8,964) indicate probes used for Southern-blot analysis. Note that ΔmtDNA4696 lost the B region by the large-scale deletion mutation. ***b***, Identification of the partially duplicated form in tails of F0–F3 generation mice. Southern-blot analysis was carried out after digestion of total DNA with either *Xho*I (X) or *Sac*I (S) using probes A (top) and B (bottom). On *Xho*I digestion, the partially duplicated mtDNA gave one 16.3- and one 11.6-kb fragment, and thus it could not be distinguished from a mixture of wild-type mtDNA and ΔmtDNA4696. On the other hand, *Sac*I digestion of the partially duplicated mtDNA produced one 27.9-kb linear fragment (filled triangles), whereas wild-type mtDNA gave one 16.3-kb linear fragment, and ΔmtDNA4696 gave one uncut band (open triangles), the mobility of which was much slower than that of the 16.3-kb linear fragment due to its circular form. These uncut bands did not correspond to the partially duplicated mtDNA or wild-type mtDNA, as they did not show any signals when the B region was used as a probe (bottom). ***c***, Distribution of ΔmtDNA4696, a partially duplicated mtDNA, and wild-type mtDNA in tissues from the same male individuals. The proportions of these three mtDNA forms were examined using ten tissues obtained from five males (two 5-week-old F1, one 17-week-old F2 and two 21-week-old F2). The amounts of the mutant mtDNA include those of ΔmtDNA4696 and the ΔmtDNA4696 region of the partially duplicated mtDNA, and are expressed as blue bars. Note that the proportion of ΔmtDNA4696 did not vary significantly in different tissues of the same individual.

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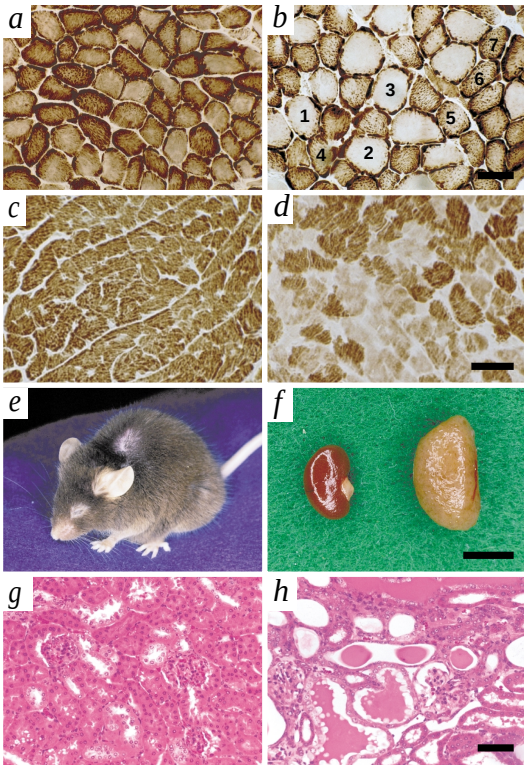

Fig. 5 Histochemical and morphological abnormalities observed in mice with predominant amounts of the mutant mtDNA. Cross-sections of skeletal muscles (*M. soleus*) (***a***,***b***) and cardiac muscles (***c***,***d***) stained for COX activity are shown. *a*,c, A 21-week-old normal male mouse; *b*,*d*, a 21-week-old F2 male with 91.0% of the mutant mtDNA (87.2% of ΔmtDNA4696 and 3.8% of the ΔmtDNA4696 region of the partially duplicated mtDNA shown by coloured boxes in Fig. 4*a*) in a muscle biopsy sample. Using serial muscle sections, the proportions of the mutant mtDNA in three COX-negative (fibres 1, 2, 3 in *b*) and four COX-positive fibres (fibres 4, 5, 6, 7 in *b*) were analysed quantitatively by PCR. Fibres 1–7 possessed 95.6, 90.0, 86.5, 81.9, 82.2, 83.2 and 77.3% of the mutant mtDNA, respectively. The average proportion of the mutant mtDNA in COX-negative fibres was 90.7±3.8%, whereas that in COX-positive fibres was 81.2±2.3%. Note that subsarcolemmal regions in the COX-negative fibres were stained, because this region usually accumulates a significant amount of mitochondria, which would provide signals of COX staining, even when each mitochondrion showed low COX activity. ***e***, A 38-week-old F1 male showing strong anaemia diagnosed from his pale ears, paws and tail. This mouse showed 65.7% of the mutant mtDNA in a muscle biopsy sample at the age of 7 weeks, and died the day after of photographing, probably of renal failure. ***f***, Kidneys from this mouse (right) and from an age-matched normal male (left). The kidney on the right is anaemic with a granulated surface, and had 88.2% of the mutant mtDNA. The other F1–F3 mice possessing predominant amounts of the mutant mtDNA in their muscle biopsy samples died of renal failure. Their kidneys possessed more than 80% of the mutant mtDNA (85.4±5.4%, n = 5). Histology is shown of the renal cortex of the kidneys. Kidney from an age-matched normal male (***g***) and a kidney with 94.0% of the mutant mtDNA (***h***). The lumen of tubules in (*h*) is dilated and contains occasional eosinophilic material. Scale bars in *b*,*d*,*f* and *h*, 5 μm, 2.5 μm, 5 mm and 5 μm, respectively.

mammalian cells interact with each other by exchanging their contents[3,19,20]. Therefore, the presence of 100% of the partially duplicated mtDNA corresponds to the presence of 50% of ΔmtDNA4696 and 50% of wild-type mtDNA, and the amount of ΔmtDNA4696 required for inducing mitochondrial dysfunction should be equivalent to the amount of the mutant mtDNA, which includes both ΔmtDNA4696 and the ΔmtDNA4696 region of the duplicated mtDNA. In fact, we found a good correlation between COX deficiency and predominance of the mutant mtDNA: all fibres possessing more than 85% of the mutant mtDNA were negative for COX, whereas those with less than 85% were positive for COX (Fig. 5*b*).

We next examined whether mouse muscle with a predominant amount of the mutant mtDNA show ragged-red fibres (RRF) on modified Gomori trichrome staining, which is a histochemical phenotypes of mitochondrial diseases. Typical RRF was not observed, although most fibres with and without COX activity showed RRF-like characteristics, suggesting the presence of a substantial amount of mitochondria in subsarcolemmal regions in most mouse fibres (data not shown). It is likely that ageing of mice with a predominant amount of the mutant mtDNA in muscle fibres is required for obtaining typical RRF.

Heart tissue of mice with predominantly mutant mtDNA also showed a mosaic distribution of COX-negative and -positive cells (Fig. 5*c*,*d*). The amount of lactic acid in peripheral blood increased with an increase in the amount of mutant mtDNA in muscle tissues (Fig. 6*a*,*b*). These observations suggest that mice with the mutant mtDNA can be used as models of mitochondrial diseases.

Most F1–F3 mice with predominant amounts of the mutant mtDNA in their muscle biopsy samples died within 200 days of birth (Fig. 5*e*–*h*). Common macroscopic abnormalities were systemic ischaemia and enlarged kidneys with a granulated surface (Fig. 5*e*,*f*). Dilatation of the cortical proximal and distal tubules was observed in renal tissues (Fig. 5*h*). Moreover, blood from these mice showed high concentrations of urea nitrogen and creatinine (Fig. 6*c*), suggesting that death was due to renal failure derived from loss of tubular function. Biochemical analyses showed that COX activity in kidney with 85% mutant mtDNA

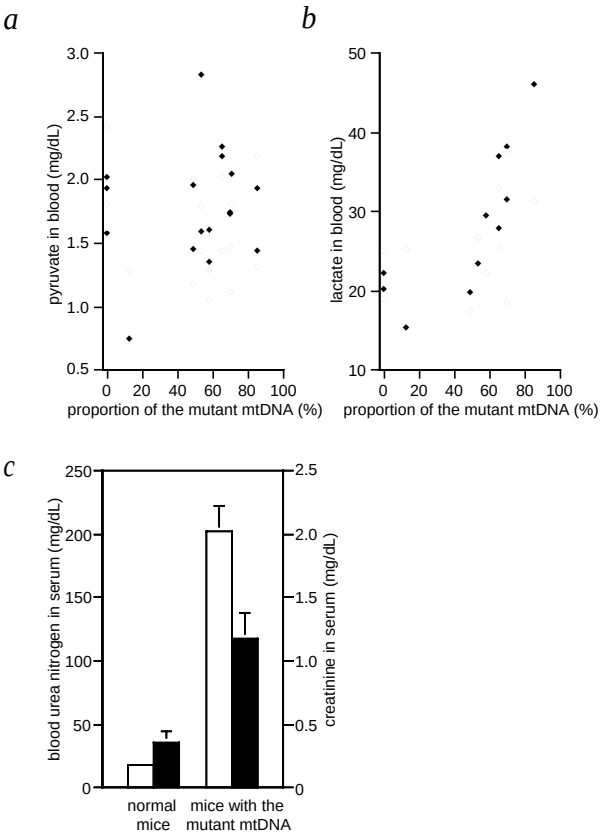

Fig. 6 Biochemical abnormalities of blood in mice with the mutant mtDNA. ***a***,***b***, Effects of the amount of the mutant mtDNA on the levels of blood pyruvate and lactate, respectively. Open and filled symbols correspond to values before and after glucose loading, respectively. Note that the correlation between lactate levels and the proportions of the mutant mtDNA in muscle biopsy samples was more evident after glucose loading. ***c***, Comparison of blood urea nitrogen (open bars) and creatinine levels (filled bars) in mice with wild-type mtDNA and mice with predominant amounts of the mutant mtDNA (85.4±5.4%, n=5) in their kidney.

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was progressively reduced to only 28% of that in normal kidney with wild-type mtDNA. These observations suggest that loss of tubular function was due to reduced respiratory activity caused by accumulation of mutant mtDNA. Although renal failure is not a common lesion in mitochondrial diseases, there have been reports of mitochondrial dysfunction of the kidney[16,21,22]. Therefore, we suggest that some human renal failures of unknown cause are due to mtDNA mutations.

Discussion

We generated mice expressing mitochondrial dysfunction by isolation of respiration-deficient cybrids with mouse ΔmtDNA4696 and its introduction into mouse zygotes by electrofusion. The introduced mutant mtDNA was transmitted maternally from the F0 generation, and its accumulation induced mitochondrial dysfunction in various tissues of the F1–F3 progenies.

Unexpectedly, we found that the exogenously introduced mitochondria with ΔmtDNA4696 from cybrids were able to escape elimination when introduced into zygotes (F0 embryos). We showed previously that mtDNA from sperm mitochondria was completely eliminated from embryos before the two-cell stage when introduced into zygotes on mouse intraspecies fertilization of *Mus musculus*, whereas some sperm mtDNA was not eliminated in mouse interspecies F1 hybrids between male *Mus spretus* and female *M. musculus*[13,14]. Using the same combination of mouse species and the same primer sets that we had used previously[13], it was reported[23,24] that *M. spretus* mtDNA from liver mitochondria escaped elimination and was transmitted when microinjected into zygotes of *M. musculus*. Therefore, introduced mitochondria from different mouse species are not eliminated from embryos, irrespective of whether they are derived from sperm[13,14] or liver[23,24]. We found here, however, that *M. musculus* mitochondria with ΔmtDNA4696 introduced from cybrids into zygotes of the same species could remain in embryos without exclusion, suggesting that exogenously introduced mitochondria of somatic cells, but not of sperm, into zygotes escape elimination. Moreover, we recently reported, by the use of quantitative PCR, that selective elimination occurred in sperm and spermatid, but not in liver, mitochondria that had been introduced into zygotes of the same mouse species[25]. Therefore, some imprinting factors ensuring preferential elimination of sperm and spermatid mitochondria are not present in somatic cell mitochondria, and generation of mice with a predominant amount of exogenous ΔmtDNA4696 is due at least in part to the escape of somatic cell mitochondria with ΔmtDNA4696 from exclusion by embryos.

Another unexpected observation was maternal transmission of the introduced mouse ΔmtDNA4696 to F1–F3 progeny. Transmission of ΔmtDNA through the female germ line to following generations has been reported in *Drosophila melanogaster*[26], but is rarely seen in patients with pathogenic ΔmtDNA (refs 1,2). Maternal inheritance of mtDNAs with point mutations[27,28] or partially duplicated mtDNAs consisting of wild-type mtDNA and ΔmtDNA (refs 16–18) was observed in patients with mitochondrial diseases. In the case of mouse ΔmtDNA4696, which was introduced from cybrids into F0 embryos, its maternal transmission to F1–F3 generations could be explained by maternal transmission of the partially duplicated form, followed by its subsequent rearrangement to reproduce ΔmtDNA4696, which may be pathogenic and may induce mitochondrial dysfunction in muscle and renal tissues. On the other hand, it is possible that ΔmtDNA4696 and the partially duplicated mtDNA could be transmitted maternally by assuming that mouse cells and tissues are resistant to respiration defects. In fact, although human mitotic tissues usually do not accumulate ΔmtDNA (ref. 29), we found a large amount of ΔmtDNA4696 in mouse mitotic tissues, reflecting the absence of preferential elimination of mouse mitotic cells with a predominant amount of ΔmtDNA4696. Therefore, apparent differences in the transmission and tissue distribution patterns of mouse and human ΔmtDNAs may be explained by assuming contribution of a partially duplicated form in our mutant mice, or by a phenotype of mouse cells resistant to energy starvation. Thus, mouse oocytes, embryos and mitotic tissues with a predominant amount of the mutant mtDNA survive, allowing maternal transmission and subsequent distribution of a predominant amount of the mutant mtDNA even in mitotic tissues.

There have been many reports of the isolation of mice with mitochondrial disorders by disruption of various factors encoded by nuclear DNA related to mitochondrial function[1,30], but they could not be used as models of diseases caused by mtDNA mutations. There have been reports of chimaeric mice with a small amount of chloramphenicol resistant (CAP^r) mtDNA (refs 31,32). As mtDNA is inherited strictly maternally, female-type embryonic stem (ES) cells were used in these experiments. Chimaeric mice were obtained by introduction of female-type ES cells with predominant CAP^r mtDNA into normal blastocyst embryos. The proportion of CAP^r mtDNA decreases very rapidly in the absence of effective CAP selection in chimaeric mice, however, resulting in failure of its germline transmission. We reported recently that congenic B6-mt^{spr} strain mice and cybrids, which are different from *M. musculus* in possessing only mtDNA of *M. spretus*, did not show any mitochondrial dysfunction in biochemical assays[33].

This is the first report of the generation of mice expressing respiration defects in various tissues by introduction of mouse mtDNA with a pathogenic mutation. These mice should be useful for studying the precise mechanisms of formation, transmission and pathogenic expression of partially duplicated mtDNA and ΔmtDNA in various tissues, and thus could be models of mtDNA-based diseases. Although most mice with a predominant amount of ΔmtDNA4696 die relatively young due to renal failures, we could test the hypothesis that accumulation of mutant mtDNAs is responsible for ageing and age-related diseases by the use of mice with lower proportion of mutant mtDNA. Moreover, samples are available from every age and every stage of pathogenesis in every tissue with various proportions of the mutant mtDNA, so that we may investigate whether human diseases of unknown cause are due to mtDNA mutations. These mice should also be valuable for screening effective drugs and testing gene therapies.

Methods

Introduction of mtDNA from somatic cells into ρ^0 mouse cells. Isolation of synaptosomal fractions from brain tissue from inbred C57BL/6J mice, enucleation of cultured cells and fusion of synaptosomes or enucleated cytoplasts with ρ^0 mouse B82 cells were carried out as described[7,8].

Southern-blot analysis. Total DNA (2–3 μg) extracted from cells and tissues was digested with the restriction endonucleases *Xho*I or *Sac*I. Restriction fragments were separated in 1.0% agarose gel, transferred to a nylon membrane and hybridized with [α-^{32}P] dATP-labelled mouse mtDNA probes. The membrane was washed and exposed to an imaging plate for 2 h and radioactivities of fragments were measured with a bioimaging analyser, Fujix BAS 2000 (Fuji Photo Film, Japan).

Analyses of mitochondrial respiratory functions. We analysed mitochondrial translation products labelled with [^{35}S] methionine as described[3]. Biochemical and histochemical analyses of COX activity were carried out by examining the rate of cyanide-sensitive oxidation of reduced cytochrome c as described[34].

Introduction of ΔmtDNA4696 from Cy4696 cybrids into mouse zygotes. We induced 8–10-week F1 female mice (B6D2F1, obtained by crossing C57BL/6J and DBA/2) to superovulate by consecutive injections of PMSG

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(pregnant mare serum gonadotropin, 5 IU) and hCG (human chorionic gonadotropin); 5 IU) with an interval of 48 h between injections. They were then caged with fertile B6D2F1 males overnight. Pronuclear stage embryos (zygotes) were collected by puncturing the oviducts 15–18 h after hCG injection. The embryos were placed in CZB medium and freed of cumulus cells with hyaluronidase. About ten cytoplasts were inserted into the perivitelline space of the embryos with a piezo-driven micromanipulator. Cytoplasts were fused with the embryos by applying an electric pulse (3,000 or 3,750 V/cm, 10 µs) with a pre- and post-pulse AC current (100 V/cm, 2 MHz, 30 s each). Most (>95%) embryos survived the electrofusion procedure and developed to the 2-cell and 4-cell stages after 24 and 48 h culture, respectively. The embryos were transferred to the oviducts of day 1 pseudopregnant ICR females (day 1 was the day after mating with vasectomized males). On day 20, the uteri of the females were examined and live pups were raised by lactating foster mothers.

Single-fibre PCR analysis. We used two serial cryosections of 10 and 20 µm of skeletal muscle (*M. soleus*) for single-fibre PCR. The thinner sections were stained with dimethylaminoazobenzene for COX activity, and COX-negative and -positive fibres were selected for PCR analysis. The thicker sections were used for dissection of cytoplasm corresponding to the COX-negative and -positive fibres with a sharp microcapillary. PCR amplification was carried out with three primers, nt 7,576–7,595, 12,260–12,280 and 12,479–12,459, of mouse mtDNA. Mutant ΔmtDNA4696 and wild-type mtDNA gave 210 bp and 220 bp fragments, respectively, and the proportion of ΔmtDNA4696 in each fibre was analysed with an FM-BIO image analyser (Hitachi). Quantitativeness was confirmed from the observations that the proportion of ΔmtDNA4696 calculated by this PCR analysis was almost equivalent to that calculated by Southern-blot analysis of the same muscle samples.

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1. Wallace, D.C. Mitochondrial diseases in man and mouse. *Science* **283**, 1482–1488 (1999).
2. Larsson, N.-G. & Clayton, D.A. Molecular genetic aspects of human mitochondrial disorders. *Annu. Rev. Genet*. **29**, 151–178 (1995).
3. Hayashi, J.-I. *et al.* Introduction of disease-related mitochondrial DNA deletions into HeLa cells lacking mitochondrial DNA results in mitochondrial dysfunction. *Proc. Natl Acad. Sci. USA* **88**, 10614–10618 (1991).
4. Chomyn, A. *et al.* In vitro genetic transfer of protein synthesis and respiration defects to mitochondrial DNA-less cells with myopathy-patient mitochondria. *Mol. Cell. Biol*. **11**, 2236–2244 (1991).
5. King, M.P., Koga, Y., Davidson, M. & Schon, E.A. Defects in mitochondrial protein synthesis and respiratory chain activity segregate with the $tRNA^{Leu(UUR)}$ mutation associated with mitochondrial myopathy encephalopathy, lactic acidosis, and strokelike episodes. *Mol. Cell. Biol.* **12**, 480–490 (1992).
6. Yoneda, M., Miyatake, T. & Attardi, G. Complementation of mutant and wild-type human mitochondrial DNAs coexisting since the mutation event and lack of complementation of DNAs introduced separately into a cell with distinct organelles. *Mol. Cell. Biol.* **14**, 2699–2712 (1994).
7. Ito, S. *et al.* Functional integrity of mitochondrial genomes in human platelets and autopsied brain tissues from elderly patients with Alzheimer's disease. *Proc. Natl Acad. Sci. USA* **96**, 2099–2103 (1999).
8. Inoue, K. *et al.* Isolation of mitochondrial DNA-less mouse cell lines and their application for trapping mouse synaptosomal mitochondrial DNA with deletion mutations. *J. Biol. Chem.* **272**, 15510–15515 (1997).
9. Inoue, K. *et al.* Isolation and characterization of mitochondrial DNA-less lines from various mammalian cell lines by application of an anticancer drug, ditercalinium. *Biochem. Biophys. Res. Commun.* **239**, 257–260 (1997).
10. Nelson, I., Gerasimov, S., Marsac, C., Lestienne, P. & Boursot, P. Sequence analysis of a deleted mitochondrial DNA molecule in heteroplasmic mice. *Mamm. Genome* **4**, 680–683 (1993).
11. Tanhauser, S.M. & Laipis, P.J. Multiple deletions are detectable in mitochondrial DNA of aging mice. *J. Biol. Chem.* **270**, 24769–24775 (1995).
12. Clayton, D.A & Vinograd, J. Circular dimer and catenate forms of mitochondrial DNA in human leukaemic leucocytes. *Nature* **216**, 652–657 (1967).
13. Kaneda, H. *et al.* Elimination of paternal mitochondrial DNA in intraspecific crosses during early mouse embryogenesis. *Proc. Natl Acad. Sci. USA* **92**, 4542–4546 (1995).
14. Shitara, H. *et al.* 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 (1998).
15. Poulton, J., Deadman, M.E. & Gardiner, R.M. Duplications of mitochondrial DNA in mitochondrial myopathy. *Lancet* **1**, 236–240 (1989).
16. Rotig, A. *et al.* Maternally inherited duplication of the mitochondrial genome in a syndrome of proximal tubulopathy, diabetes mellitus, and cerebellar ataxia. *Am. J. Hum. Genet*. **50**, 364–370 (1992).
17. Dunbar, D.R. *et al.* Maternally transmitted partial direct tandem duplication of mitochondrial DNA associated with diabetes mellitus. *Hum. Mol. Genet*. **10**, 1619–1624 (1993).
18. Ballinger, S.W., Shoffner, J.M., Gebhart, S., Koontz, D.A. & Wallace, D.C. Mitochondrial diabetes revisited. *Nature Genet*. **7**, 458–459 (1994).
19. Hayashi, J.-I., Takemitsu, M., Goto, Y.-i. & Nonaka, I. Human mitochondria and mitochondrial genome function as a single dynamic cellular unit. *J. Cell Biol.* **125**, 43–50 (1994).
20. Takai, D. *et al.* Interorganellar interaction between distinct human mitochondria with deletion mutant mtDNA from a patient with mitochondrial disease and with HeLa mtDNA. *J. Biol. Chem.* **272**, 6028–6033 (1997).
21. Jansen, J.J. *et al.* Mutation in mitochondrial tRNA(Leu(UUR)) gene associated with progressive kidney disease. *J. Am. Soc. Nephrol.* **8**,1118–1124 (1997).
22. Szabolcs, M.J. *et al.* Mitochondrial DNA deletion: a cause of chronic tubulointerstitial nephropathy. *Kidney Int*. **45**, 1388–1396 (1994).
23. Pinkert, C.A., Irwin, M.H., Johnson, L.W. & Moffatt, R.J. Mitochondria transfer into mouse ova by microinjection. *Transgenic Res.* **6**, 379–383 (1997).
24. Irwin, M.H., Johnson, L.W. & Pinkert, C.A. Isolation and microinjection of somatic cell derived mitochondria and germline heteroplasmy in transmitochondrial mice. *Transgenic Res*. **8**, 119–123 (1999).
25. Shitara, H. *et al.* Selective and continuous elimination of mitochondria microinjected into mouse eggs from spermatids, but not from liver cells, occurs throughout embryogenesis. *Genetics* (in press).
26. Volz-Lingenhohl, A., Solignac, M. & Sperlich, D. Stable heteroplasmy for a large-scale deletion in the coding region of *Drosophila subobscura* mitochondrial DNA. *Proc. Natl Acad. Sci. USA* **89**, 11528–11532 (1992).
27. Goto, Y.-i., Nonaka, I. & Horai, S. A mutation in the $tRNA^{Leu(UUR)}$ gene associated with the MELAS subgroup of mitochondrial encephalomyopathies. *Nature* **348**, 651–653 (1990).
28. Shoffner, J.M. *et al.* Myoclonic epilepsy and ragged-red fiber disease (MERRF) is associated with a mitochondrial DNA $tRNA^{Lys}$ mutation. *Cell* **61**, 931–937 (1990).
29. Holt, I.J., Harding, A. & Morgan-Hughes, J.A. Deletions of muscle mitochondrial DNA in patients with mitochondrial myopathies. *Nature* **331**, 717–719 (1988).
30. Wang, J. *et al.* Dilated cardiomyopathy and atrioventricular conduction blocks induced by heart-specific inactivation of mitochondrial DNA gene expression. *Nature Genet*. **21**, 133–137 (1999).
31. Marchington, D.R., Barlow, D. & Poulton, J. Transmitochondrial mice carrying resistance to chloramphenicol on mitochondrial DNA: developing the first mouse model of mitochondrial DNA disease. *Nature Med*. **5**, 957–960 (1999).
32. Levy, S.E., Waymire, K.G., Kim, Y.L., MacGregor, G.R. & Wallace, D.C. Transfer of chloramphenicol-resistant mitochondrial DNA into the chimeric mouse. *Transgenic Res*. **8**, 137–145 (1999).
33. Yamaoka, M. *et al.* Complete repopulation of mouse mitochondrial DNA-less cells with rat mitochondrial DNA restores mitochondrial translation but not mitochondrial respiratory functions. *Genetics* **155**, 301–307 (2000).
34. Miyabayashi, S. *et al.* Cytochrome c oxidase deficiency in two siblings with Leigh encephalomyelopathy. *Brain Dev.* **6**, 362–372 (1984).

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