# Transmitochondrial mice as models for primary prevention of diseases caused by mutation in the *tRNA$^{Lys}$* gene

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**We generated transmitochondrial mice (mito-mice) that carry a mutation in the *tRNA$^{Lys}$* gene encoded by mtDNA for use in studies of its pathogenesis and transmission profiles. Because patients with mitochondrial diseases frequently carry mutations in the mitochondrial *tRNA$^{Lys}$* and *tRNA$^{Leu(UUR)}$* genes, we focused our efforts on identifying somatic mutations of these genes in mouse lung carcinoma P29 cells. Of the 43 clones of PCR products including the *tRNA$^{Lys}$* or *tRNA$^{Leu(UUR)}$* genes in mtDNA of P29 cells, one had a potentially pathogenic mutation (G7731A) in the *tRNA$^{Lys}$* gene. P29 subclones with predominant amounts of G7731A mtDNA expressed respiration defects, thus suggesting the pathogenicity of this mutation. We then transferred G7731A mtDNA into mouse ES cells and obtained $F_0$ chimeric mice. Mating these $F_0$ mice with C57BL/6J (B6) male mice resulted in the generation of $F_1$ mice with G7731A mtDNA, named "mito-mice-tRNA$^{Lys7731}$." Maternal inheritance and random segregation of G7731A mtDNA occurred in subsequent generations. Mito-mice-tRNA$^{Lys7731}$ with high proportions of G7731A mtDNA exclusively expressed respiration defects and disease-related phenotypes and therefore are potential models for mitochondrial diseases due to mutations in the mitochondrial *tRNA$^{Lys}$* gene. Moreover, the proportion of mutated mtDNA varied markedly among the pups born to each dam, suggesting that selecting oocytes with high proportions of normal mtDNA from affected mothers with *tRNA$^{Lys}$*-based mitochondrial diseases may be effective as a primary prevention for obtaining unaffected children.**

mutated mtDNA segregation | mtDNA heteroplasmic mutation | preimplantation genetic diagnosis | selection of oocytes

Mitochondrial DNA (mtDNA) carrying a large-scale deletion (ΔmtDNA) and single-point mutations in the *tRNA$^{Lys}$* gene and in the *tRNA$^{Leu(UUR)}$* gene causes chronic progressive external ophthalmoplegia (CPEO); myoclonic epilepsy with ragged-red fibers (MERRF); and mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS), respectively—the three most prevalent mitochondrial diseases (1–3). However, there are slight differences among the three disease phenotypes, even though these pathogenic mtDNA mutations all induce mitochondrial respiration defects. Considering that mitochondrial respiratory function is controlled by both mitochondrial and nuclear genomes (1–3), this controversial issue can be clarified by generating transmitochondrial mice (mito-mice) that share the same nuclear genetic background but carry different pathogenic mtDNA mutations corresponding to the mutations found in the three prevalent mitochondrial diseases. However, no well-established, effective protocols are available for introducing mutagenized mtDNA into the mitochondria of mammalian cells.

In our previous studies (4–8) we found mtDNAs carrying pathogenic mutations in mouse cell lines, transferred them into mouse female germ lines, and generated several types of mito-mice, including mito-mice-Δ (4, 5) which harbor ΔmtDNA and therefore are disease models for CPEO. However, mito-mice harboring mtDNA with pathogenic mutations in the *tRNA$^{Lys}$* and *tRNA$^{Leu(UUR)}$* genes—and therefore prospective disease models of MERRF and MELAS, respectively—have not previously been established owing to the unavailability of mouse cell lines with corresponding *tRNA* mutations in mtDNA.

To complement the paucity of effective technologies required for introducing mutagenized mtDNA into mitochondria of living mouse cells, we developed an alternative strategy involving cloning and sequence analysis to detect small amounts of mtDNA with somatic mutations in the mitochondrial *tRNA* genes. Because pathogenic mutations responsible for mitochondrial diseases occur preferentially in the *tRNA$^{Leu(UUR)}$* and *tRNA$^{Lys}$* genes of humans (1–3), we sequenced 43 clones generated from PCR products carrying the mitochondrial *tRNA$^{Leu(UUR)}$* and *tRNA$^{Lys}$* genes of P29 mouse lung carcinoma cells (9). One of the 43 clones had a somatic G7731A mutation in *tRNA$^{Lys}$*, which enabled the generation of transmitochondrial mito-mice expressing respiration defects for their use as models for diseases caused by mutations in the mitochondrial *tRNA$^{Lys}$* gene.

## Results

**Cloning and Sequence Analysis of PCR Products Including *tRNA* Genes.** To detect the small proportion of mtDNA with somatic and possibly pathogenic mutations in the *tRNA$^{Leu(UUR)}$* and *tRNA$^{Lys}$* genes, we used two sets of primers so that the resulting PCR products would include the *tRNA$^{Leu(UUR)}$* or *tRNA$^{Lys}$* gene of P29

### Significance

We generated transmitochondrial mito-mice-tRNA$^{Lys7731}$ as models for precise examination of the pathogenesis and transmission profiles of mtDNA mutations in the *tRNA$^{Lys}$* genes and have obtained important information regarding primary prevention of the diseases caused by the mtDNA mutations. Although nuclear transplantation from oocytes of affected mothers into enucleated oocytes of unrelated women has been suggested, the methodology carries the technical risk of inducing nuclear abnormalities and prompts ethical concerns regarding the production of three-parent babies with normal mtDNA from unrelated oocyte donors. The current study suggests that the selection of oocytes with high proportions of normal mtDNA from affected mothers can be used to avoid these issues and therefore provides insights into mitochondrial genetics and medicine.

Author contributions: A.S., K.N., and J.-I.H. designed research; A.S., T.M., C.H., E.O., R.K., and I.N. performed research; K.T. contributed new reagents/analytic tools; A.S., T.M., and E.O. analyzed data; and J.-I.H. wrote the paper.

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AP014540 and AP014541).

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cells (Fig. S1). After cloning the PCR products, we sequenced all 43 clones obtained and compared the resulting sequences with that of mtDNA from P29 cells (Table 1). We found one and two somatic mutations in the mitochondrial $tRNA^{Leu(UUR)}$ and $tRNA^{Lys}$ genes, respectively. Given that each somatic mutation was present in only 1 of the 43 clones, the proportion of each in the mtDNA population of P29 cells would be about 2.2%.

The T7728C and G7731A mutations in the $tRNA^{Lys}$ gene both occurred in conserved sites (Table S1) and may correspond to pathogenic mutations that induce respiration defects by their accumulation. Moreover, an orthologous mutation to mouse G7731A has been reported to occur in human mtDNA from patients with mitochondrial diseases (10, 11).

Therefore, we selected these mutations in mtDNA for the generation of mito-mice. In an attempt to detect the T7728C and G7731A mutations in P29 mtDNA, we performed XmnI and DraI digestions of the PCR products of mtDNA, because the T7728C and G7731A mutations create an XmnI site and a DraI site, respectively (*SI Materials and Methods*). However, the T7728C and G7731A mutations were undetectable owing to their insufficient amounts in P29 cells.

**Concentration of G7731A mtDNA in Subclones of P29 Cells.** We previously showed that two mtDNA haplotypes with different mutations in single cells segregate stochastically during cell division (12). Therefore, some individual cells in the P29 population may possess detectable amounts of the mutated mtDNA. To obtain some individual cells that had accumulated either T7728C mtDNA or G7731A mtDNA from the P29 cell population, we isolated 100 subclones from P29 cells. Their mtDNA genotyping showed that two subclones, P29-42 and P29-69, possessed 34% and 48% G7731A mtDNA, respectively. However, we did not obtain any subclones carrying detectable amounts of T7728C mtDNA.

In the case of human mitochondrial *tRNA* gene mutations found in patients with mitochondrial diseases, respiration defects were apparent only when the mutated mtDNA had accumulated to more than 90% (13). To isolate P29 cells with more than 90% G7731A mtDNA, we cultured subclone P29-69, which had 48% G7731A mtDNA, for an additional 3 mo to allow further amplification of G7731A mtDNA through stochastic segregation; we then isolated more than 200 subclones from the P29-69 cells. We obtained one subclone, P29-69-183, which contained 92% G7731A mtDNA (Fig. 1*A*)—a level likely to be sufficient for the expression of respiration defects, if the G7731A mutation indeed is a pathogenic mutation.

**Table 1. Somatic mutations in P29 mtDNA according to cloning and sequence analysis of the PCR products including the $tRNA^{Leu(UUR)}$ and $tRNA^{Lys}$ genes**

| | tRNA gene | | | | | |
|---|---|---|---|---|---|---|
| No. of clones | $Leu^{UUR}$ | *Ile* | *Met* | $Ser^{CUN}$ | *Lys* | *Lys* |
| Nucleotide position | 2721 | 3760 | 3883 | 6931 | 7728* | 7731† |
| P29 sequence‡ | T | G | — | T | T | G |
| No. of clones | | | | | | |
| 1 | del | — | — | — | — | — |
| 1 | — | A | — | — | — | — |
| 1 | — | — | insA | — | — | — |
| 1 | — | — | — | C | — | — |
| 1 | — | — | — | — | C | — |
| 1 | — | — | — | — | — | A |
| 37 | — | — | — | — | — | — |

*The T7728C mutation in the $tRNA^{Lys}$ gene occurred in a site highly conserved throughout animals and fungi (Table 2). The mutation affects the first base of an anticodon triplet (TTT), resulting in an anticodon swap from TTT to CTT (Lys to Glu).

†The mutation equivalent to the G7731A mutation in the $tRNA^{Lys}$ gene of mice has been reported to occur in the mtDNA from human patients with mitochondrial diseases (10, 11).

‡Registered under GenBank accession no. EU312160.

**Determination of the Pathogenicity of G7731A mtDNA.** Comparison of the $O_2$ consumption rates between parental P29 cells and the P29-69-183 cells revealed the expression of respiration defects in the P29-69-183 cells (Fig. 1*B*). Moreover, P29-69-183 cells demonstrated slight overproduction of reactive oxygen species (Fig. 1*C*), indicating the pathogenicity of the G7731A mutation. Whole-sequence analysis of mtDNA in P29-69-183 cells showed that the G7731A mutation is the only mutation in those cells that is capable of inducing respiration defects (Table S2). However, we had to resolve two important issues before generation of the mito-mice carrying G7731A mtDNA.

First, we had to confirm that the respiration defects in P29-69-183 cells were due to G7731A mtDNA and not to the selection of cells with mutations in nuclear DNA that were acquired during repeated recloning. Second, P29-69-183 cells could not be used as mtDNA donors to isolate mouse ES cells with G7731A mtDNA

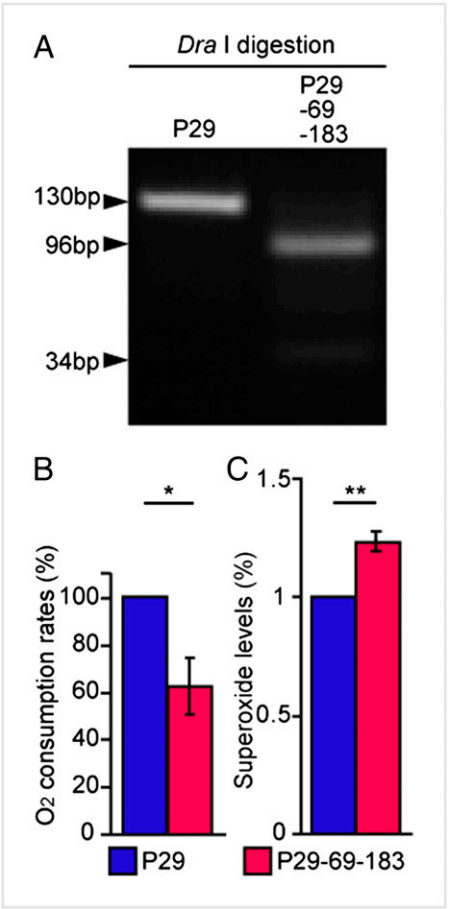


**Fig. 1.** Characterization of P29-69-183 cells to determine the pathogenicity of a G7731A mutation in the mitochondrial $tRNA^{Lys}$ gene. (*A*) Estimation of the proportion of G7731A mtDNA in P29 and P29-69-183 cells by DraI digestion of the PCR products. The G7731A mtDNA produced 96-bp and 34-bp fragments owing to the gain of a DraI site through G7731A substitution in the $tRNA^{Lys}$ gene, whereas mtDNA without the mutation produced a 130-bp fragment. Quantitative estimation of G7731A mtDNA showed that P29-69-183 cells contained 92% G7731A mtDNA. (*B*) Estimation of $O_2$ consumption rates of P29 and P29-69-183 cells. *$P < 0.05$. (*C*) Estimation of mitochondrial superoxide levels in P29 and P29-69-183 cells after their treatment with MitoSOX Red. **$P < 0.01$.

because of the difficulty of excluding unenucleated P29-69-183 cells from fusion mixtures of ES and enucleated P29-69-183 cells.

To simultaneously resolve these issues, we cytoplasmically transferred G7731A mtDNA from P29-69-183 cells into mtDNA-less ($\rho^0$) B82 cells (6). B82 cells are mouse fibrosarcoma cells that are resistant to BrdU and sensitive to hypoxanthine–aminopterin–thymidine (HAT) owing to their deficiency of thymidine kinase activity (6). Moreover, $\rho^0$ B82 cells are unable to grow in the absence of uridine and pyruvate owing to their complete lack of mtDNA. Using selection medium containing BrdU and lacking uridine and pyruvate, we isolated two colonies, B82mt7731-1 and B82mt7731-2. Genotyping of mtDNA showed that B82mt7731-1 and -2 possessed 70% and 95% G7731A mtDNA, respectively (Fig. 2*A*), indicating the transfer of G7731A mtDNA from P29-69-183 cells into $\rho^0$ B82 cells.

We then examined the respiratory function of these two cybrids by estimating $O_2$ consumption rates and the amounts of reactive oxygen species. The B82mt7731-2 cybrids showed decreased $O_2$ consumption rates (Fig. 2*B*) and increased production of reactive oxygen species (Fig. 2*C*) compared with those of B82mtB6 cybrids containing normal mtDNA from B6 mice. Therefore, respiration defects were transferred to B82mt7731 cybrids concurrently with the transfer of G7731A mtDNA from P29-69-183 cells into $\rho^0$ B82 cells, suggesting that the somatic G7731A mutation in mtDNA is a pathogenic mutation that can induce mitochondrial respiration defects by its predominant accumulation. Furthermore, B82mt7731 cybrids are effective as donors of G7731A mtDNA to ES cells, because unenucleated B82mt7731 cybrids can be excluded by using selection medium containing HAT.

**Isolation of ES Cells Containing G7731A mtDNA and Their Chimeric Mice.** Our previous study showed that no chimeric mice were obtained from ES cells carrying predominant amounts of ΔmtDNA, because the significant respiration defects induced by ΔmtDNA inhibited differentiation of ES cells to various tissues and germ cells (14). Therefore, we were concerned that the transfer of mtDNA from B82mt7731-2 cybrids containing 95% G7731A mtDNA to ES cells would inhibit generation of chimeric mice, and instead we used B82mt7731-1 cybrids containing lower proportions of G7731A mtDNA (Fig. 2*A*) as mtDNA donors.

Female (XO)-type ES cells (TT2 cells) were pretreated with rhodamine 6G (R6G) to eliminate endogenous mitochondria and mtDNA. They then were used as recipients of G7731A mtDNA and fused with enucleated B82mt7731-1 cybrids. The fusion mixture was cultured in selection medium containing HAT to exclude unenucleated B82mt7731-1 cybrids. Seven ES clones grew in the selective medium, two of which—clones ESmt7731-4 and ESmt7731-7—contained G7731A mtDNA (Fig. S2). The absence of G7731A mtDNA in the remaining five clones may be due to incomplete elimination of endogenous mtDNA in ES cells during R6G pretreatment.

We then aggregated the ESmt7731-4 and -7 cybrid clones with eight-cell-stage mouse embryos (ICR strain) and obtained 35 $F_0$ chimeric mice. Because mouse mtDNA is inherited strictly maternally (15, 16), we selected 15 $F_0$ chimeric female mice with 13–76% G13997A mtDNA in their tails as founder mice (Table 2) and mated them with B6 male mice to generate $F_1$ mice that carried G7731A mtDNA owing to its transfer through the female germ line.

**Generation of Mito-Mice-tRNA$^{Lys7731}$ via Female Germ-Line Transfer of G7731A mtDNA.** Of the 15 $F_0$ female chimeras, 11 produced a total of 121 $F_1$ pups, 63 of which had G7731A mtDNA in their tails (Table 2). This finding suggests that G7731A mtDNA was transmitted maternally from $F_0$ female mice to $F_1$ progeny. Mice that carried G7731A mtDNA derived from lung carcinoma P29 cells were named "mito-mice-tRNA$^{Lys7731}$." Because the proportion of G7731A mtDNA did not differ significantly between tissues of the same $F_1$ male mito-mouse-tRNA$^{Lys7731}$ (Fig. S3), we can deduce the approximate overall proportion of G7731A mtDNA in mito-mice-tRNA$^{Lys7731}$ by estimating the proportion of G7731A mtDNA in the tail, which we can do without killing them.

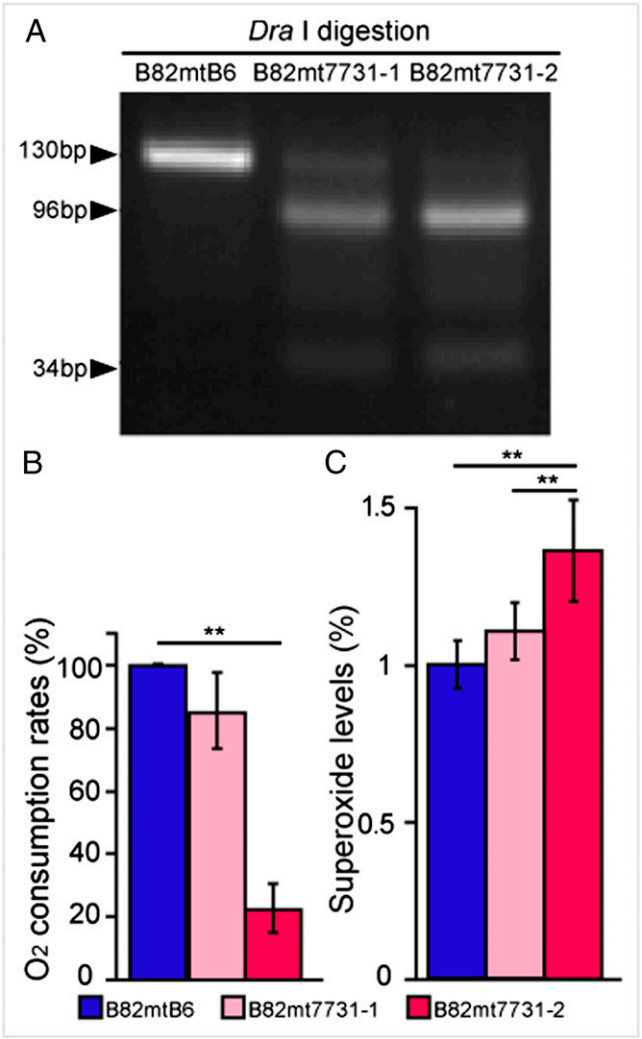


**Fig. 2.** Characterization of transmitochondrial B82mt7731 cybrids for their use as G7731A mtDNA donors to ES cells. (*A*) Estimation of the proportion of G7731A mtDNA in cybrids clones B82mt7731-1 and B82mt7731-2 by DraI digestion of the PCR products. Quantitative estimation of G7731A mtDNA showed that B82mt7731-1 and B82mt7731-2 had 70% and 95% G7731A mtDNA, respectively. (*B*) Estimation of $O_2$ consumption rates. $^{**}P < 0.01$. (*C*) Estimation of mitochondrial superoxide levels after treatment with MitoSOX Red. $^{**}P < 0.01$.

Of the 63 $F_1$ mice with G7731A mtDNA in their tails, seven $F_1$ female mice carrying high proportions of G7731A mtDNA were mated with B6 male mice to obtain subsequent generations ($F_2$–$F_5$) of mito-mice-tRNA$^{Lys7731}$ with sufficient G7731A mtDNA for the expression of respiration defects and resultant disorders. The proportions of G7731A mtDNA varied significantly among the pups born to each dam, but none of the pups carried more than 85% G7731A mtDNA (Fig. 3*A*).

To examine the reasons for the lack of mito-mice-tRNA$^{Lys7731}$ with more than 85% G7731A mtDNA, we estimated the proportion of G7731A mtDNA in the oocytes obtained by ovarian hyperstimulation of $F_5$ female mito-mice-tRNA$^{Lys7731}$ with high proportions of G7731A mtDNA. The results again showed significant variation in G7731A mtDNA proportions among the oocytes and the absence of oocytes with more than 85% G7731A mtDNA (Fig. 3*B*), indicating that lethality of oocytes with high

**Table 2. Generation of $F_0$ chimeric mice and their $F_1$ progeny that carry G7731A mtDNA in their tails**

| | | 15 $F_0$ chimeric female mice | | |
|---|---|---|---|---|
| ES clones | % G7731A mtDNA | % Chimerism* | % G7731A mtDNA in tails | No. of $F_1$ pups with G7731A mtDNA/no. of $F_1$ pups |
| ESmt7731-4 | 49.8 | 95 | 68 | 4/4 |
| | | 95 | 53 | 2/12 |
| | | 90 | 45 | 24/24 |
| | | 50 | 48 | 0/17 |
| | | 40 | 16 | 0/0 |
| | | 35 | 21 | 0/8 |
| ESmt7731-7 | 48.8 | 95 | 72 | 19/19 |
| | | 95 | 76 | 4/4 |
| | | 95 | 72 | 2/12 |
| | | 95 | 68 | 8/8 |
| | | 80 | 52 | 0/8 |
| | | 60 | 61 | 0/5 |
| | | 40 | 26 | 0/0 |
| | | 15 | 13 | 0/0 |
| | | 10 | 19 | 0/0 |
| | | | | Total: 63/121 |

*Chimerism was judged by coat color.

levels of G7731A mtDNA is responsible for the absence of mito-mice-tRNA$^{Lys7731}$ carrying more than 85% G7731A mtDNA.

**Expression of Disorders in Mito-Mice-tRNA$^{Lys7731}$ with Predominant G7731A mtDNA.** We used B6 mice as controls and three groups of $F_5$ mito-mice-tRNA$^{Lys7731}$ with different heteroplasmic conditions (low, intermediate, and high levels of G7731A mtDNA) in their tails to examine various phenotypes relevant to mitochondrial diseases (Fig. 4).

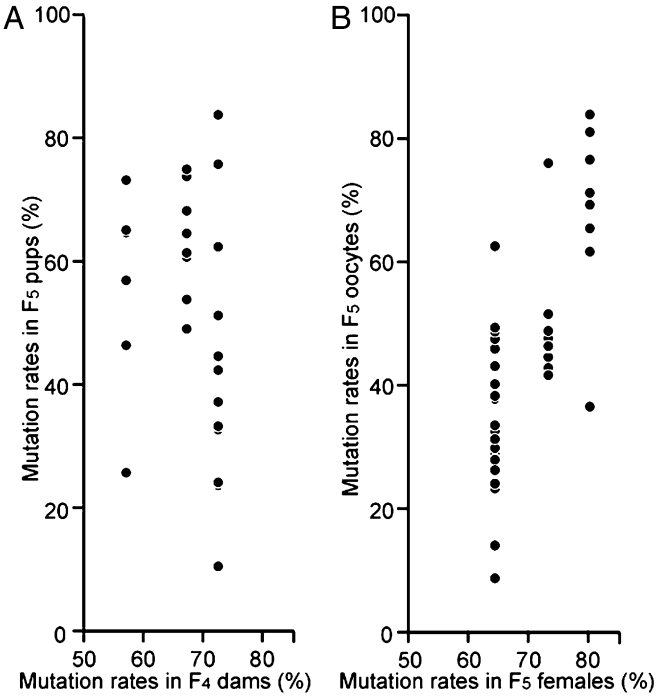


**Fig. 3.** Variation of G7731A mtDNA proportions among pups or oocytes from individual female mice. (*A*) Variation of G7731A mtDNA proportions among $F_5$ pups born to three $F_4$ dams. G7731A mtDNA proportions were estimated by using tails from $F_5$ pups that were obtained from three $F_4$ dams with high proportions of G7731A mtDNA. (*B*) Variation of G7731A mtDNA proportions among oocytes obtained from three $F_5$ female mice. G7731A mtDNA proportions were estimated by using oocytes obtained by ovarian hyperstimulation of $F_5$ female mito-mice-tRNA$^{Lys7731}$ with high proportions of G7731A mtDNA.

First, we analyzed body length (Fig. 4*A*) and muscle strength (Fig. 4*B*), because abnormalities in these characteristics frequently occur in patients with mitochondrial diseases (10, 11), and these parameters can be examined without killing the mice. Unlike mito-mice tRNA$^{Lys7731}$ with low and intermediate levels, those with high levels of G7731A mtDNA showed short body length (Fig. 4*A*) and muscle weakness (Fig. 4*B*), which are closely associated with the clinical phenotypes caused by mutations in the mitochondrial *tRNA$^{Lys}$* gene (2, 10, 11).

We then quantitatively estimated mitochondrial respiratory function and revealed respiration defects in skeletal muscle and kidney from mito-mice-tRNA$^{Lys7731}$ with high levels of G7731A mtDNA (Fig. 4*C*). Therefore, accumulation of G7731A mtDNA likely is responsible for the respiration defects in mito-mice-tRNA$^{Lys7731}$ with high levels of G7731A mtDNA (Fig. 4*C*). These respiration defects in the skeletal muscle subsequently result in the expression of muscle weakness (Fig. 4*B*), which corresponds to a phenotype relevant to mitochondrial diseases (2).

In contrast, other metabolic parameters relevant to mitochondrial diseases were normal (Fig. S4). Histochemical analysis showed that ragged-red fibers frequently observed in MERRF patients (2) and renal failures frequently observed in mito-mice-Δ (4, 5) were not found in mito-mice-tRNA$^{Lys7731}$ (Figs. S5 and S6). Absence of these disorders in mito-mice-tRNA$^{Lys7731}$ may be due in part to the fact that G7731A mtDNA proportions in the mice were not sufficient to induce significant respiration defects. For example, G8344A mtDNA proportions exceed 85% in patients with MERRF (2). Our failure to obtain mito-mice-tRNA$^{Lys7731}$ with more than 85% G7731A mtDNA can be explained by the lethality of mouse oocytes carrying these levels of G7731A mtDNA (Fig. 3*B*).

These observations suggest that mito-mice-tRNA$^{Lys7731}$ at least in part can serve as models to investigate pathogenesis of mitochondrial diseases that arise owing to mutations in the mitochondrial *tRNA* genes. In addition, the transmission profiles of G7731A mtDNA showed that selecting oocytes with lower levels of the mutated mtDNA likely would be effective to prevent maternal transmission of the disease phenotypes to the progeny.

## Discussion

The current study generated mito-mice-tRNA$^{Lys7731}$ carrying G7731A mtDNA with a pathogenic G7731A mutation in the mitochondrial *tRNA$^{Lys}$* gene. Specifically, we concentrated a small proportion of mtDNA with a somatic G7731A mutation

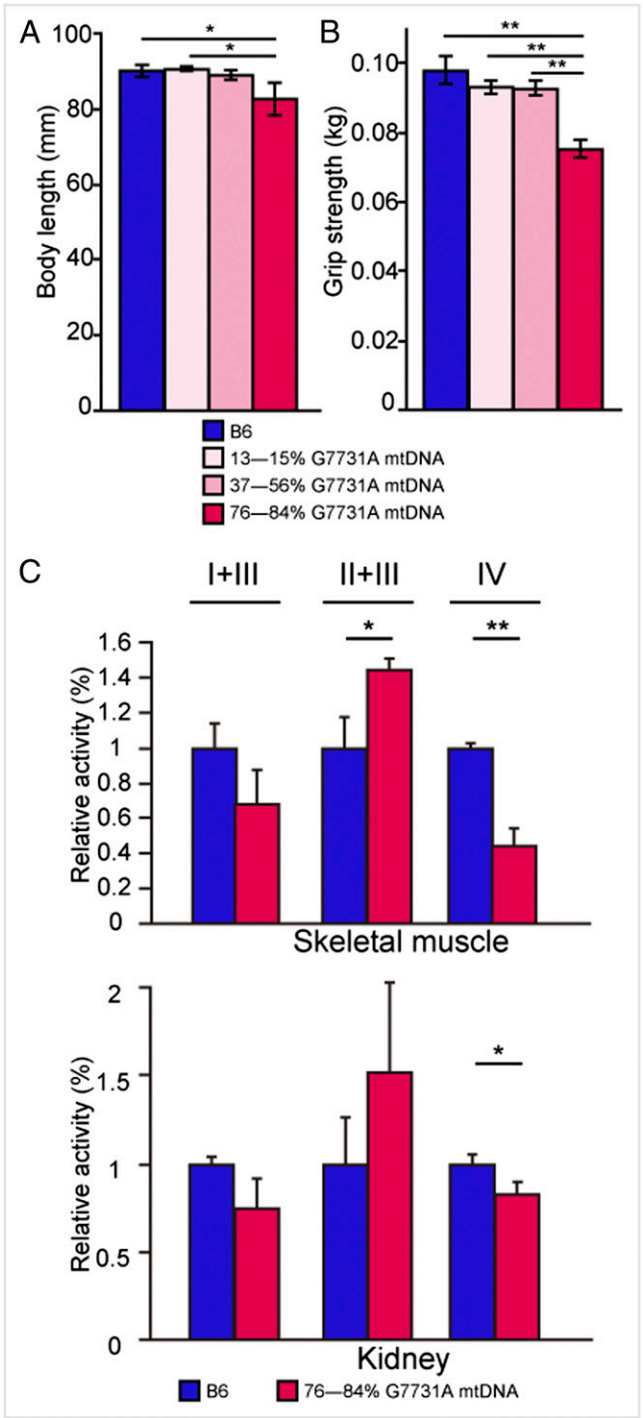


**Fig. 4.** Characterization of $F_5$ male mito-mice-tRNA$^{Lys7731}$ according to phenotypes associated with mitochondrial diseases. Comparison of (*A*) body length and (*B*) muscle (grip) strength between B6 mice ($n = 3$) and $F_5$ mito-mice-tRNA$^{Lys7731}$ carrying low (13–15%; $n = 3$), intermediate (37–56%; $n = 3$), and high (76–84%; $n = 3$) proportions of G7731A mtDNA. Body length and grip strength were measured at 4 mo after birth. Data are presented as means ± SD. $*P < 0.05$; $**P < 0.01$. (*C*) Comparison of activities of mitochondrial respiratory complexes (I + III, II + III, and IV) between B6 mice and $F_5$ mito-mice-tRNA$^{Lys7731}$ carrying high proportions (76–84%) of G7731A mtDNA in the skeletal muscle and the kidney at 4 mo after birth. Respiratory complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (cytochrome c reductase), and complex IV (cytochrome *c* oxidase) are components of the electron-transport chain. Enhanced activity of complex II + III in mito-mice-tRNA$^{Lys7731}$ would be due to compensatory activation of complex II, which is controlled exclusively by nuclear DNA. Data are presented as means ± SD ($n = 3$). $*P < 0.05$; $**P < 0.01$.

that was present in P29 cells and then introduced it into ES cells to generate mito-mice-tRNA$^{Lys7731}$. The resulting mice were used to investigate the pathogenesis and transmission profiles of G7731A mtDNA.

Regarding the pathogenesis of G7731A mtDNA, mito-mice-tRNA$^{Lys7731}$ with 74–84% G7731A mtDNA demonstrated respiration defects and the resultant muscle weakness and short body length (Fig. 4). These abnormalities in our mice are very similar to those found in patients with mitochondrial diseases owing to human orthologous G8328A mutation (10, 11) and in MERRF patients carrying the G8344A mutation in the *tRNA$^{Lys}$* gene (2, 17–19). Although these abnormalities were expressed in mito-mice-tRNA$^{Lys7731}$ with 74–84% G7731A mtDNA (Fig. 4) and in the patients with 57% (10) and 82% G8328A mtDNA (11), more than 85% G8344A mtDNA was required for the onset of severe abnormalities in patients with MERRF (2, 17–19). However, we could not obtain mito-mice-tRNA$^{Lys7731}$ with more than 85% G7731A mtDNA (Fig. 3*A*), which would be expected to induce significant respiration defects and severe abnormalities corresponding to MERRF, owing to the lethality of mouse oocytes with more than 85% G7731A mtDNA (Fig. 3*B*). Therefore, the expression of the significant respiration defects and severe clinical disorders in MERRF patients can be explained by supposing the absence of lethality in human oocytes even at G8344A mtDNA levels exceeding 85%. In contrast, our previous studies (4, 5) showed that mito-mice-Δ possessed more than 85% ΔmtDNA with a large-scale deletion and expressed significant respiration defects and severe abnormalities, even though their oocytes did not have more than 80% ΔmtDNA (20). The increase in the proportion of ΔmtDNA after birth may be due to its replication advantage (4, 5).

The absence of abnormalities in mito-mice-tRNA$^{Lys7731}$ with intermediate levels (37–56%) of G7731A mtDNA (Fig. 4 *A* and *B*) seems to be different from what is observed for mtDNA with human orthologous G8328A mutation, because the patient with only 57% G8328A mtDNA in the skeletal muscles expressed disorders (10). This apparent discrepancy may be due to the aging effects in the patient with 57% G8328A mtDNA, considering that the phenotypes of the mito-mice-tRNA$^{Lys7731}$ were examined 4 mo after birth (Fig. 4) and that the patient expressed disorders 45 y after birth (10). Thus, we have to examine whether mito-mice-tRNA$^{Lys7731}$ with intermediate levels of G7731A mtDNA express abnormalities along with aging.

Regarding transmission profiles, those of mito-mice-tRNA$^{Lys7731}$ are highly similar to those of MERRF patients, in that the mutated mtDNA is inherited by subsequent generations through the female germ line and the proportion of mutated mtDNA varied markedly among the pups born to each dam (Fig. 3*A*). The pups always carry wild-type mtDNA (Fig. 3*A*), owing to the significant pathogenicity of G7731A mtDNA and resultant induction of oocyte lethality in the absence of wild-type mtDNA (Fig. 3*B*). In contrast, we previously generated mito-mice-COI$^{6589}$ (6) and mito-mice-ND6$^{13997}$ (7, 8), which carry a 100% (homoplasmic) T6589C mtDNA mutation and a homoplasmic G13997A mtDNA mutation in the structural genes *COXI* and *ND6*, respectively, owing to the mild pathogenicity of the mutations. Because patients with MERRF and mito-mice-tRNA$^{Lys7731}$ always carry both wild-type mtDNA and mutated mtDNA, mito-mice-tRNA$^{Lys7731}$ are appropriate models for further investigating the transmission profiles of mutated mtDNA with marked pathogenicity.

The heteroplasmic mtDNA (wild-type mtDNA and mutated G7731A mtDNA) of our mito-mice-tRNA$^{Lys7731}$ segregated stochastically, like those derived from different mouse strains (BALB and NZB) with polymorphic mutations (21) and those with and without point mutations (22). The significant variation in the G7731A mtDNA proportions among pups (Fig. 3*A*) may reflect "bottleneck effects" with (23, 24) or without decrease in the mtDNA copy number (25, 26) during female germ-line transmission of the heteroplasmic mtDNAs.

Regarding primary prevention of mitochondrial diseases, the results in Figs. 3 and 4 indicate that the selection of oocytes with

low proportions of G7731A mtDNA likely would yield phenotypically normal mice. In contrast, our previous study (20) showed that nuclear transplantation from zygotes of transmitochondrial mito-mice-Δ into enucleated zygotes with normal mtDNA is effective as germ-line gene therapy. Many recent reports similarly have noted that the use of the nuclear transplantation from human oocytes of affected mothers into enucleated oocytes donated by unrelated women would prevent the transmission of mitochondrial diseases caused by mtDNA mutations to their children (27–29). However, this technology includes the risk of inducing nuclear abnormalities, even though it excludes the risk of mitochondrial abnormalities. This problem would be resolved by the selection of oocytes with low proportions of mutated mtDNA. A previous study showed that polar bodies are effective for preimplantation genetic diagnosis (PGD) to deduce the proportion of mutated mtDNA in mouse oocytes (20). However, subsequent studies using polar bodies from human oocytes (30) and blastomeres from human embryos (31, 32) indicated that blastomeres are more appropriate than are polar bodies for PGD to deduce the proportion of mutated mtDNA. Although this procedure would not completely exclude the mutated mtDNA from the affected mothers, our previous studies (33, 34) showed the presence of intermitochondrial complementation to maintain normal respiratory function in the presence of the mutated mtDNA. Therefore, in light of the results in Figs. 3 and 4, we propose that the selection of embryos with low proportions of mutated mtDNA from affected mothers would be effective for obtaining unaffected children.

The risk of late-onset disorders in subjects with low proportions of mutated mtDNA seems to be negligible, because no subjects with less than 40% MERRF G8344A mutation in the blood expressed any abnormal symptoms throughout their lifetimes (17). Moreover, mito-mice-COI$^{6589}$ carrying homoplasmic T6589C mtDNA in the *COX1* gene and expressing mild respiration defects showed normal lifespans and no late-onset disorders (6, 8). Although mito-mice-ND6$^{13997}$ carrying homoplasmic G13997A mtDNA in the *ND6* gene developed late-onset and age-related disorders (8), the onset was not due to respiration defects but to overproduction of reactive oxygen species, which can be suppressed by the administration of antioxidants (8). Because high proportions of G7731A mtDNA also induced slight overproduction of reactive oxygen species (Fig. 2*C*), we will examine whether mito-mice-tRNA$^{Lys7731}$ with high proportions of G7731A mtDNA express late-onset disorders owing to overproduction of reactive oxygen species.

## Materials and Methods

Detailed information on materials and methods in this study is provided in *SI Materials and Methods*. Information on materials includes cell line and mice. Technical information on methods contains: cloning and sequencing of PCR products; genotyping of mtDNA; isolation of B82mt7731 and ESmt7731 cybrids; generation of chimeric mice and mitomice-tRNALys7731; oxygen consumption; measurement of reactive oxygen species in mitochondria; histopathological analyses; grip strength test; biochemical measurements of respiratory enzyme activity; measurement of blood glucose, lactate and blood urea nitrogen; measurement of hematocrit values; sequence analysis; and statistical analysis.

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1. Larsson N-G, Clayton DA (1995) Molecular genetic aspects of human mitochondrial disorders. *Annu Rev Genet* 29:151–178.
2. Wallace DC (1999) Mitochondrial diseases in man and mouse. *Science* 283(5407): 1482–1488.
3. Taylor RW, Turnbull DM (2005) Mitochondrial DNA mutations in human disease. *Nat Rev Genet* 6(5):389–402.
4. Inoue K, et al. (2000) Generation of mice with mitochondrial dysfunction by introducing mouse mtDNA carrying a deletion into zygotes. *Nat Genet* 26(2):176–181.
5. Nakada K, et al. (2001) Inter-mitochondrial complementation: Mitochondria-specific system preventing mice from expression of disease phenotypes by mutant mtDNA. *Nat Med* 7(8):934–940.
6. Kasahara A, et al. (2006) Generation of trans-mitochondrial mice carrying homoplasmic mtDNAs with a missense mutation in a structural gene using ES cells. *Hum Mol Genet* 15(6):871–881.
7. Yokota M, et al. (2010) Generation of trans-mitochondrial mito-mice by the introduction of a pathogenic G13997A mtDNA from highly metastatic lung carcinoma cells. *FEBS Lett* 584(18):3943–3948.
8. Hashizume O, et al. (2012) Specific mitochondrial DNA mutation in mice regulates diabetes and lymphoma development. *Proc Natl Acad Sci USA* 109(26):10528–10533.
9. Ishikawa K, et al. (2008) ROS-generating mitochondrial DNA mutations can regulate tumor cell metastasis. *Science* 320(5876):661–664.
10. Houshmand M, Lindberg C, Moslemi AR, Oldfors A, Holme E (1999) A novel heteroplasmic point mutation in the mitochondrial tRNA(Lys) gene in a sporadic case of mitochondrial encephalomyopathy: De novo mutation and no transmission to the offspring. *Hum Mutat* 13(3):203–209.
11. Blakely EL, et al. (2007) Sporadic myopathy and exercise intolerance associated with the mitochondrial 8328G>A tRNALys mutation. *J Neurol* 254(9):1283–1285.
12. Hayashi J-I, Tagashira Y, Yoshida MC, Ajiro K, Sekiguchi T (1983) Two distinct types of mitochondrial DNA segregation in mouse-rat hybrid cells. Stochastic segregation and chromosome-dependent segregation. *Exp Cell Res* 147(1):51–61.
13. Chomyn A, et al. (1992) MELAS mutation in mtDNA binding site for transcription termination factor causes defects in protein synthesis and in respiration but no change in levels of upstream and downstream mature transcripts. *Proc Natl Acad Sci USA* 89(10):4221–4225.
14. Ishikawa K, et al. (2005) Application of ES cells for generation of respiration-deficient mice carrying mtDNA with a large-scale deletion. *Biochem Biophys Res Commun* 333(2):590–595.
15. Kaneda H, et al. (1995) Elimination of paternal mitochondrial DNA in intraspecific crosses during early mouse embryogenesis. *Proc Natl Acad Sci USA* 92(10):4542–4546.
16. Shitara H, Hayashi J-I, Takahama S, Kaneda H, 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(2):851–857.
17. Hammans SR, et al. (1993) The mitochondrial DNA transfer RNA$^{Lys}$ A → G$^{(8344)}$ mutation and the syndrome of myoclonic epilepsy with ragged red fibres (MERRF). *Brain* 116(Pt 3):617–632.
18. Hammans SR, et al. (1995) The mitochondrial DNA transfer RNALeu(UUR) A—>G (3243) mutation. A clinical and genetic study. *Brain* 118(Pt 3):721–734.
19. Chinnery PF, Howell N, Lightowlers RN, Turnbull DM (1998) MELAS and MERRF. The relationship between maternal mutation load and the frequency of clinically affected offspring. *Brain* 121(Pt 10):1889–1894.
20. Sato A, et al. (2005) Gene therapy for progeny of mito-mice carrying pathogenic mtDNA by nuclear transplantation. *Proc Natl Acad Sci USA* 102(46):16765–16770.
21. Jenuth JP, Peterson AC, Fu K, Shoubridge EA (1996) Random genetic drift in the female germline explains the rapid segregation of mammalian mitochondrial DNA. *Nat Genet* 14(2):146–151.
22. Freyer C, et al. (2012) Variation in germline mtDNA heteroplasmy is determined prenatally but modified during subsequent transmission. *Nat Genet* 44(11):1282–1285.
23. Cree LM, et al. (2008) A reduction of mitochondrial DNA molecules during embryogenesis explains the rapid segregation of genotypes. *Nat Genet* 40(2):249–254.
24. Wai T, Teoli D, Shoubridge EA (2008) The mitochondrial DNA genetic bottleneck results from replication of a subpopulation of genomes. *Nat Genet* 40(12):1484–1488.
25. Cao L, et al. (2007) The mitochondrial bottleneck occurs without reduction of mtDNA content in female mouse germ cells. *Nat Genet* 39(3):386–390.
26. Cao L, et al. (2009) New evidence confirms that the mitochondrial bottleneck is generated without reduction of mitochondrial DNA content in early primordial germ cells of mice. *PLoS Genet* 5(12):e1000756.
27. Craven L, et al. (2010) Pronuclear transfer in human embryos to prevent transmission of mitochondrial DNA disease. *Nature* 465(7294):82–85.
28. Tachibana M, et al. (2013) Towards germline gene therapy of inherited mitochondrial diseases. *Nature* 493(7434):627–631.
29. Paull D, et al. (2013) Nuclear genome transfer in human oocytes eliminates mitochondrial DNA variants. *Nature* 493(7434):632–637.
30. Gigarel N, et al. (2011) Poor correlations in the levels of pathogenic mitochondrial DNA mutations in polar bodies versus oocytes and blastomeres in humans. *Am J Hum Genet* 88(4):494–498.
31. Steffann J, et al. (2006) Analysis of mtDNA variant segregation during early human embryonic development: a tool for successful NARP preimplantation diagnosis. *J Med Genet* 43(3):244–247.
32. Tajima H, et al. (2007) The development of novel quantification assay for mitochondrial DNA heteroplasmy aimed at preimplantation genetic diagnosis of Leigh encephalopathy. *J Assist Reprod Genet* 24(6):227–232.
33. Hayashi J-I, Takemitsu M, Goto Y-i, Nonaka I (1994) Human mitochondria and mitochondrial genome function as a single dynamic cellular unit. *J Cell Biol* 125(1):43–50.
34. Ono T, Isobe K, Nakada K, Hayashi J-I (2001) Human cells are protected from mitochondrial dysfunction by complementation of DNA products in fused mitochondria. *Nat Genet* 28(3):272–275.