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Generation of trans-mitochondrial mice carrying homoplasmic mtDNAs with a missense mutation in a structural gene using ES cells

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Generation of various kinds of trans-mitochondrial mice, mito-mice, each carrying mtDNAs with a different pathogenic mutation, is required for precise investigation of the pathogenesis of mitochondrial diseases. This study used two respiration-deficient mouse cell lines as donors of mtDNAs with possible pathogenic mutations. One cell line expressed 45–50% respiratory activity due to mouse mtDNAs with a T6589C missense mutation in the *COI* gene (T6589C mtDNA) and the other expressed 40% respiratory activity due to rat (*Rattus norvegicus*) mtDNAs in mouse cells. By cytoplasmic transfer of these mtDNAs to mouse ES cells, we isolated respiration-deficient ES cells. We obtained chimeric mice and generated their F_6 progeny carrying mouse T6589C mtDNAs by its female germ line transmission. They were respiration-deficient and thus could be used as models of mitochondrial diseases caused by point mutations in mtDNA structural genes. However, chimeric mice and mito-mice carrying rat mtDNAs were not obtained, suggesting that significant respiration defects or some deficits induced by rat mtDNAs in mouse ES cells prevented their differentiation to generate mice carrying rat mtDNAs.

INTRODUCTION

Human mtDNAs with large-scale deletions (ΔmtDNAs) and with point mutations have been identified to be closely associated with mitochondrial diseases expressing respiratory defects in various tissues (for reviews, see 1,2). Moreover, these mtDNA mutations have also been identified in association with human aging and with various age-related disorders including diabetes and neurodegenerative diseases (1,2). However, possible involvement of nuclear DNA mutations in expression of respiration defects was not excluded, as respiratory function is controlled by both nuclear DNA and mtDNA (1,2). Subsequent studies resolved this issue by providing direct evidence for the pathogenicity of mtDNA mutations in patients by co-transmission of the mutated mtDNAs and respiration defects from the patients to mtDNA-less human cells (3–6).

However, even though mutated mtDNAs induced respiration defects, there is no direct evidence that respiration defects are responsible for the disease phenotypes found in patients with mitochondrial diseases. Therefore, it was still possible that nuclear DNA mutations induced expression of disease phenotypes. Our recent studies (7–9) excluded this possibility by generating trans-mitochondrial mice, mito-mice, sharing the same nuclear-genome background but carrying various proportions of ΔmtDNAs with a 4696 bp deletion

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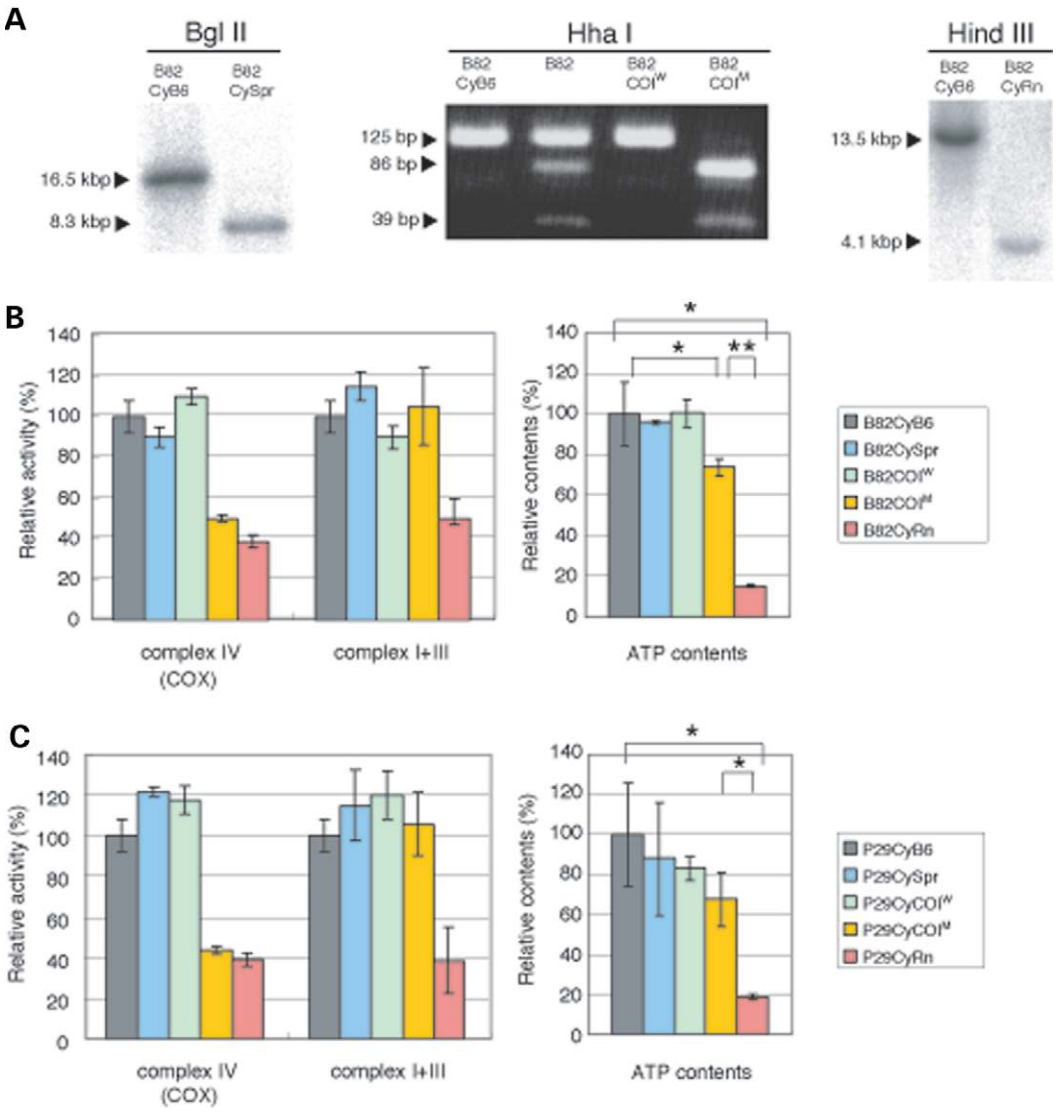

Figure 1. Characterization of mtDNA donor cell lines carrying possible pathogenic mtDNAs. B82 cybrids of mtDNA donors to ES cells share the same nuclear-genome background as that of mouse B82 cells derived from C3H strain mice (*M. m. domesticus*), but possess various mtDNAs. B82CyB6, B82CySpr and B82CyRn cells possess wild-type mtDNAs from *M. m. domesticus*, *M. spretus* and *R. norvegicus*, respectively. Parental B82 cells possess heteroplasmic *M. m. domesticus* mtDNAs with and without a missense T6589C mutation in the *COI* gene. B82COI^{W} and B82COI^{M} cells were obtained by recloning B82 cells and possess homoplasmic wild-type mtDNAs without T6589C mutation and homoplasmic T6589C-mutated mtDNAs, respectively. The genome composition of mouse cell lines used in this experiment is summarized in Table 1. (**A**) Analyses of mtDNA genotypes. On Southern blot analysis of *Bgl*II digests, B82CyB6 cells with *M. m. domesticus* mtDNAs from B6 strain mice (*M. m. domesticus*) and B82CySpr cells with *M. spretus* mtDNAs gave a 16.5 and an 8.3 kb fragment, respectively. On *Hha*I digestion of 125 bp PCR products, B82COI^{W} cells with mouse wild-type mtDNAs gave a 125 bp fragment, whereas B82COI^{M} cells with mouse T6589C mtDNAs gave 86 and 39 bp fragments due to gain of an *Hha*I site by a T6589C mutation in the *COI* gene. Parental mouse B82 cells with both the wild-type and mutated mtDNAs gave all three fragments. On Southern blot analysis of *Hin*dIII digests, B82CyB6 cells with mouse (*M. m. domesticus*) mtDNAs and B82CyRn cells with rat (*R. norvegicus*) mtDNAs gave a 13.5 and a 4.1 kb fragment, respectively. (**B**) Analyses of respiratory function in B82 cells and their cybrids. B82CyB6 cells carrying nuclear genome from C3H strain mice and mtDNAs from B6 strain mice (*M. m. domesticus*) were used as standards expressing normal respiratory function. Asterisks indicate a *P*-value less than 0.05 and double asterisks indicate a *P*-value less than 0.005. (**C**) Analyses of respiratory function in P29 cybrids (cf. Table 1). P29CyB6 cells carrying nuclear genome and mtDNAs from B6 strain mice (*M. m. domesticus*) were used as standards expressing normal respiratory function. Asterisks indicate a *P*-value less than 0.05.

mutation, as they expressed disease phenotypes only when ΔmtDNAs accumulated sufficiently for inducing respiration defects.

Disease phenotypes of the patients appeared to vary slightly depending on whether pathogenic mtDNA mutations are created in tRNA genes or in structural genes. For example, the former mutations are associated with CPEO, MELAS and MERRF, and the latter with Leber's disease, NARP, and Leigh syndrome, although all the mutations eventually induced respiration defects (1,2). Therefore, mito-mice carrying different pathogenic mutations in various genes of mtDNAs have to be generated to resolve the issue of whether differences in the mutation sites in mtDNAs are responsible for expression of different disease phenotypes. However,

Table 1. Genome composition of mouse cell lines, founder mice and mito-mice

Cells and mice	Nuclear backgrounds (strains)	Breeding status	mtDNA species
B82 cybrids			
B82CyB6	C3H/An		*M. m. domesticus*
B82CySpr	C3H/An		*M. spretus*
B82COI^{W}	C3H/An		*M. m. domesticus*
B82COI^{M}	C3H/An		*M. m. domesticus*
B82CyRn	C3H/An		*R. norvegicus*
P29 cybrids			
P29CyB6	B6		*M. m. domesticus*
P29CySpr	B6		*M. spretus*
P29CyCOI^{W}	B6		*M. m. domesticus*
P29CyCOI^{M}	B6		*M. m. domesticus*
P29CyRn	B6		*R. norvegicus*
ES (TT2-F) cybrids			
ESSpr	B6/CBAF1		*M. spretus*
ESCOI^{M}	B6/CBAF1		*M. m. domesticus*
ESRn	B6/CBAF1		*R. norvegicus*
Founder (F_0) mice			
Founder Spr	B6/CBAF1 + ICR	F_0	*M. spretus*
Founder COI^{M}	B6/CBAF1 + ICR	F_0	*M. m. domesticus*
Mito-mice			
F_6 mito-mouse Spr	B6 (99.2%)	F_6	*M. spretus*
F_6 mito-mouse COI^{M}	B6 (99.2%)	F_6	*M. m. domesticus*
F_6 mito-mouse Δ87	B6 (99.2%)	F_6	*M. m. domesticus*

The chromosome numbers obtained from 30 metaphase B82 cybrids and ES cybrids were 53 and 39, respectively, and were very stable irrespective of whether mouse mtDNA was replaced by rat mtDNA.

there are significant problems in generating mito-mice carrying various pathogenic mtDNAs.

One is the problem of isolating donor cells carrying mtDNAs with pathogenic mutations equivalent to those found in patients with mitochondrial diseases, because no procedures are thus far available for introduction of artificially mutagenized mtDNAs into mitochondria in living cells or even into isolated mitochondria. Therefore, there have been attempts to obtain mouse cell lines possessing mtDNAs with naturally occurring pathogenic mutations for use as mtDNA donors (7,10,11). In addition to using such mutated mouse mtDNAs, in this study we tried to use rat mtDNA as a possible pathogenic mtDNA, because complete repopulation of mtDNAs in mouse cells by rat mtDNAs induced significant reduction of mitochondrial respiratory function (12–14), probably due to incompatibilities between mouse nuclear and rat mitochondrial genomes.

Another problem is the recipients of pathogenic mtDNAs for generation of mito-mice. In a previous study (7), we used mouse zygotes as ΔmtDNA recipients to generate mito-mice carrying ΔmtDNAs, as it has the replication advantage of being predominant due to being smaller than recipient wild-type mtDNAs. However, mouse zygotes possess 5×10^4 to 5×10^5 copies of endogenous wild-type mtDNAs (15) and thus are not appropriate for use as recipients of mutated mtDNAs, particularly when pathogenic mtDNAs without replication advantage are to be introduced for generation of mito-mice carrying them. This problem could be resolved using ES cells as recipients of pathogenic mtDNAs, as they possess 3×10^3 mtDNA genomes, which could be excluded by their pretreatment with the mitochondrial toxin rhodamine-6G (R6G) (16).

As inheritance of mtDNAs is strictly maternal (17,18), in this study we used XO-type mouse ES cells as recipients, and isolated respiration-deficient ES cells by cytoplasmic transfer of possible pathogenic mtDNAs from respiration-deficient mouse cell lines. Although karyotypic instability of XO-type ES cells used in this study could be a major problem for generation of trans-mitochondrial models, we obtained chimeric mice and generated mito-mice expressing respiration defects by germ line transmission of a mutated mtDNA from female chimeric mice to their progeny.

RESULTS

Characterization of mtDNA donor cell lines carrying mtDNAs with point mutations

For generation of respiration-deficient mito-mice carrying mtDNAs with point mutations, we used cell fusion techniques to isolate ES cells with mtDNAs from two respiration-deficient mouse B82 cell lines and from one respiration-competent mouse B82 cell line. As these cells with nuclear genomes from B82 cells could not survive in the selection medium with hypoxanthine/aminopterin/thymidine (HAT), unfused cells were effectively eliminated from the fusion mixture and thus could be used as mtDNA donors for mouse ES cells.

A respiration-deficient mouse cell line B82COI^{M} with homoplasmic T6589C-mutated mtDNAs in the *COI* gene (T6589C mtDNA) and a respiration-competent mouse cell line B82COI^{W} with homoplasmic wild-type mtDNAs were obtained by recloning parental mouse B82 cells possessing 35.3% T6589C mtDNAs (Fig. 1A). The T6589C missense mutation, which changes the amino acid of the COI subunit from Val 421 to Ala, has been reported to be pathogenic (19). Direct sequencing of whole mtDNAs in B82COI^{M} and B82COI^{W} cells showed that they shared the same mtDNA sequences except for a T6589C mutation in the *COI* gene.

Using B82CyB6 cells carrying B6 mtDNAs as normal standards of respiratory function and adenosine triphosphate (ATP) contents, we carried out biochemical analysis of respiratory function and found that B82COI^{M} cells expressed 50% complex IV (cytochrome *c* oxidase; COX) activity, but expressed normal activities of other respiratory complexes (Fig. 1B). Their ATP contents were 70% of those in B82CyB6 cells (Fig. 1B), whereas B82COI^{W} cells possessed normal ATP contents and expressed normal activities in all the respiratory complexes we tested (Fig. 1B). Considering that COI is one of the COX subunits, all these observations suggest that respiration defects in B82COI^{M} cells should be due to a pathogenic T6589C mutation in the *COI* gene.

Another respiration-deficient cell line B82CyRn, which carried nuclear DNA from mouse B82 cells (*Mus musculus domesticus*) and mtDNA from rat (*Rattus norvegicus*) (Fig. 1A and Table 1), expressed 40% COX activity and contained 15% ATP (Fig. 1B). Therefore, rat mtDNAs could also be used as mtDNAs with possible pathogenic mutations in mouse cells. Such respiration defects observed in B82CyRn cells may be due to incompatibilities between the mouse

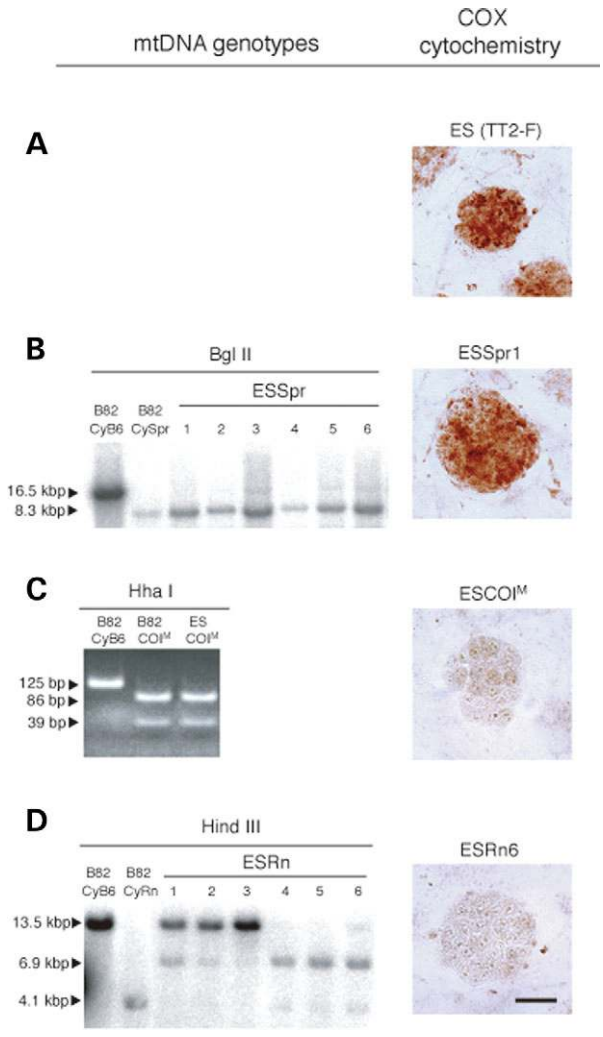

Figure 2. Characterization of ES cell lines repopulated by various exogenous mtDNAs. Repopulation of mtDNAs in ES cells was attained by pretreating ES cells with R6G to exclude internal mitochondria and mtDNAs, and their subsequent rescue by cell-fusion-mediated introduction of exogenous mitochondria and mtDNAs. Complete replacement of mtDNAs in ES cells by exogenously introduced mtDNAs was confirmed by Southern blot or PCR analysis (left panels). Resultant expression of respiration defects was examined by COX cytochemistry (right panels). (**A**) Parental ES (TT2-F) cells. Colonies of ES cells stained normally on COX cytochemistry. (**B**) mtDNA-repopulated ESSpr cells isolated by pretreatment of ES cells with R6G and subsequent fusion with enucleated B82CySpr cells. On Southern blot analysis of *Bgl*II digests, 16.5 and 8.3 kb fragments represented wild-type mtDNAs of *M. m. domesticus* and *M. spretus*, respectively. All six ESSpr clones possessed *M. spretus* mtDNAs derived from B82CySpr cells (left panel) and one clone ESSpr1 was used for COX cytochemistry (right panel). (**C**) mtDNA-repopulated ESCOI^{M} cells isolated by pretreatment of ES cells with R6G and their subsequent fusion with enucleated B82COI^{M} cells. On *Hha*I digestion of the PCR products, wild-type mtDNAs of *M. m. domesticus* gave one 125 bp fragment, whereas T6589C mtDNAs gave two fragments of 86 and 39 bp due to gain of an *Hha*I site by T6589C mutation (left panel). One ES clone, ESCOI^{M}, possessing homoplasmic T6589C mtDNAs was used for COX cytochemistry (right panel). (**D**) mtDNA-repopulated ESRn cells isolated by pretreatment of ES cells with R6G and their subsequent fusion with enucleated B82CyRn cells. On Southern blot analysis of *Hin*dIII digests, we obtained three fragments of 13.5, 6.9 and 4.1 kb, which corresponded to mtDNAs of *M. m. domesticus*, *M. spretus* and *R. norvegicus* (rat), respectively. We used mouse embryonic fibroblasts carrying *M. spretus* mtDNAs as feeder cells for distinguishing host *M. m. domesticus* mtDNAs in ES cells. Thus, 13.5, 6.9 and 4.1 kb fragments corresponded to remaining mtDNAs in host ES cells, *M. spretus* mtDNAs in feeder fibroblasts and rat mtDNAs from mtDNA-donor B82CyRn cells, respectively. A faint signal of a 4.1 kb fragment of rat mtDNAs is due to *M. m. domesticus* mtDNAs used as a probe for confirming the absence of *M. m. domesticus* mtDNAs in ESRn cells. Of six ESRn clones, three clones possessed homoplasmic rat mtDNAs, whereas the other three possessed only host *M. m. domesticus* mtDNAs (left panel). ESRn6 cells carrying rat mtDNAs were selected for COX cytochemistry (right panel). Bar, 30 μm.

nuclear and rat mitochondrial genomes, because most respiratory complexes including COX consist of both nuclear DNA- and mtDNA-coded polypeptides.

A respiration-competent mouse cell line B82CySpr with nuclear DNA from mouse B82 cells (*M. m. domesticus*) and mtDNAs from a different mouse species *M. spretus* (Fig. 1A) retained normal respiratory function and normal ATP contents (Fig. 1B) and was used as a donor of normal mtDNAs with polymorphic mutations.

Statistically significant correlation coefficients between cybrids with T6589C mtDNAs and rat mtDNAs were not observed in COX activity, but observed in ATP contents (Fig. 1B). Similar results were obtained when we used mouse P29 cybrids sharing B6 nuclear background different from that of B82 cybrids (Table 1 and Fig. 1C).

Isolation of respiration-deficient ES cells by introduction of mutated mtDNAs

The ES cells (XO-type TT2-F cells) used as recipients of exogenous mtDNAs were pretreated with R6G to eliminate endogenous mitochondria and mtDNAs (16), and fused with enucleated mtDNA donor cells (B82CySpr cells, B82COI^{M} cells and B82CyRn cells; cf. Table 1) to obtain mtDNA-repopulated ES cells. Then, the fusion mixture was cultivated in HAT medium to eliminate mtDNA donor cells, which possess HAT-sensitive nuclear genomes. Unfused ES cells could not survive due to the absence of mitochondria owing to their pretreatment with R6G. Thus, we could obtain either mtDNA-repopulated ES cells with exogenous mtDNAs from enucleated mtDNA donor cells, or parental ES cells, which restored remaining endogenous mtDNAs.

Growing colonies were cloned for examination of mtDNA genotypes (Fig. 2). From the fusion mixture of R6G-pretreated ES cells and enucleated B82CySpr cells, we obtained six clones and found that they all possessed predominant amounts of *M. spretus* mtDNAs (Fig. 2B). From the fusion mixture of R6G-pretreated ES cells and enucleated B82COI^{M} cells, one clone, ESCOI^{M} cells, which carried homoplasmic T6589C mtDNAs was isolated (Fig. 2C). We also obtained six clones from the fusion mixture of R6G-pretreated ES cells and enucleated B82CyRn cells. Of the six clones, three clones (ESRn4–6) possessed only rat mtDNAs, whereas the other three (ESRn1–3) possessed host mouse mtDNAs, which would be derived from remaining mouse mtDNAs in ES cells even after R6G pretreatment (Fig. 2D).

We selected three mtDNA-repopulated ES clones, ESSpr1, ESCOI^{M} and ESRn6 cells, in which mtDNAs were replaced

by *M. spretus* mtDNAs, mouse T6589C mtDNAs and rat mtDNAs, respectively, for further examination. We examined their respiratory function by histochemical analysis (COX cytochemistry), but not by biochemical analysis, because it was difficult to prepare a sufficient number of mtDNA-repopulated ES cells (5×10^6 cells) without contamination with the feeder cells for biochemical assay.

As expected, ESCOI^M and ESRn6 cells showed respiration defects, whereas ESSpr1 cells had normal activity (Fig. 2). These observations indicated that mtDNA genotypes and respiration phenotypes were transferred simultaneously from mtDNA donor cell lines to ES cells, and the resultant ESCOI^M and ESRn6 cells could be used for generation of respiration-deficient mito-mice, whereas ESSpr1 cells could be used as normal controls (Fig. 2).

Generation of founder mice and mito-mice carrying exogenous mutated mtDNAs

Three mtDNA-repopulated ES cell lines were introduced into 8-cell-stage embryos of ICR strain mice (*M. m. domesticus*). We obtained F_0 (founder) mice showing 100% chimerism from ESSpr1 and ESCOI^M cells, but not from ESRn6 cells (Table 2). On Southern blot (Fig. 3A) and polymerase chain reaction (PCR) analyses of tail mtDNAs (Fig. 3B), founder mice showing 100% chimerism possessed 100% exogenous mtDNAs and were named founder Spr and founder COI^M.

Then, females of founder Spr and founder COI^M showing 100% chimerism were mated with B6 males. As expected, all F_1 mice born from these founder mice showed the agouti-coat color of the ES-derived phenotype (Table 2). Moreover, mtDNA analysis showed that they possessed 100% *M. spretus* mtDNAs (Fig. 3A) or 100% T6589C mtDNAs in all tissues tested (Fig. 3B). These observations provided convincing evidence that the F_1 mice corresponded to mito-mice carrying 100% exogenous mtDNAs. Then, F_1 female mito-mice were back-crossed five times with B6 males and the resultant F_6 mito-mice carrying 100% T6589C mtDNAs and 100% *M. spretus* mtDNAs were named F_6 mito-mice COI^M and F_6 mito-mice Spr, respectively (Table 1).

However, on introduction of ESRn6 cells into 8-cell-stage embryos of ICR strain mice, no chimerism was found in any of the 24 individuals of founder mice (Table 2). The absence of rat mtDNAs was confirmed by Southern blot analysis of tail mtDNAs from three founder mice (Fig. 3C). One founder mouse, however, showed slight PCR signals for rat mtDNAs in the heart, kidney and tail (Fig. 3C), although its coat color did not show chimerism. Direct sequencing of the PCR products showed that they corresponded to rat mtDNAs (Table 3), suggesting the presence of a very small number of ESRn6-derived somatic cells. As they may also have trace amounts of oocytes derived from ESRn6 cells, and produce F_1 individuals carrying rat mtDNAs, we tried to generate mice carrying rat mtDNAs by mating founder females obtained from ESRn6 cells with B6 males. However, none of the 118 F_1 progeny (45 females and 73 males) examined had the agouti-coat color (Table 2). These observations suggested that respiration defects of ESRn6 cells with rat mtDNAs inhibited their differentiation to oocytes or that their respiration defects inhibited subsequent embryogenesis to F_1 progeny, even if they had been able to differentiate into oocytes carrying rat mtDNAs.

Table 2. Generation of F_0 (founder mice) and their F_1 progeny carrying exogenous mtDNAs (mito-mice)

ES cells	No. of F_0 (founder mice) born (% chimerism)[a]	No. of F_1 mito-mice/ No. of F_1 mice born
ESSpr1	3 (0)	—
	6 (100): founder Spr	15/15
ESCOI^M	1 (10)	0/6
	1 (30)	0/11
	2 (100): founder COI^M	22/22
ESRn6	24 (0)	0/118

[a]The percentage of the coat color that appeared agouti.

Examination of respiratory function and disease phenotypes in F_6 mito-mice COI^M

F_6 mito-mice COI^M were used for further investigation of the pathogenesis, whereas B6 mice and F_6 mito-mice Spr were used as normal controls. As pathogenic controls, we used F_6 mito-mice Δ87, possessing 87% mtDNAs and co-expressing respiration defects and mitochondrial disease phenotypes. They were obtained by five times back-crossing of F_1 mito-mice carrying ΔmtDNAs (7) with B6 males. Mito-mice Δ100 possessing homoplasmic ΔmtDNAs were not obtained due to its strong pathogenicity to induce complete loss of respiratory function (7), whereas F_6 mito-mice COI^M possessing 100% T6589C mtDNAs survived, probably due to the weaker pathogenicity of the latter.

Cross-sections of heart from 6-month-old B6, F_6 mito-mouse Spr, F_6 mito-mouse Δ87 and F_6 mito-mouse COI^M were stained histochemically for examination of COX activity (Fig. 4A). All cardiac cells in the heart from an F_6 mito-mouse COI^M showed lower staining of COX activity than those from an F_6 mito-mouse Spr and a normal B6 mouse (Fig. 4A). In contrast to an F_6 mito-mouse COI^M, mosaic staining of COX activity was observed in the heart of an F_6 mito-mouse Δ87: ~65% of cardiac cells did not stain for COX activity, whereas the remaining 35% showed normal staining. Quantitative PCR analysis of ΔmtDNAs in serial microdissection samples showed that the former cells possessed 89% ΔmtDNAs and the latter possessed 77% (data not shown). Variation in the amount of ΔmtDNAs and resultant variation of COX activity in an F_6 mito-mouse Δ87 would be due to random segregation of ΔmtDNAs (9). In contrast, homoplasmic T6589C mtDNAs in an F_6 mito-mouse COI^M resulted in the uniform distribution of reduced COX activity in their cardiac cells (Fig. 4A). Biochemical analysis of COX activity also decreased in the brain, heart, liver and skeletal muscles from F_6 mito-mice COI^M (Fig. 4B).

Therefore, F_6 mito-mice COI^M expressing respiration defects were used for the investigation of whether respiration defects caused by a point mutation of the *COI* gene in mtDNAs are responsible for the pathogenesis of mitochondrial diseases. As patients with respiration defects due to similar missense mutations in the *COI* (20) and *COII* genes (21)

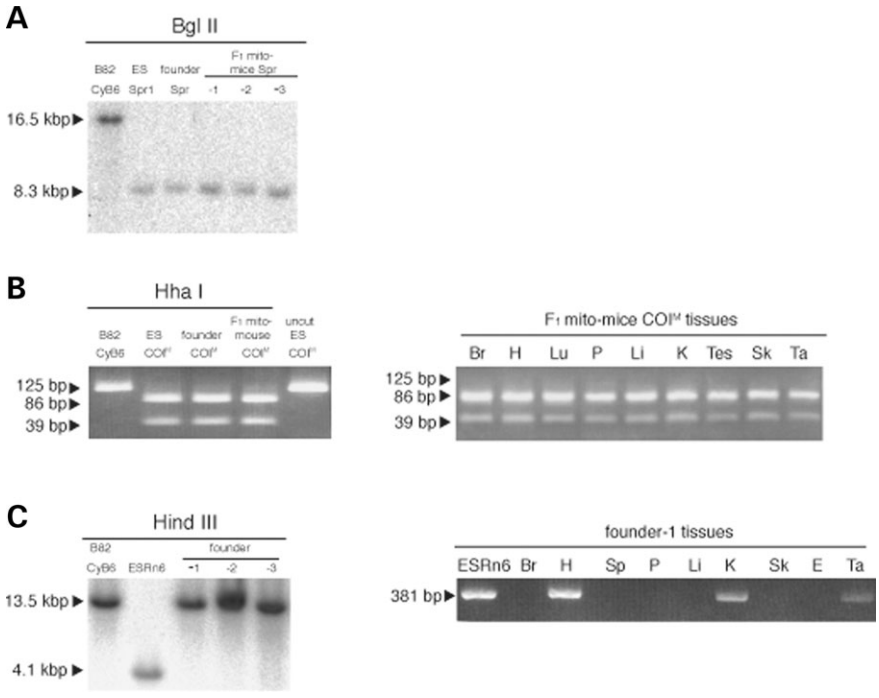

Figure 3. Identification of exogenous mtDNAs in F_0 (founder) mice and their F_1 progeny (mito-mice). DNA samples prepared from tails of founder mice and their F_1 progeny were used for mtDNA genotype analyses to confirm germ line transmission of exogenous mtDNAs from founder mice to their F_1 progeny. (**A**) Examination of *M. spretus* mtDNAs in tails of a founder Spr showing 100% chimerism and its F_1 progeny (F_1 mito-mice Spr-1, -2 and -3) by Southern blot analysis of *Bgl*II digests. Fragments of 16.5 and 8.3 kb represent those of *M. m. domesticus* mtDNAs and *M. spretus* mtDNAs, respectively. (**B**) Examination of T6589C mtDNAs in a founder COI^M showing 100% chimerism and its F_1 progeny (F_1 mito-mouse COI^M) using *Hha*I digests of PCR products. One fragment of 125 bp and two fragments of 86 and 39 bp, respectively, represented wild-type mtDNAs and T6589C mtDNAs of *M. m. domesticus*. The 39 bp *Hha*I fragments did not represent primer dimers, because uncut PCR products did not show signals of the primer dimers (left panel). For further identification of homoplasmic T6589C mtDNAs in an F_1 mito-mouse COI^M, PCR analysis was carried out using various tissues (right panel). Br, brain; H, heart; Lu, lung; P, pancreas; Li, liver; K, kidney; Tes, testis; Sk, skeletal muscles; Ta, tail. (**C**) Examination of rat mtDNAs in founder mice showing 0% chimerism (founder-1, -2, and -3) by Southern blot analysis of *Hin*dIII digests. No signal of a 4.1 kb fragment representing rat mtDNAs was observed in the tail of founder mice (left panel). However, PCR analysis using specific primers for selective amplification of rat mtDNAs gave signals of a 381 bp fragment corresponding to rat mtDNAs in the heart, kidney and tail of one founder mouse (founder-1), indicating the presence of a very small number of somatic cells carrying rat mtDNAs in the founder mouse showing 0% chimerism (right panel). Br, brain; H, heart; Sp, spleen; P, pancreas; Li, liver; K, kidney; Sk, skeletal muscles; E, ear lobe; Ta, tail.

commonly expressed lactic acidosis, we examined this phenotype and observed increase in the amount of lactate in the blood of F_6 mito-mice COI^M (Fig. 4C). Moreover, F_6 mito-mice COI^M showed growth retardation (Fig. 4D), suggesting that they can be used as models of mitochondrial diseases induced by point mutations in structural genes.

Effects of respiration defects of ES cells induced by mutated mtDNAs on their differentiation

The success in obtaining chimera and mito-mice from $ESCOI^M$ cells but not from ESRn6 cells suggested that rat mtDNAs preferentially inhibited multipotent ES cells from differentiating into various somatic cells and germ line cells. For testing this idea, the mtDNA-repopulated ES cells, ESSpr1, $ESCOI^M$ and ESRn6 cells, were inoculated subcutaneously into the back of nude mice.

All mtDNA-repopulated ES cells, except for ESRn6 cells with rat mtDNAs, formed tumors by 4 weeks after their inoculation. ESRn6 cells formed a small tumor in one of the three nude mice, but the growth of tumors decreased progressively in ESRn6 cells (Fig. 5, A1–D1). Moreover, histological analyses of their tumors showed that parental ES cells, ESSpr1 cells and $ESCOI^M$ cells differentiated into various tissues, such as secretory cells (Fig. 5, A2–C2), bone cells (Fig. 5, A3–C3), striated muscles (Fig. 5, A4–C4) and hair follicles (Fig. 5, A5–C5). Thus, $ESCOI^M$ cells did not lose abilities for multipotent differentiation (Fig. 5, C1–C5), even though they were respiration-deficient (Fig. 2C). However, no tissues, except for secretory cells, were differentiated from ESRn6 cells (Fig. 5, D2). These observations suggest that rat mtDNAs or rat mtDNA-induced respiration defects in ES cells inhibited their proper differentiation and should be responsible for the failure to generate chimeric mice from ESRn6 cells (Table 2).

DISCUSSION

Point mutations in the *COI* (20) and *COII* genes (21) of human mtDNAs were reported in patients with respiration defects and appeared to be commonly associated with lactic acidosis. In addition, one patient suffered epilepsy (20) and the other muscle weakness (21). In human cases, however, patients carry various nuclear-genome backgrounds, which may affect disease phenotypes by inducing respiration defects or directly induce disease phenotypes. Therefore, it was difficult

Table 3. Comparison of the sequence of the PCR products with rat mtDNA sequence

Nucleotide positions	5745	5778	5780
R. norvegicus[a]	T	G	T
PCR products[b]			
From ESRn6	C	A	C
From founder heart	C	A	C

[a]*R. norvegicus* sequence from Wister strain rat (GenBank Accession No. X14848).
[b]PCR products (381 bp) were obtained using primer sets for selective amplification of rat mtDNAs.

to determine whether mtDNA mutations in the *COI* and *COII* genes are responsible for respiration defects. Moreover, even if mtDNA mutations in the *COI* and *COII* genes are responsible for respiration defects, it is also difficult to determine whether the respiration defects caused by the mtDNA mutations are responsible for disease phenotypes. However, mito-mice share the same nuclear-genome background and their genetic variations are restricted to their mtDNA mutations. Therefore, mito-mice could provide unambiguous evidence that the disease phenotypes, which were observed exclusively in the mito-mice, were due to respiration defects caused by the mtDNA mutations.

We generated F_6 mito-mice COI^M using ES cells, and statistical analysis of the amounts of lactate in their blood showed that a missense T6589C mutation in the mtDNA *COI* gene alone is sufficient for clinical expression of lactic acidosis. However, these mice did not express epilepsy or muscle weakness, which were reported in patients carrying missense mutations in the *COI* gene (20) and *COII* gene (21), respectively. Probably, mtDNAs with less pathogenic mutations, such as the T6589C mutation, require cooperation with nuclear gene mutations for expression of disorders, such as epilepsy and muscle weakness.

Chimeric mice were not obtained from respiration-deficient mouse ESRn6 cells carrying rat mtDNAs (Fig. 3C). Moreover, ESRn6 cells did not differentiate into various somatic tissues, whereas $ESCOI^M$ cells maintained multipotent properties when they were inoculated into the back of nude mice (Fig. 5). Although cybrids with rat mtDNAs and cybrids with mouse T6589C mtDNAs showed similar reduction of COX activity, rat mtDNAs provided only 15–20% ATP contents in B82CyRn and P29CyRn cybrids, whereas mouse T6589C mtDNAs provided 70% ATP contents in $B82CyCOI^M$ and $P29CyCOI^M$ cells (Fig. 1B and C). Preferential reduction of ATP contents in cybrids with rat mtDNAs would be due to multiple amino acid replacement induced by rat mtDNAs and resultant multiple mitochondrial dysfunction. Thus, it is possible to suppose that more than 20% of ATP contents is necessary for ES cells to show their proper differentiation and development into chimeric mice and for germ line transmission of the mutated mtDNAs in ES cells.

However, it is also possible that rat mtDNAs specifically induced deficit in cell growth or predisposition to aneuploidy in mouse ES cells, resulting in the prevention of generation of mice carrying rat mtDNAs. The possible involvement of rat cytoplasmic factors encoded by rat nuclear genome derived from rat platelets in B82CyRn cells (14) was excluded because of their long-term cultivation after the fusion and resultant complete replacement by mouse cytoplasmic factors before introduction of their rat mtDNAs into mouse ES cells.

One approach to the question of whether the significant reduction of ATP contents was the main factor in the non-transmission of the rat mtDNAs would be to make cybrids with intermediate levels of rat mtDNAs. Previously, we introduced mouse mtDNAs into B82CyRn cybrids with only rat mtDNA and examined whether rat mtDNAs could propagate in mouse cells even in the presence of mouse mtDNAs (22). The results showed that rat mtDNAs, which propagated stably and expressed mitochondrial dysfunction in mouse cells, disappeared rapidly on exogenous introduction of mouse mtDNAs, suggesting that rat mtDNAs could not propagate in the presence of mouse mtDNAs in mouse cells. Probably, mouse mtDNAs replicated preferentially under control of the mouse nuclear genome and the rejection of rat mtDNAs from mouse cells was due to the incompatibility between nuclear and mitochondrial genomes of different species.

Recently, maternal transmission of a homoplasmic mtDNA with a significant pathogenic mutation in $tRNA^{Val}$ gene was reported to be responsible for multiple neonatal deaths in a family expressing mitochondrial diseases (23). These observations suggested that female germ line cells with pathogenic mtDNAs could differentiate into various tissues. However, this study showed that mouse ES cells with rat mtDNAs did not differentiate into various tissues (Fig. 5). This discrepancy could be explained by supposing that female germ line cells are more resistant to respiration defects than ES cells for their differentiation and this is why neonates are born from their respiration-deficient mother (23).

Respiration-deficient mito-mice similar to F_6 mito-mice COI^M have been generated from ES cells carrying a homoplasmic mitochondrial rRNA gene mutation, which gave a phenotype for resistance to chloramphenicol (CAP^r) and was responsible for 50% activity of normal respiratory function even in the absence of CAP (24). Respiration-deficient CAP^r mice showed various disorders, such as growth retardation and dilated cardiomyopathy, and died within 11 days after their birth (11), although there have been no reports of patients expressing mitochondrial diseases caused by CAP^r mutations in mtDNAs, probably due to the instability of CAP^r mutations. However, our F_6 mito-mice COI^M showed lactic acidosis and this abnormality was also observed in patients carrying similar missense mutations to T6589C (20,21). Therefore, it appears to be arguable whether significant abnormalities were preferentially observed in CAP^r mice, although both CAP^r and T6589C point mutations in mtDNAs resulted in ~50% activity of respiratory function.

This problem might be explained by supposing that differences in mutation sites in human mtDNA genes sometimes resulted in the expression of very close but different abnormalities. For example, mutations in tRNA genes are responsible for MELAS, MERRF and cardiomyopathy, whereas mutations in structural genes induce Leigh syndrome and Leber's disease (1,2). Another explanation is that the difference of nuclear-genome background of ES cells may affect disease phenotypes. The XO-type ES cells (CC9.3.1 cells) used for

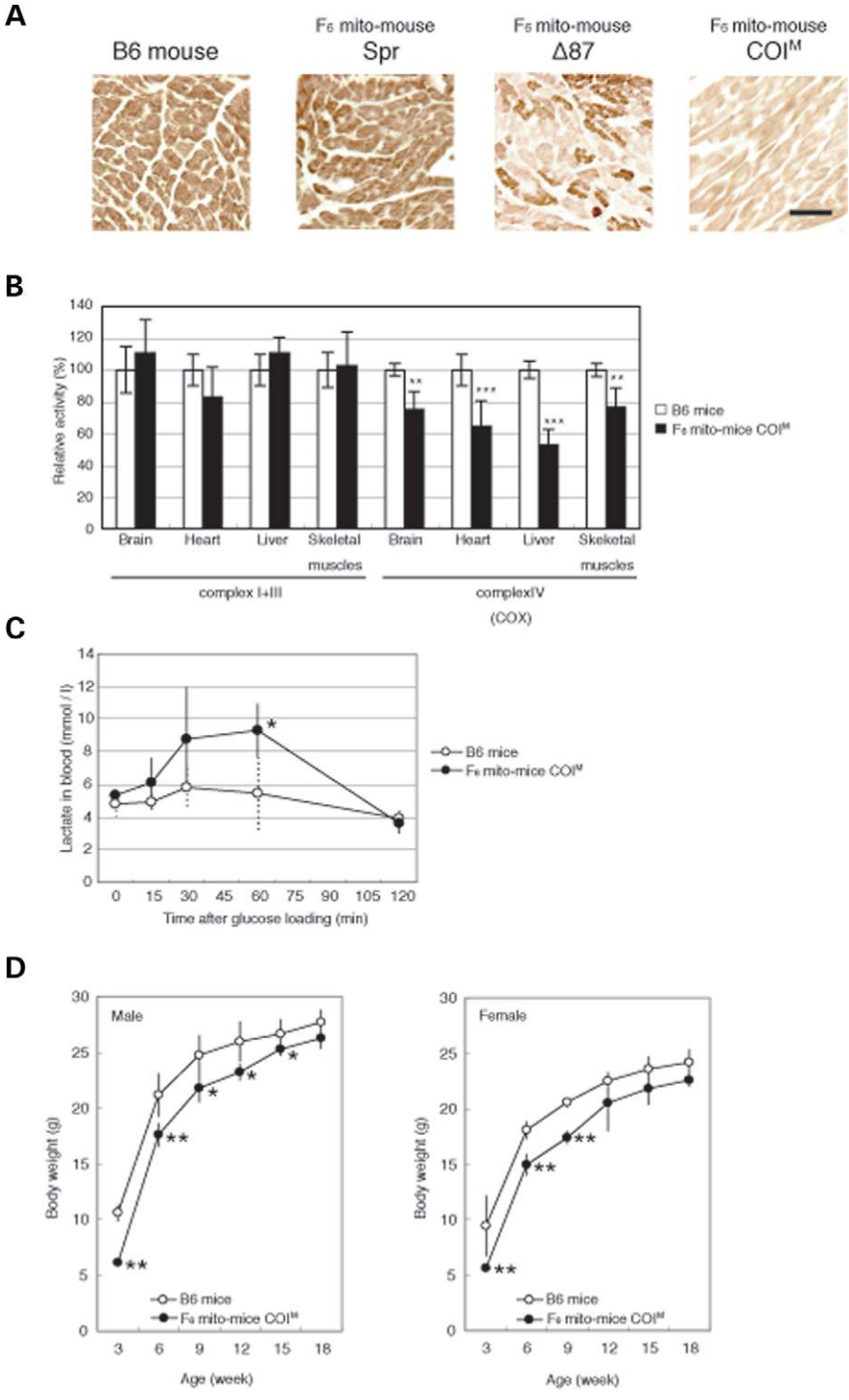

Figure 4. Examination of respiration defects and resultant expression of clinical abnormalities in F_6 mito-mice COI^M. (**A**) Histochemical analysis of COX activity in cardiac cells from 6-month-old F_6 mito-mice carrying various exogenous mtDNAs. One B6 mouse carrying wild-type mtDNAs of *M. m. domesticus* and an F_6 mito-mouse Spr carrying *M. spretus* mtDNAs were used as normal controls, whereas an F_6 mito-mouse Δ87 carrying 87% ΔmtDNAs in its tail was used as a pathogenic control. Uniform reduction of COX activity was observed in cardiac cells of an F_6 mito-mouse COI^M. Bar, 50 μm. (**B**) Biochemical analysis of respiratory complexes in brain, heart, liver and skeletal muscles from 6-month-old F_6 mito-mice COI^M. B6 mice were used as normal controls. Double asterisks indicate a *P*-value less than 0.005 and triple asterisks indicate a *P*-value less than 0.0005. (**C**) Estimation of the amount of lactate in blood of B6 mice and F_6 mito-mice COI^M. The amount of lactate in blood was examined before and after glucose loading. An increase in the amount of lactate was observed in F_6 mito-mice COI^M. Open and closed circles indicate control B6 mice and F_6 mito-mice COI^M, respectively (n=4 and 12 weeks). Asterisk indicates a *P*-value less than 0.05. (**D**) Estimation of body weights of B6 mice and F_6 mito-mice COI^M. Growth retardation was observed in F_6 mito-mice COI^M until 18 weeks after birth. Open and closed circles indicate control B6 mice and F_6 mito-mice COI^M, respectively. Asterisks indicate a *P*-value less than 0.05 and double asterisks indicate a *P*-value less than 0.005.

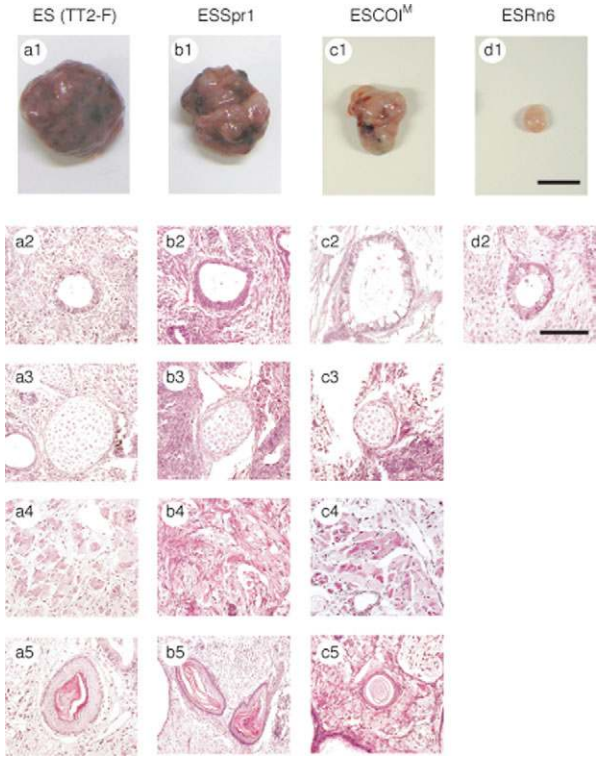

Figure 5. Effect of respiration defects on differentiation of ES cells. ES cells carrying various exogenous mtDNAs were inoculated into nude mice and the resultant tumors were used for histological analysis. All ES cells, except for ESRn6 cells, formed tumors. ESRn6 cells formed a small tumor in one of the three nude mice, whereas none of the three nude mice formed tumors on inoculation of ESRn4 cells. (**A1**–**A5**) parental ES cells; (**B1**–**B5**) ESSpr cells; (**C1**–**C5**) ESCOI^{M} cells; (**D1** and **D2**) ESRn6 cells. A1–D1 represent tumor masses formed in nude mice. A2–D2, A3–C3, A4–C4, A5–C5 correspond to secretory cells, bone cells, striated muscles and hair follicles, respectively. The proportions of secretory cells were 67.0, 62.0, 64.7 and 100%, bone cells were 5.2, 8.2, 6.0 and 0%, striated muscles were 13, 10.4, 9.5 and 0% and hair follicles were 14.8, 19.4, 19.8 and 0% in parental ES cells, ESSpr, ESCOI^{M} and ESRn cells, respectively. Bar in D1, 1 cm; bar in D2, 100 μm.

generation of CAP^{r} mice (11) were derived from 129 strain mice, whereas the XO-type ES cells (TT2-F cells) used in this study were from a B6CBAF1 embryo. Differences in the nuclear-genome backgrounds of ES cells could also explain why McKenzie *et al*. (25) did not isolate XO-type ES cells (CC9.3.1) carrying rat mtDNAs, but we obtained them (Fig. 2D).

There are many disorders requiring both nuclear and mtDNA mutations for their expression. For example, the homoplasmic missense T3394C mutation in the *ND1* gene of mtDNAs from a patient expressing diabetes and mitochondrial diseases (26) was also observed in a pedigree expressing maternally inherited cardiac abnormalities, but not expressing diabetes (27). Thus, the specificity of disease phenotypes is determined and controlled by mutations in nuclear genes. Moreover, Johnson *et al*. (28) reported that thresholds of auditory-evoked brainstem response were slightly increased in A/J strain mice possessing nuclear DNA mutations in the age-related hearing loss (*ahl*) locus by addition of a mutation in the mtDNA tRNA^{Arg} gene. Similar cases of nuclear and mitochondrial cooperation for expression of hearing loss were reported in maternally inherited and non-syndromic congenital deafness (29,30) and in mito-mice carrying more than 80% ΔmtDNAs (31). These observations suggest that hearing loss caused by nuclear gene mutation was enhanced by the addition of mtDNA mutations.

Therefore, mito-mice carrying various pathogenic mtDNA mutations and various nuclear-genome backgrounds have to be generated for precise investigation of the pathogenesis of mitochondrial diseases. We are now generating mito-mice COI^{M} with different nuclear-genome backgrounds to prove the hypothesis that mtDNA mutations and resultant expression of respiration defects simply enhances the expression of disease phenotypes caused by nuclear DNA mutations (31). We are also investigating aging effects on respiration defects and morphological abnormalities in aged F_6 mito-mice COI^{M}.

MATERIALS AND METHODS

Cell culture and media

Mouse mtDNA-less (ρ^0) cell lines (32) and their mtDNA-repopulated cybrids were grown in normal medium: RPMI 1640 (Nissui Seiyaku) containing 10% fetal calf serum, uridine (50 ng/ml) and pyruvate (0.1 μg/ml). Owing to the absence of thymidine kinase activity, mouse cells carrying nuclear genome from B82 cells could not survive in the presence of HAT. The ES cells used to generate mutant mice were TT2-F, an XO subline established from XY TT2 cells (33). The ES cells and mtDNA-repopulated ES cells were cultivated on mitomycin C-inactived feeder cells derived from fetal fibroblasts. For their cultivation, we used ES medium consisting of Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 15% knockout serum replacement (Invitrogen), 1× non-essential amino acids (ICN Biomedicals), leukemia inhibitory factor (10^5 U/ml, Invitrogen), 100 μM 2-mercaptoethanol and gentamicin. The nuclear backgrounds of the mouse cells used in this study are summarized in Table 1.

Production of mtDNA-repopulated ES cells

To isolate mtDNA-repopulated ES cells by exogenously introduced mtDNAs, we pretreated ES cells with R6G (0.75–1.5 μg/ml 3% ethanol) for 48 h in ES medium supplemented with uridine (50 ng/ml) and pyruvate (0.1 μg/ml) to exclude endogenous mtDNAs. After R6G pretreatment, ES cells were washed with phosphate-buffered saline (PBS) and suspended in R6G-free medium for 2 h for recovery. Enucleated cells, i.e. cytoplasts, of mtDNA donor cells were prepared by their pretreatment with cytochalasin B (10 μg/ml) for 10 min and centrifugation at 13 000*g* for 38 min at 37°C. The resultant cytoplasts were washed with PBS and incubated for 30 min. Then, the cytoplasts were fused with R6G-pretreated ES cells using polyethylene glycol. Fusion mixtures were plated onto fresh feeders and exposed to selective ES medium with HAT. Seven days after fusion, growing colonies were picked up for further examination.

Chromosome analysis

Chromosome numbers in cybrids and ES cells were analyzed after colcemide (0.02 μg/ml) treatment and Giemsa staining. The chromosome numbers were obtained from 30 metaphase B82 cybrids and ES cybrids.

Generation of founder chimeric mice and mito-mice

Jcl:ICR females (6-week-old, Crea Japan) were superovulated by consecutive injections of pregnant mare serum gonadotropin (7.5 IU) and human chorionic gonadotropin (7.5 IU) with a 48 h interval between injections. They were caged overnight with fertile ICR males, and 8-cell-stage embryos were collected in M16 medium. About 15 mtDNA-repopulated ES cells were injected into the 8-cell-stage embryos. After cultivation in ES medium for 24 h, the embryos were transferred into pseudo-pregnant ICR females. The resultant progeny was identified by their coat color chimerism. For generation of F_1 progeny, founder (F_0) chimera females were mated with B6 males. F_1 progeny was back-crossed five times with B6 males, and F_6 generations were obtained for further analyses. The nuclear backgrounds of the mice used in this study are summarized in Table 1. As a pathogenic control, we used F_6 mito-mouse Δ87, which was generated by five times back-crossing of F_1 mito-mice carrying ΔmtDNAs (7) with B6 males.

Southern blot analysis

Restriction fragments of total DNA or PCR products were separated on 1.0% agarose gel, transferred to a nylon membrane and hybridized with [α^{32}P] dATP-labeled mouse or rat mtDNA probes. The membrane was washed and exposed to an imaging plate for 2 h. The radioactivities of the restriction fragments were measured with a bioimaging analyzer, Fujix BAS 2000 (Fuji PhotoFilm).

Analyses of mitochondrial respiratory function

Biochemical and histochemical analyses of complex IV (COX) activity were carried out by examining the rate of cyanide-sensitive oxidation of reduced cytochrome as reported (34) with slight modifications (35). In histochemical analysis of COX activity, hearts were excised from the animals and cryosections of 10 μm thickness from the tissues were stained for COX activity. Biochemical analyses of complex I + III activities were carried out as described previously (35).

ATP measurement

Total cellular ATP contents were measured using 1×10^4 cells plated in 12-well plates. Cells were lysed with 100 μl lysis buffer (Toyo ink) and placed directly into the chamber of a luminometer (Berthold). Light emission was recorded after addition of 100 μl of luciferin-luciferase solution (Toyo ink).

PCR analysis and sequencing

Total cellular DNA (0.2 μg) extracted from cultivated cells and tissues was used as a template. For detection of rat mtDNAs, the nucleotide sequences of the *COI* gene encoded by rat mtDNAs (GenBank Accession No. X14848) were used to make the oligonucleotide primers: 5′-GCTT CGGAAACTGACTTGTACC-3′ and 5′-GCTGCTAATACT GGCAGTGAGA-3′, which corresponded to the nucleotide sequences from positions 5538 to 5559 and from 5919 to 5898, respectively. The cycle times were 60 s for denaturation at 94°C, 60 s for annealing at 52°C and 60 s for extension at 72°C for 30 cycles. PCR products were separated by electrophoresis in 3% agarose gels containing ethidium bromide (0.1 μg/ml). PCR products were analyzed by direct sequencing in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, California, MA, USA) using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Detection of the T6589C mutation in *COI* gene was achieved by restriction fragment length polymorphism (RFLP) analysis. A 125 bp fragment was amplified by PCR with the following primers reported previously (1): 5′-CATGA GCAAAAGCCCACTTCGCCATCATATTCGTAGGcG-3′ (a mismatch indicated by the lower case c) and 5′-TGTGGTGTAAGCATCTGGGTAG-3′, which corresponded to mouse mtDNA (GenBank Accession No. AY172335) sequences from positions from 6550 to 6588 and from 6674 to 6653, respectively. The cycle times were 60 s for denaturation at 94°C, 60 s for annealing at 60°C and 60 s for extension at 72°C for 30 cycles. The combination of the PCR-generated mutation together with the mutant version at the 6589 site (6589C) creates a recognition site for *Hha*I. Thus, the presence of the 6589T (wild-type) disrupts the restriction site. Restriction fragments were separated by electrophoresis in 3% agarose gels containing ethidium bromide (0.1 μg/ml).

Analysis of multipotency of mtDNA-repopulated ES cells

The mtDNA-repopulated ES cells (1×10^6 cells) were inoculated subcutaneously into the back of 6-week-old nude mice (JCL, BALB/c-nu/nu; Clea Japan) for testing their multipotency. The resulting teratomas (tumors) were fixed in 30% neutral-buffered formalin, embedded in paraffin and examined histologically after hematoxylin and eosin staining.

Lactate measurement

For determination of fasting blood lactate concentrations, blood was collected from the tail vein after overnight starvation. After oral administration of glucose (1.5 g/kg body weight) to these mice, blood was again collected and lactate concentrations were measured using an automatic blood lactate test meter (Lactate Pro, ARKRAY).

Statistical analysis

We analyzed data with the (unpaired or paired) Student's *t*-test. Values with $P < 0.05$ were considered significant.

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Conflict of Interest statement. The authors have no conflict of interest to declare.

REFERENCES

1. Larsson, N.-G. and Clayton, D.A. (1995) Molecular genetic aspects of human mitochondrial disorders. *Annu. Rev. Genet.*, **29**, 151–178.
2. Wallace, D.C. (1999) Mitochondrial diseases in man and mouse. *Science*, **283**, 1482–1488.
3. Chomyn, A., Meola, G., Bresolin, N., Lai, S.T., Scarlato, G. and Attardi, G. (1991) *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.
4. Hayashi, J.-I., Ohta, S., Kikuchi, A., Takemitsu, M., Goto, Y.-I. and Nonaka, I. (1991) 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.
5. King, M.P., Koga, Y., Davidson, M. and Schon, E.A. (1992) 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.
6. Yoneda, M., Miyatake, T. and Attardi, G. (1994) 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.
7. Inoue, K., Nakada, K., Ogura, A., Isobe, K., Goto, Y.-I., Nonaka, I. and Hayashi, J.-I. (2000) Generation of mice with mitochondrial dysfunction by introducing mouse mtDNA carrying a deletion into zygotes. *Nat. Genet.*, **26**, 176–181.
8. Shoubridge, E.A. (2000) A debut for mito-mouse. *Nat. Genet.*, **26**, 132–134.
9. Nakada, K., Inoue, K., Ono, T., Isobe, K., Ogura, A., Goto, Y.-I., Nonaka, I. and Hayashi, J.-I. (2001) Inter-mitochondrial complementation: mitochondria-specific system preventing mice from expression of disease phenotypes by mutant mtDNA. *Nat. Med.*, **7**, 934–940.
10. Marchington, D.R., Barlow, D. and Poulton, J. (1999) Transmitochondrial mice carrying resistance to chloramphenicol on mitochondrial DNA: developing the first mouse model of mitochondrial DNA disease. *Nat. Med.*, **8**, 957–960.
11. Sligh, J.E., Levy, S.E., Waymire, K.G., Allard, P., Dillehay, D.L., Nusinowitz, S., Heckenlively, J.R., MacGregor, G.R. and Wallace, D.C. (2000) Maternal germ-line transmission of mutant mtDNAs from embryonic stem cell-derived chimeric mice. *Proc. Natl Acad. Sci. USA*, **97**, 14461–14466.
12. Dey, R., Barrientos, A. and Moraes, C.T. (2000) Functional constraints of nuclear-mitochondrial DNA interactions in xenomitochondrial rodent cell lines. *J. Biol. Chem.*, **275**, 31520–31527.
13. McKenzie, M. and Trounce, I. (2000) Expression of *Rattus norvegicus* mtDNA in *Mus musculus* cells results in multiple respiratory chain defects. *J. Biol. Chem.*, **275**, 31514–31519.
14. Yamaoka, M., Isobe, K., Shitara, H., Yonekawa, H., Miyabayashi, S. and Hayashi, J.-I. (2000) Complete repopulation of mouse mitochondrial DNA-less cells with rat mitochondrial DNA restores mitochondrial translation but not mitochondrial respiratory function. *Genetics*, **155**, 301–307.
15. Shitara, H., Kaneda, H., Sato, A., Inoue, K., Ogura, A., Yonekawa, H. and Hayashi, J.-I. (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.
16. Ziegler, M.L. and Davidson, R.L. (1981) Elimination of mitochondrial elements and improved viability in hybrid cells. *Somatic Cell Genet.*, **7**, 73–88.
17. Kaneda, H., Hayashi, J.-I., Takahama, S., Taya, C., Fischer Lindahl, K. and Yonekawa, H. (1995) Elimination of paternal mitochondrial DNA in intraspecific crosses during early mouse embryogenesis. *Proc. Natl Acad. Sci. USA*, **92**, 4542–4546.
18. Shitara, H., Hayashi, J.-I., Takahama, S., Kaneda, H. and Yonekawa, H. (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.
19. Acin, P.R., Bayona, B.M.P., Bueno, M., Machicado, C., Fernandez, S.P., Perez, M.A., Montoya, J., Lopez, P.M.J., Sancho, J. and Enriquez, J.A. (2003) An intragenic suppressor in the cytochrome *c* oxidase I gene of mouse mitochondrial DNA. *Hum. Mol. Genet.*, **12**, 329–339.
20. Varlamov, D.A., Kudin, A.P., Vielhaber, S., Schroder, R., Sassen, R., Becker, A., Kunz, D., Haug, K., Rebstock, J., Heils, A. *et al*. (2002) Metabolic consequences of a novel missense mutation of the mtDNA *COI* gene. *Hum. Mol. Genet.*, **16**, 1797–1805.
21. Rahman, S., Taanman, J.W., Cooper, J.M., Nelson, I., Hargreaves, I., Meunier, B., Hanna, M.G., Garcia, J.J., Capaldi, R.A., Lake, B.D. *et al*. (1999) A missense mutation of cytochrome oxidase subunit II causes defective assembly and myopathy. *Am. J. Hum. Genet.*, **65**, 1030–1039.
22. Yamaoka, M., Mikami, T., Ono, T., Nakada, K. and Hayashi, J.-I. (2001) Mice with only rat mtDNA are required as models of mitochondrial diseases. *Biochem. Biophys. Res. Commun.*, **282**, 707–711.
23. McFarland, R., Clark, K.M., Morris, A.A., Taylor, R.W., Macphail, S., Lightowlers, R.N. and Turnbull, D.M. (2002) Multiple neonatal deaths due to a homoplasmic mitochondrial DNA mutation. *Nat. Genet.*, **30**, 145–146.
24. Levy, S.E., Waymire, K.G., Kim, Y.L., MacGregor, G.R. and Wallace, D.C. (1999) Transfer of chloramphenicol-resistant mitochondrial DNA into the chimeric mouse. *Transgenic Res.*, **8**, 137–145.
25. McKenzie, M., Trounce, I.A., Cassar, C.A. and Pinkert, C.A. (2004) Production of homoplasmic xenomitochondrial mice. *Proc. Natl Acad. Sci. USA*, **101**, 1685–1690.
26. Hirai, M., Suzuki, S., Onoda, M., Hinokio, Y., Ai, L., Hirai, A., Ohtomo, M., Komatsu, K., Kasuga, S., Satoh, Y. *et al*. (1996) Mitochondrial DNA 3394 mutation in the NADH dehydrogenase subunit 1 associated with non-insulin-dependent diabetes mellitus. *Biochem. Biophys. Res. Commun.*, **219**, 951–955.
27. Matsuoka, R., Furutani, M., Hayashi, J.-I., Isobe, K., Akimoto, K., Shibata, T., Imamura, S., Tatsuguchi, M., Furutani, Y., Takao, A. *et al*. (1999) A mitochondrial DNA mutation cosegregates with the pathophysiological U wave. *Biochem. Biophys. Res. Commun.*, **257**, 228–233.
28. Johnson, K.R., Zheng, Q.Y., Bykhovskaya, Y., Spirina, O. and Fischel-Ghodsian, N. (2001) A nuclear-mitochondrial DNA interaction affecting hearing impairment in mice. *Nat. Genet.*, **27**, 191–194.
29. Jaber, L., Shohat, M., Bu, X., Fischel-Ghodsian, N., Yang, H.Y., Wang, S.J. and Rotter, J.I. (1992) Sensorineural deafness inherited as a tissue specific mitochondrial disorder. *J. Med. Genet.*, **29**, 86–90.
30. Prezant, T.R., Agapian, J.V., Bohlman, M.C., Bu, X., Oztas, S., Qiu, W.Q., Arnos, K.S., Cortopassi, G.A., Jaber, L., Rotter, J.I. *et al*. (1993) Mitochondrial ribosomal RNA mutation associated with both antibiotic-induced and non-syndromic deafness. *Nat. Genet.*, **4**, 289–294.
31. Nakada, K., Sato, A., Sone, H., Kasahara, A., Ikeda, K., Kagawa, Y., Yonekawa, H. and Hayashi, J.-I. (2004) Accumulation of pathogenic ΔmtDNA induced deafness but not diabetic phenotypes in mito-mice. *Biochem. Biophys. Res. Commun.*, **323**, 175–184.
32. Inoue, K., Takai, D., Hosaka, H., Ito, S., Shitara, H., Isobe, K., LePecq, J.B., Segal-Bendirdjian, E. and Hayashi, J.-I. (1997) 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.
33. Yagi, T., Tokunaga, T., Furuta, Y., Nada, S., Yoshida, M., Tsukada, T., Saga, Y., Takeda, N., Ikawa, Y. and Aizawa, S. (1993) A novel ES cell line, TT2, with high germline-differentiating potency. *Anal. Biochem.*, **214**, 70–76.
34. Seligman, A.M., Karnovsky, M.J., Wasserkrug, H.L. and Hanker, J.S. (1968) Nondroplet ultrastructural demonstration of cytochrome oxidase activity with a polymerizing osmophilic reagent, diaminobenzidine (DAB). *J. Cell. Biol.*, **38**, 1–14.
35. Miyabayashi, S., Haginoya, K., Hanamizu, H., Iinuma, K., Narisawa, K. and Tada, K. (1989) Defective pattern of mitochondrial respiratory enzymes in mitochondrial myopathy. *J. Inher. Metab. Dis.*, **12**, 373–377.