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Over-expression of *Tfam* improves the mitochondrial disease phenotypes in a mouse model system

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ABSTRACT

The phenotypes of mitochondrial diseases caused by mutations in mitochondrial DNA (mtDNA) have been proposed to be strictly regulated by the proportion of wild-type and pathogenically mutated mtDNAs. More specifically, it is thought that the onset of the disease phenotype occurs when cells cannot maintain the proper mitochondrial function because of an over-abundance of pathological mtDNA. Therapies that cause a decrease in the pathogenic mtDNA population have been proposed as a treatment for mitochondrial diseases, but these therapies are difficult to apply in practice. In this report, we present a novel concept: to improve mitochondrial disease phenotypes via an increase in the absolute copy number of the wild-type mtDNA population in pathogenic cells even when the relative proportion of mtDNA genotypes remains unchanged. We have succeeded in ameliorating the typical symptoms of mitochondrial disease in a model mouse line by the over-expression of the mitochondrial transcription factor A (*Tfam*) followed by an increase of the mtDNA copy number. This new concept should lead to the development of a novel therapeutic treatment for mitochondrial diseases.

1. Introduction

Mitochondria play several essential roles in cell functions, and the accumulation of mitochondrial DNA (mtDNA) with pathogenic mutations causes mitochondrial diseases, including mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS); myoclonus epilepsy with ragged red fibers (MERRF); Kearns–Sayre syndrome (KSS); and chronic progressive external ophthalmoplegia (CPEO) [1,2].

One general feature of mitochondrial diseases is that at least two types (wild-type and mutated) of mtDNA exist in a single cell, which is a genetic status called heteroplasmy. Several mouse models have been generated to study human mitochondrial diseases, including heteroplasmic mtDNA genetics. These mice possess mtDNAs derived from two strains [3,4] containing either point-mutated mtDNA [5,6] or mtDNA that has a large deletion [7]. These mice have been used to study mtDNA transmission, pathogenic mechanisms, and the development of effective therapies for diseases. Effective treatment procedures for diseases have not yet been established, although several attempts have been reported, including a study in which one type of mtDNA (presumably pathogenic) was specifically digested by a mitochondrial-targeted restriction enzyme [8]. However, this treatment is difficult to apply practically to medical care because the restriction sites specific to the pathogenic mutant on the mtDNA are rare.

Mitochondrial diseases are caused by the accumulation of mtDNA with pathogenic mutations in single cells followed by the reduction of mitochondrial respiration activity. Interestingly, this reduction of activity does not simply and linearly correlate with the proportion of mutated mtDNA but is caused only when the proportion of the mutated mtDNA exceeds a certain threshold level [7,9,10]. This phenomenon is thought to be caused by the occurrence of inter-mitochondrial complementation through the mixing of genetic contents [10] due to mitochondrial dynamics, such as fusion, fission and transportation [11] that makes mitochondria

Abbreviations: mtDNA, mitochondrial DNA; *Tfam*, mitochondrial transcription factor A; mito-mouse, mitochondrial diseases model mouse; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes; MERRF, myoclonus epilepsy with ragged red fibers; KSS, Kearns–Sayre syndrome; CPEO, chronic progressive external ophthalmoplegia; MDS, mtDNA depletion syndrome; PGC-1α, peroxisome proliferative-activated receptor γ coactivator-1α.

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function as a single cellular unit [12]. The accumulation of mutated mtDNA causes a decrease in wild-type mtDNA, which results in a reduction of genetic factors and defects in respiratory activity.

The depletion of mtDNA copy number in humans [13] and mice [14] has caused severe diseases, such as mtDNA depletion syndrome (MDS). Furthermore, the down-regulation of mtDNA replication or transcription by knockout [15] or knockdown [16] of the *Tfam* gene, which encodes the mtDNA binding protein, results in decreased respiratory activity. These data suggest that an appropriate copy number of normal mtDNA is essential for the maintenance of mitochondrial function and that cells carrying mutated mtDNA may be rescued by the increase of wild-type mtDNA sufficient to supply normal rRNAs, tRNAs and proteins.

Based on this view, we generated transgenic mice that over-express *Tfam* to increase the total mtDNA copy number [17,18], and we mated these mice to the mitochondrial diseases model mice (mito-mice) with mutant mtDNA carrying a deletion of 4696 bp (ΔmtDNA) [7]. The increase in total mtDNA copy number due to the over-expression of *Tfam* in the mito-mice improved the severe symptoms of mitochondrial disease exhibited by the mito-mice, resulting in a prolonged life span. From this study, we propose a novel therapeutic treatment for mitochondrial diseases. We propose that an increase in the absolute copy number of healthy mtDNA restores the normal mitochondrial function even under conditions where the proportion of pathogenic mtDNA is unchanged.

2. Materials and methods

2.1. Mouse strains

The C57BL/6 J (CLEA Japan), mito-mouse [7] and *Tfam*/EGFP-Tg strains were used in this research. The Institutional Animal Experiment Committee approved all of the animal experiments.

2.2. Estimation of mtDNA copy number by comparative real-time PCR

Comparative real-time PCR for estimating the copy number of mtDNA in the tissues of mito-mice and Tg-mito-mice was performed using a QuantiTect SYBR Green PCR Kit (Qiagen) with *Gapdh* as the control for the nuclear genome copy number. The following primers were used: 5′- AAC TCA AAG GAC TTG GCG GTA CTT TAT ATC-3′ and 5′- GAT GGC GGT ATA TAG GCT GAA TTA GCA AGA G-3′ to amplify the region common in both the wild-type and the deleted mtDNA, and 5′-AAC GAC CCC TTC ATT GAC-3′ and 5′-TCC ACG ACA TAC TCA GCA C-3′ for *Gapdh* [19].

The total DNA samples purified from the tissues were diluted to a concentration of 100–1000 pg/μl and amplified using an ABI Prism 7900 (Applied Biosystems). The thermal cycling conditions were as follows: 50 °C for 2 min; 95 °C for 15 min; and 40 cycles of 95 °C for 20 s followed by 60 °C for 60 s.

2.3. Estimation of ΔmtDNA proportions by real-time PCR

Real-time PCR was used to estimate the proportions of ΔmtDNA, as previously described [20].

2.4. Northern blot for mitochondrial tRNA

Total RNA was purified from homogenized tissue samples using ISOGEN (NIPPON GENE), and Northern blots were then carried out as previously described [21,22]. The DNA sequences for the specific probes were as follows: 5′- ATG TTT TTA AAC ATG GAA GCA TGA A-3′ for $tRNA^{Ser(AGY)}$ and 5′- GTC TCC CAT CCA AGT ACT AAC CAA G-3′ for 5S rRNA.

2.5. BUN measurement

Blood was obtained from a tail vein, and blood urea nitrogen (BUN) concentrations were measured using a Urea N B kit (Wako Pure Chemical) as previously described [23].

2.6. Histochemical analyses of COX and SDH activity

Histochemical analyses of cytochrome *c* oxidase (COX) and succinate dehydrogenase (SDH) activity were carried out as previously described [7,10]. Hearts and kidneys were excised from the animals, and 10-μm cryosections from the tissues were stained for COX and SDH activity.

2.7. Single-fiber PCR analysis

Two serial 10-μm cryosections of heart muscle were used for single-fiber PCR. One of the sections was stained for COX and SDH activity, and the COX-positive fibers were selected for PCR analysis. Another section was used for the dissection of the cytoplasm corresponding to the COX-positive fibers with an LMD6000 (Leica). The total DNA was extracted from dissected samples and used directly for PCR analysis as previously described [24]. The PCR conditions were also carried out as described above.

3. Results

3.1. Tfam/EGFP over-expression increased the amount of mtDNA

We generated a transgenic (Tg) mouse strain that over-expressed the mtDNA binding protein, TFAM, combined with EGFP (C57BL/6 J-Tg(CAG-*Tfam*/EGFP)38Rin, *Tfam*/EGFP-Tg) (Fig. S1a). Compared with wild-type mice, the *Tfam*/EGFP-Tg mice displayed 9.10-fold (heart) and 5.67-fold (kidney) levels of total *Tfam* mRNA, but the same levels of endogenous *Tfam* (Fig. S1b). The TFAM/EGFP protein expression level was measured by Western blot using an antibody specific to GFP. The left ventricle, kidney and pancreas had a relatively higher expression of TFAM/EGFP, but the spleen and stomach had low levels. In Western blot analysis using cell fractionation of the heart and kidney, EGFP was detected in the mitochondrial fraction (Fig. S1c), and it was also found to be localized to the mitochondrial matrix using immunoelectron microscopy in the *Tfam*/EGFP-Tg mice (Fig. S1d). A gel shift assay showed a band shift of a linear DNA fragment with an mtDNA sequence dependent on the concentration of TFAM and TFAM/EGFP proteins, indicating that the TFAM/EGFP protein had DNA binding capacity (Fig. S1e).

We assayed the amount of mtDNA in 17 tissues from the *Tfam*/EGFP-Tg and wild-type mice using quantitative real-time PCR. Compared with their wild-type littermates, most tissues of the *Tfam*/EGFP-Tg mice had increased mtDNA copy numbers: cerebral cortex (2.51- and 1.61-fold in female and male, respectively), cerebellum (1.59- and 1.34-fold), heart (2.32- and 2.03-fold), thymus (1.52- and 2.67-fold), lung (4.28- and 2.43-fold), liver (0.92- and 1.03-fold), kidney (1.11- and 1.32-fold), adrenal body (1.45- and 1.69-fold), spleen (2.02- and 0.69-fold), pancreas (1.89- and 1.45-fold), stomach (2.14- and 1.89-fold), small intestine (1.87- and 1.60-fold), large intestine (2.09- and 1.06-fold), extensor digitorum longus (EDL) (2.83- and 1.90-fold), soleus (1.48- and 1.82-fold), ovary (1.59-fold), testis (1.10-fold) and tail (2.65- and 2.19-fold) (Fig. 1A). There was no significant difference between the amount of mtDNA in the tails of the wild-type mice and those of mtGFP-Tg mice that expressed mitochondrial-targeted EGFP (data not shown) [24], suggesting that the increase in mtDNA in the *Tfam*/EGFP-Tg mice was due to the effect of the *Tfam* over-expression.

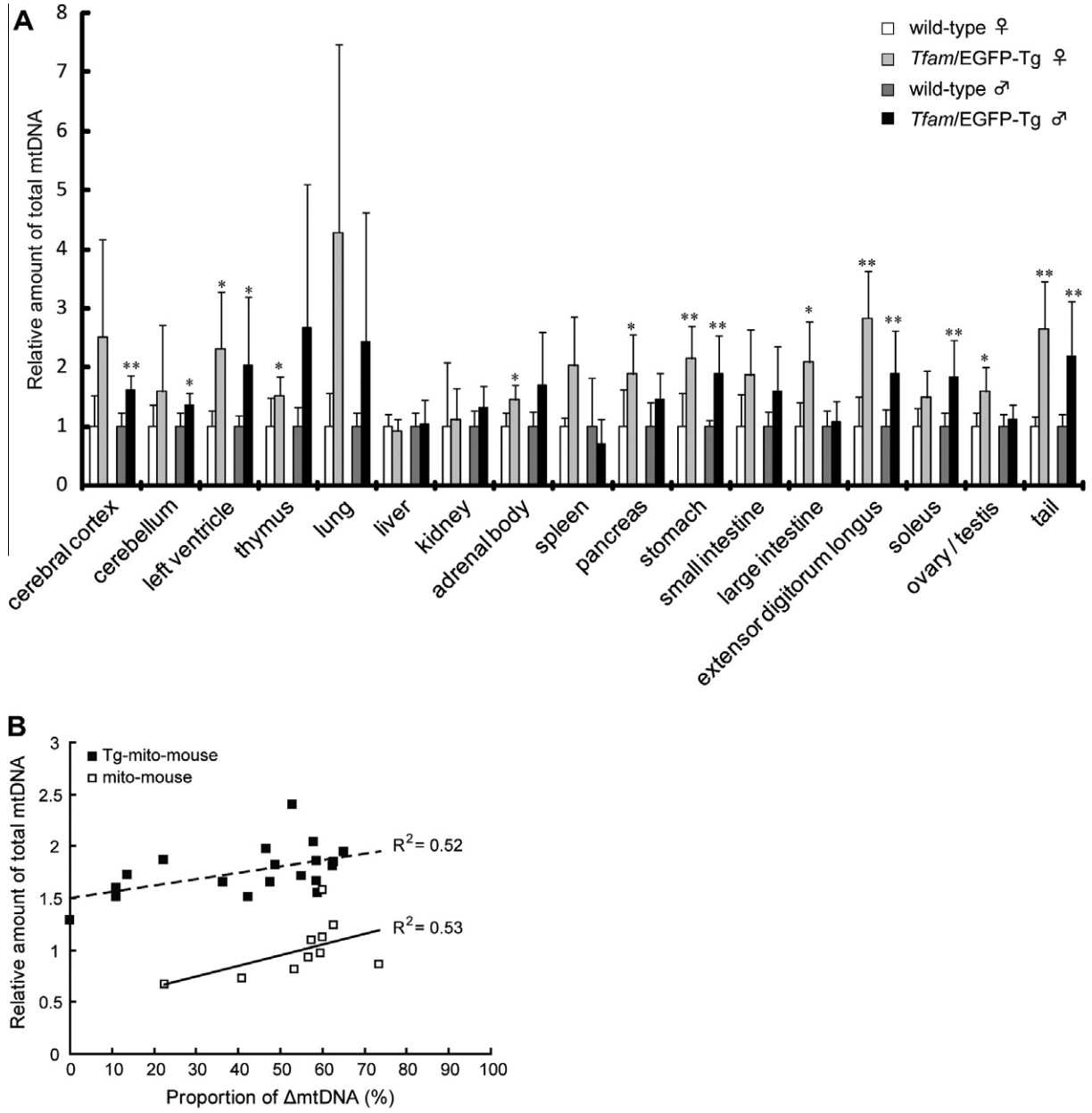

Fig. 1. The effect of Tfam/EGFP over-expression on the amount of mtDNA. (A) The amount of mtDNA in tissues of wild-type and *Tfam*/EGFP-Tg mice. The vertical scales indicate the relative amounts of mtDNA compared with the mean of the wild-type samples in each sex. The white bar, lighter dark bar, dark bar and black bar show wild-type females ($n = 10$), Tg females ($n = 5$), wild-type males ($n = 7$) and Tg males ($n = 8$), respectively. $^{*}P < 0.05$ and $^{**}P < 0.01$. (B) The amounts of total mtDNA in the tail of mito-mice and Tg-mito-mice were measured by the comparative real-time PCR method. On average, the Tg-mito-mice contained 1.74 times as many mtDNA copies as the mito-mice in the four-week-old tail. Both mouse strains had positive correlations between the proportion of ΔmtDNA and mtDNA copy number. The solid and dashed lines indicate the regression lines for the mito-mice and Tg-mito-mice, respectively.

3.2. Analysis of mitochondrial disease symptoms in Tfam/EGFP over-expressing mito-mice

To investigate whether over-expression of *Tfam* improves the mitochondrial disease phenotypes, *Tfam*/EGFP over-expressing mito-mice (Tg-mito-mice) were generated by *in vitro* fertilization. Then the amounts of mtDNA in tail were estimated and we found that the Tg-mito-mice possessed 1.74 times as many as the average copy number of mtDNAs in their littermate mito-mice (Fig. 1B).

Among several littermates of Tg-mito-mice and mito-mice, the mice with more than 50% ΔmtDNA were examined for their survival. Compared with mito-mice, the life spans of the Tg-mito-mice were significantly extended ($P = 0.01$ in Mantel–Cox test), and the median survival periods were 54% prolonged, from 247 days in the mito-mice to 381 days in the Tg-mito-mice (Fig. 2A).

Because most mito-mice show a high concentration of blood urea nitrogen (BUN) (reflecting renal dysfunction) and ultimately die due to renal failure [7], we measured the level of BUN in the mito-mice and Tg-mito-mice at eight months of age (Fig. 2B). An increased concentration of BUN was observed only in the mito-mice with more than 65% ΔmtDNA in the tail, which was consistent with previous reports [7,23]. In the Tg-mito-mice group, the increase in BUN concentration was remarkably suppressed. However, there was one exception. A mito-mouse with 75.5% ΔmtDNA had a normal BUN concentration, and it had a 1.58 times as much mtDNA copy number as the average of the control mito-mice at

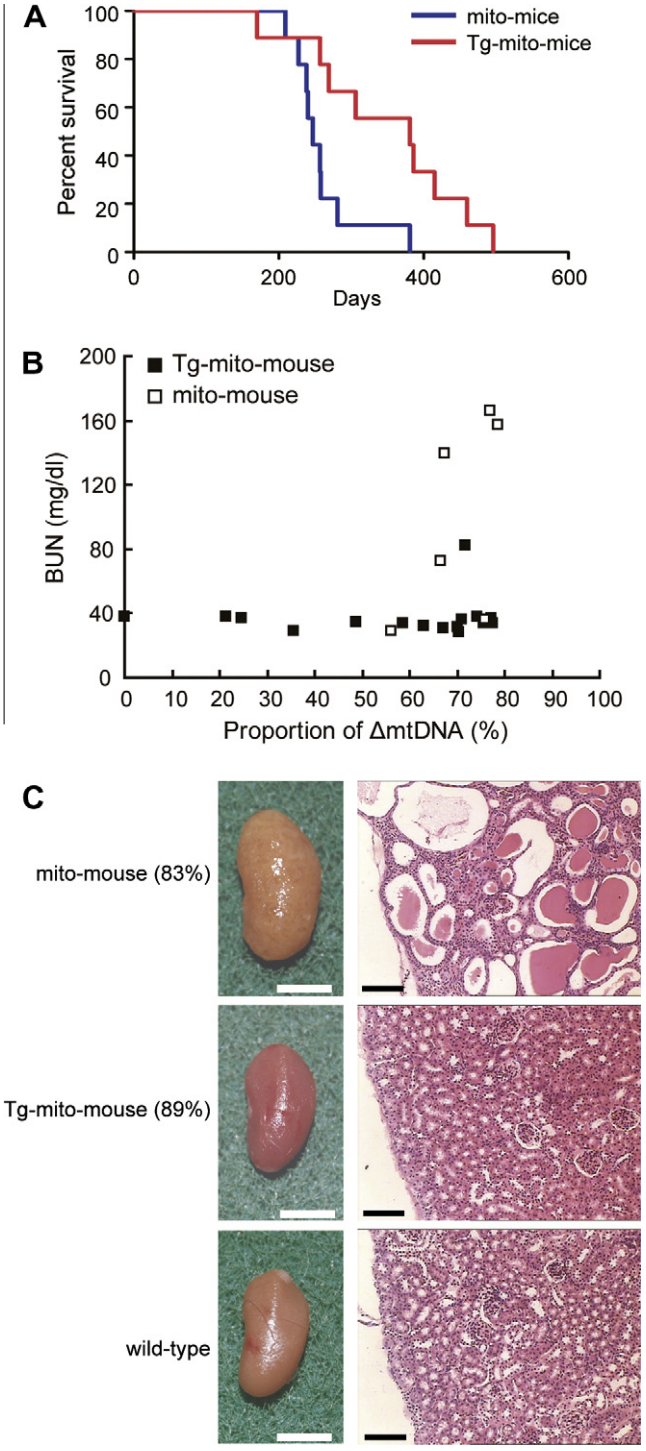

Fig. 2. The effects of *Tfam*/EGFP over-expression on the disease phenotypes of mito-mice. (A) Kaplan–Meier survival curves of mito-mice ($n = 9$; blue line) and Tg-mito-mice ($n = 9$; red line) with more than 50% ΔmtDNA. (B) An association between the proportion of ΔmtDNA and blood urea nitrogen (BUN) in eight-month-old mice. The black squares indicate Tg-mito-mice and the white squares indicate mito-mice. (C, left panels) The morphology of the kidneys from a mito-mouse, a Tg-mito-mouse and a wild-type mouse at eight months of age. The Tg-mito-mouse contained 2.99 times as many mtDNA copies in the kidney as the mito-mouse. Bar, 5 mm. (C, right panels) The kidney sections were stained with hematoxylin and eosin to observe the histopathology of the renal cortex. Bar, 0.1 mm.

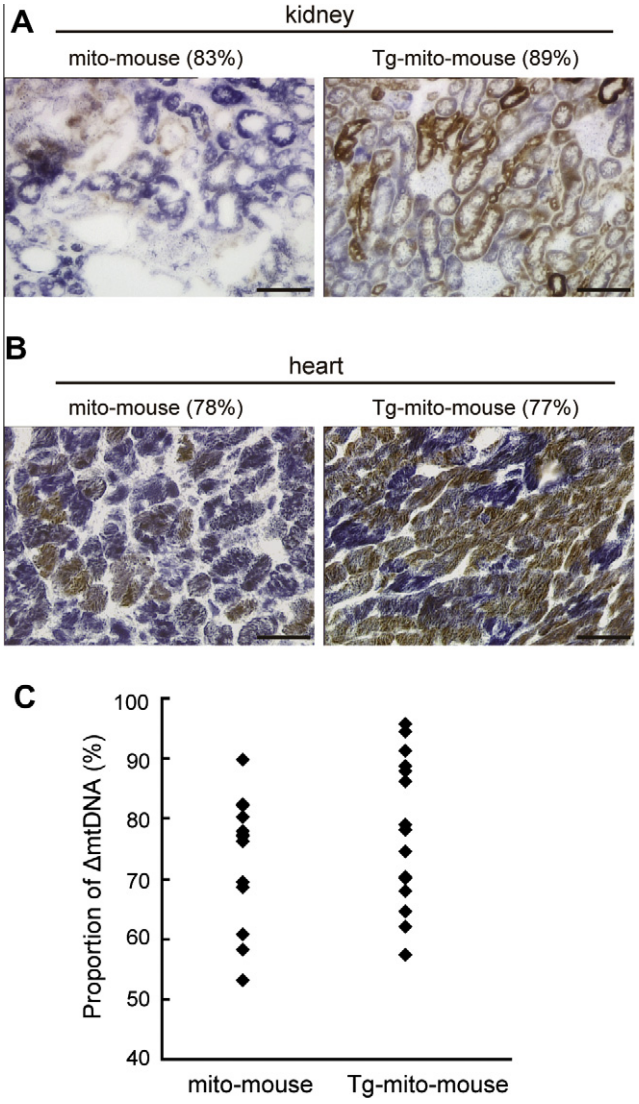

Fig. 3. The tissue distribution of COX-positive and COX-negative cells in mito-mice and Tg-mito-mice. (A, B) The tissue sections were sequentially stained for the activity of the COX (brown) and SDH (blue) of the electron transport chain. The brown staining depicts cells with intact COX activity. The blue-stained cells have lost their COX activity but have retained SDH activity, which is only encoded by nuclear genes. The mito-mouse and the Tg-mito-mouse displayed 83% and 89% ΔmtDNA in the kidney, respectively (A), and 78% and 77% in the heart, respectively (B). The Tg-mito-mouse displayed 2.99 and 2.27 times as many mtDNA copies as the mito-mouse in the kidney and heart, respectively. Bar, 100 μm in kidney and 50 μm in heart. (C) The proportion of ΔmtDNA in a COX-positive single fiber of heart muscle.

four weeks of age. These results indicate that the increased mtDNA copy number resulted in the recovery of kidney function. The kidneys of mito-mice with more than 80% ΔmtDNA showed ischemia and increased size with a granulated surface [7]. In contrast, the Tg-mito-mouse kidney with 89% ΔmtDNA, which contained 2.99 times as many mtDNA copies compared to the mito-mouse control kidney, showed a mild enlargement with a normal surface (Fig. 2C; left panel). A histological analysis showed dilatation of the cortical proximal and distal tubules in the renal tissues of the mito-mouse, which was recovered in the Tg-mito-mouse (Fig. 2C; right panels).

3.3. Histochemical analyses of cytochrome c oxidase

Histochemical staining was used to evaluate the COX activity in the kidney (Fig. 3A) and heart (Fig. 3B). The kidneys of mito-mice with 83% ΔmtDNA showed dilation in the cortical proximal and

distal tubules and a reduction of COX activity with a compensatory up-regulation of SDH activity [25]. The kidneys of the Tg-mito-mice with 89% ΔmtDNA, however, had recovered both the COX activity and the histological characteristics of these tissues.

To confirm the effect of TFAM over-expression on the recovery of the mitochondrial activity in a single cell, cardiac muscle samples from the mito-mice and Tg-mito-mice (78% and 77%, respectively, ΔmtDNA in the heart tissue block) were subjected to histochemical and quantitative single-fiber PCR analyses. Serial sections were used to compare the COX activity and proportion of ΔmtDNA in a single muscle fiber. Consistent with a previous study [7], we found that there were fibers with variable proportions of ΔmtDNA in the mito-mouse heart and that COX-positive fibers exhibited less than 85% ΔmtDNA. In contrast, there were COX-positive fibers with more than 85% ΔmtDNA in the Tg-mito-mouse heart (Fig. 3C). Although it is difficult to estimate the amount of mtDNA in single fibers because there is no effective internal control, the elevation of the threshold percentage of the ΔmtDNA for maintaining COX activity was likely caused by the increase of mtDNA (2.27-fold higher than the mito-mouse control) due to the over-expression of TFAM.

3.4. The expression of mitochondrial tRNA was increased in Tg-mito-mice

In cells with ΔmtDNA, the amounts of mtDNA-coded proteins concurrently decreased regardless if the gene position was in the deleted region. The decrease in mtDNA-coded proteins should be due to the shortage of tRNAs[9], and compensation for this shortage was thought to be responsible for prolongation of life span (Fig. 2A), improved kidney function (Fig. 2B, C) and COX activity (Fig. 3A, B) in Tg-mito-mice. Therefore, we examined the amount of $tRNA^{Ser(AGY)}$ that coded in the deleted region.

Compared with mito-mouse tissues, the amounts of $tRNA^{Ser(AGY)}$ were increased as follows: 1.56-fold in the heart of the Tg-mito-mouse (78% ΔmtDNA), which contained 2.27 times as many mtDNA copies as that of the mito-mouse (77%); 1.52-fold in the kidney of the Tg-mito-mouse (89%) which contained 2.99 times as many mtDNAs as that of the mito-mouse (83%); 2.42-fold in the soleus of the Tg-mito-mouse (70%) which contained 2.01 times as many mtDNAs as that of the mito-mouse (89%) (Fig. 4). The comparable result was observed using an additional set of mice (Fig. S2). These results showed that the amount of tRNA was moderately restored in the Tg-mito-mouse, although it did not completely recover.

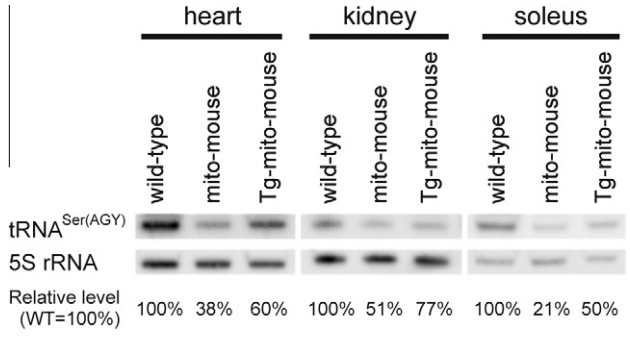

Fig. 4. The amount of mitochondrial $tRNA^{Ser(AGY)}$ in heart, kidney and soleus measured by Northern blot analysis. Relative amounts of $tRNA^{Ser(AGY)}$ compared with wild-type tissues are indicated. The proportions of ΔmtDNA in each mouse were as follows: heart, 78% in Tg-mito-mouse and 77% in mito-mouse; kidney, 89% in Tg-mito-mouse and 82% in mito-mouse; and soleus, 70% in Tg-mito-mouse and 89% in mito-mouse.

4. Discussion

In this study we showed that *Tfam* over-expression improved the symptoms caused by mitochondrial deficiency with large deleted mtDNA. To elucidate the mechanism of this improvement, we created a new model for the pathogenicity of mutant mtDNA. The accumulation of ΔmtDNA causes the simultaneous decrease of wild-type mtDNA, and a reduction in the tRNA supply. Consequently, the total protein levels of the respiratory complexes decrease as previously reported using cybrids with various proportions of ΔmtDNA [7]. A certain level of respiratory complexes is required to maintain normal activity, so a loss of mitochondrial function occurs when the wild-type mtDNA decreases below the threshold. When the copy number of the wild-type mtDNA increases under the sufficient condition of gene expression, the tRNA supply and protein synthesis should increase, resulting in the improvement of mitochondrial function.

It has been recognized commonly that the proportion of pathogenic mutant mtDNA is responsible for the occurrence of mitochondrial dysfunction. However, our model proposes that the amount of wild-type mtDNA is important to maintain the mitochondrial function irrespective of the proportion of mutant mtDNA. There was a strong correlation between the amount of wild-type mtDNA and the life spans of both mito-mice and Tg-mito-mice (Fig. S3), indicating that the amount of wild-type mtDNA directly affects the life span of these mice.

However, there is a limitation for this therapeutic treatment. In mito-mice, ΔmtDNA increases age-dependently [20], and mice born with a lower proportion of ΔmtDNA may develop the mitochondrial diseases within their life span. In Tg-mito-mice, although the threshold percentage was elevated (Fig. 3C) by the increase in mtDNA levels, the mitochondrial dysfunction occurred when the proportion of ΔmtDNA exceeded the threshold level. Thus, the mice could not be completely recovered even though the occurrence of the disease phenotype was extensively delayed and the life span was elongated (Fig. 2A).

Considering the model further, the mtDNA up-regulating factor does not need to be applied with a transgenic technique in the zygote. Viral vectors, gene guns or other undiscovered medicines might instead be used to increase the copy number of mtDNA. Furthermore, the method of increasing mtDNA copy number would be used to improve mitochondrial dysfunction in adults. There are several reports in which the copy number of mtDNA has been increased using a non-transgenic approach. For instance, rats fed a high-fat diet and injected with heparin had increased levels of plasma free fatty acids and increased respiratory capacity, which is accompanied by an increase in the mtDNA copy number in skeletal muscle [26]. Raising the cytosolic Ca^{2+} with caffeine results in the increased expression of the peroxisome proliferative-activated receptor γ coactivator-1α (PGC-1α) gene [27], which increases mitochondrial biogenesis by activating the expression of *Tfam* and the subunits of the respiratory chain. It has also been reported that several cytokines, such as IL-1β and combinations of TNFα and IL-1α, stimulate PGC-1α activity through the activation of p38 MAPK, which is accompanied by increased mitochondrial gene expression and cellular respiration [28]. Pursuing the mechanisms underlying these phenomena may provide clues for the discovery of potent medicines for the treatment of mitochondrial diseases.

This is the first study to find that increase in the amount of total mtDNA rescues the impairment caused by pathogenic mtDNA, which is the cause of mitochondrial diseases. The therapeutic treatment in this study may be applied to human mitochondrial diseases, such as CPEO and KSS, especially in cases where the causal mtDNA mutation is the common deletion in which the deleted

region extends from the *mt-Atp8* to the *mt-Nd5* gene [29], which is the same as the ΔmtDNA in mito-mice.

5. Conflict of interest

None declared.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.08.143.

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