

FAST TRACKS

Maintenance of Mitochondrial DNA Copy Number and Expression Are Essential for Preservation of Mitochondrial Function and Cell Growth

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Abstract To examine whether a reduction in the mtDNA level will compromise mitochondrial biogenesis and mitochondrial function, we created a cell model with depleted mtDNA. Stable transfection of small interfering (si)RNA of mitochondrial transcription factor A (*Tfam*) was used to interfere with *Tfam* gene expression. Selected stable clones showed 60–95% reduction in *Tfam* gene expression and 50–90% reduction in cytochrome *b* (Cyt *b*) gene expression. *Tfam* gene knockdown clones also showed decreased mtDNA-encoded cytochrome *c* oxidase subunit I (COX I) protein expression. However, no significant differences in protein expression were observed in nuclear DNA (nDNA)-encoded mitochondrial respiratory enzyme subunits. The cell morphology changed from a rhombus-like to a spindle-like form as determined in clones with decreased expressions of *Tfam*, mtRNA, and mitochondrial proteins. The mitochondrial respiratory enzyme activities and ATP production in such clones were significantly lower. The proportions of mtDNA mutations including 8-hydroxy-2'-deoxyguanosine (8-OHdG), a 4,977-bp deletion, and a 3,243-point mutation were also examined in these clones. No obvious increase in mtDNA mutations was observed in mitochondrial dysfunctional cell clones. The mitochondrial respiratory activity and ATP production ability recovered in cells with increased mtDNA levels after removal of the specific siRNA treatment. These experimental results provide direct evidence to substantiate that downregulation of mtDNA copy number and expression may compromise mitochondrial function and subsequent cell growth and morphology. *J. Cell. Biochem.* 103: 347–357, 2008. © 2007 Wiley-Liss, Inc.

Key words: mitochondrial DNA; mitochondrial transcription factor; small interfering RNA

Mitochondria-related proteins are synthesized from two cellular locations. A minor proportion of mitochondrial proteins is encoded by mtDNA and is transcribed/translated by their own machinery. The other predominant fractions of mitochondrial proteins are encoded

by nuclear DNA (nDNA) and are translated in the cytoplasm. Human mtDNA is a circular, histone-free molecule composed of 16.6 kb of DNA, present in one or more copies in every mitochondria. It encodes 13 out of a total of about 80 protein subunits constituting the oxidative phosphorylation system, the remainder being encoded by nuclear genes and imported into mitochondria. Human mtDNA also encodes two ribosome RNAs and 22 transfer RNAs. The oxidative phosphorylation capacity of mitochondria is determined by the interplay between nuclear and mitochondrial genes. While mtDNA encodes 13 polypeptides that are components of the respiratory system, nDNA encodes the majority of respiratory chain proteins and all of the proteins and enzymes that regulate replication and transcription of mtDNA [Taanman, 1999].

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In mammals, an essential component of the mitochondrial transcription initiation complex is Tfam, a nuclear-encoded 25-kDa protein which bends and unwinds mtDNA upon binding and which belongs to the high-mobility-group (HMG)-box family of proteins [Chang and Clayton, 1984; Fisher and Clayton, 1988]. Tfam regulates both mitochondrial transcription and replication by permitting commencement of transcription of the RNA primer from the unwinding L-strand DNA [Montoya et al., 1997]. The *Tfam* gene has been isolated in humans and is composed of seven exons and six introns [Larsson et al., 1997].

Decreased mitochondrial content and reduced mRNA expression of mtDNA resulting in mitochondrial dysfunction and altered cellular characteristics have been reported in various kinds of cells. A marked decrease in cellular mtDNA and its transcribed mRNAs in ethidium bromide (EtBr)-treated MIN6 cells showed impaired insulin secretion induced by glucose [Tsuruzoe et al., 1998]. A decreased mtDNA copy number in renal cancer [Selvanayagam and Rajaraman, 1996] and hepatocellular carcinoma [Lee et al., 2004] has also been observed. Additionally, Lewis et al. [2007] showed that mutant polymerase gamma caused mtDNA depletion and subsequent mitochondrial oxidative stress and cardiomyopathy.

Creation of mtDNA-depleted cells for fusion with cells harboring disease-related mitochondria provides a powerful tool for investigating the pathogenesis of dysfunctional mitochondria [King and Attardi, 1989; Chomyn et al., 1994; Sligh et al., 2000]. Disruption of mitochondrial biogenesis through chemical or genetic methods, including EtBr treatment, negative dominant polymerase γ expression, and knockout of the *Tfam* gene, has also contributed to the study of mitochondria's roles in multiple degenerative diseases [Biswas et al., 1999; Silva et al., 2000; Jazayeri et al., 2003]. Powerful gene silencing by siRNAs was developed in *Caenorhabditis elegans* and *Drosophila* [Fire et al., 1998; Kennerdell and Carthew, 1998], with double-stranded (ds) RNA initiating a potent sequence-specific degradation of cytoplasmic mRNAs containing the same sequence as the dsRNA trigger. siRNAs found in nature are derived from the cytoplasmic processing of long dsRNAs by an RNase-III-type enzyme termed Dicer [Bernstein et al., 2001]. An siRNA expression vector for mammalian cells has been developed, and siRNA

is produced through the transcription of hairpin RNA that structurally mimics a microRNA precursor, allowing it to be processed into siRNA inside the cell, thus silencing the target gene [Brummelkamp et al., 2002; Lee et al., 2002]. In this study, stably expressed siRNA for Tfam was constructed, and knockdown of *Tfam* gene expression and the consequent inhibition of mitochondrial transcription and translation were carried out.

MATERIALS AND METHODS

Cell Culture and Drug Treatment

Human 143B TK⁻ cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 50 μ g/ml uridine supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μ g/ml streptomycin. Cells were grown at 37°C in a humidified atmosphere with 5% CO₂ for 3 days before the experimental treatment began. 143B cells were transfected with a plasmid by the calcium-phosphate precipitation method [Chen and Okayama, 1988], and several transfected cell lines were isolated by screening in DMEM with 10% FBS and 0.8 mg/ml G418 (gentamycin). EtBr-treated cells were cultured in DMEM containing 100 ng/ml EtBr for 3 months.

siRNA Design

siRNA was designed to interfere with *Tfam* expression, using technical information from Ambion (Austin, TX). Two sets of 19-mer oligonucleotides, called TFA1 and TFA2, were selected from the human *Tfam* open reading frame (ORF) sequence. The sequence of TFA1 originating from the 21 bases before the start codon (ATG) consisted of ATAACACACGCCG-GAGGGT. The sequence of TFA2 from the 26 bases after the start codon consisted of AT-CTGTCTTGGCAAGTTGT. The 19-mer sense and antisense siRNA sequences were linked by a nine-nucleotide spacer (TTCAAGAGA) as a loop. Six T bases and six A bases were added as termination signals to the 3' end of the forward oligonucleotides and the 5' end of the reverse oligonucleotides, respectively. Then nucleotides corresponding to the *Bam*HI and *Hind*III restriction sites were respectively added to the 5' end of the forward and reverse oligonucleotides. Forward and reverse oligonucleotides were incubated in DNA annealing buffer (100 mM K-acetate, 30 mM HEPES-KOH, and

2 mM Mg-acetate); the mixture was heated to 90°C for 3 min, cooled to 37°C, and incubated for 1 h. The annealed DNA for siRNA was ligated with the linearized pSilencer-neo siRNA expression vector (Ambion).

RNA Extraction and Semiquantitative Reverse-Transcription Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was extracted from cells with Trizol reagent according to the manufacturer's instructions. Total RNA (1–5 µg) was reverse-transcribed into cDNA using oligo (dT) 18 as a primer and into MMLV reverse transcriptase (Ambion), and 1 µl of the cDNA template was separately used to amplify different mRNAs. RT-PCR amplifications were performed with 1 µl of cDNA in a total volume of 25 µl of amplification buffer, 10 pmol of specific primers, and 2.5 units of Taq DNA polymerase (Life Technologies, Grand Island, NY). The sequences of the oligonucleotide primers used in this study were: Tfam (forward, CCGGAGGGTCG-CACGCGGGT and reverse, CAGATGAAAAC-CACCTCAAT), NADH dehydrogenase subunit I (NDI) (forward, GACCTTAGCTCTCACCA-TCG and reverse, GGCAGGAGTAATCAGAG-GTG), cytochrome b (Cyt b) (forward, GACAAT-TATACCCTAGCCAA and reverse, GTCCAA TGATGGTAAAAGGG), cytochrome c oxidase subunit I (COX I) (forward, GTCCATCAATA-GGAGCTGT and reverse, TTCGAAGCGAAG-GCTTCTC), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward, ACCACA-GTCCATGCCATCA and reverse, TCCACCAC-CCTGTTGCTGTA). The predicted sizes of the Tfam, ND1, Cyt b, COX I, and GAPDH RT-PCR products were 235, 305, 294, 299, and 453 bp, respectively. For semiquantitative amplification, each cycle was carried out at 92°C for 30 s, 58°C for 30 s, and 72°C for 60 s. The reactions were analyzed after 15, 20, 25, 30, 35, and 40 cycles, respectively, to optimize the linear range of amplification. The PCR reactions were optimized with respect to the annealing temperature and number of PCR cycles [Hsieh et al., 2004]. Each PCR product was run through a 2% agarose gel and was visualized with EtBr staining. Cycle-dependent amplification of the housekeeping GAPDH mRNA was almost identical in different clones, which allowed semiquantitative comparison of the mtDNA PCR products obtained with each sample by densitometric analysis. The relative expression

levels of the PCR products were determined using an imaging densitometer, and results are expressed as a ratio of analyzed genes divided by GAPDH.

Determination of the mtDNA Copy Number, the 4,977-bp Deletion, and 3,243-Point Mutation

The levels of mtDNA were determined by amplification of the ND1 gene (5'-GGAGTAA-TCCAGGTTCGGT-3' and 5'-TGGGTACAATG-AGGAGTAGG-3') and GAPDH gene (which served as the internal standard) (5'-ATCAA-GAAGGTGGTGAAGC-3' and 5'-CTGTAGCCA-AATTCGTTGTC-3'). The 4,977-bp deletion was determined by amplified primers (3'-GCCCC-AACTAAATACTACCG-3' and 5'-GGCTCAGG-CGTTTGTGTATG-3'). The PCR amplification profile was as follows: 1 cycle of 94°C for 10 min; 35 cycles of 94°C for 1 s, 62°C for 5 s, and 72°C for 10 s; and 1 cycle of 65°C for 15 s, then it was stored at 4°C. The LightCycler PCR machine (Roche Diagnostics, Mannheim, Germany) was used to perform the quantitative PCR. The DNA content of the ND1 gene was normalized with that of the GAPDH gene to calculate the copy number of mtDNA. The proportions of the 4,977-bp deletion were determined by normalized 4,977 DNA content to quantify the mtDNA. A 286-bp mtDNA fragment, encompassing the putative mutation site at bp 3,243, was amplified by PCR using a pair of primers (5'-TAC-GTGATCTGAGTTCAGAC-3' and 5'-TGGGTACAATGAGGAGTAGG-3'). The target mtDNA fragment was generally amplified in a 100 µl reaction mixture containing 2–5 ng DNA, 200 µM of each dNTP, 20 pmol of each primer, 1.5 units of Taq DNA polymerase, 50 mM KCl, 1.5 mM MgCl₂, and 10 mM Tris-HCl. The A3243G mutation in the mtDNA creates an additional *Apa*I restriction site in this region. The PCR fragments were digested with *Apa*I and electrophoretically separated in a 1.5% agarose gel. The proportion of the 3,243-point mutation was determined by the ratio of the band intensities of the DNA fragments amplified from mutant and wild-type mtDNAs [Hsieh et al., 2001].

Enzyme Digestion of DNA and ELISA for 8-OHdG

The presence of 8-OHdG was detected as reported previously [Hsieh et al., 2005]. Aliquots of DNA equivalent to 100 µg were freeze-dried and reconstituted in 20 mM sodium acetate (pH 4.8) containing 45 mM zinc chloride.

Samples were heated in a boiling water bath for 3 min and quickly cooled on ice prior to the addition of nuclease P1 to give 0.1 U/ μ g DNA, and the samples were incubated at 37°C for 1 h. Samples were made alkaline by the addition of 1.5 M Tris-HCl (pH 8.0), and alkaline phosphatase was added to yield 0.05–0.075 U/ μ g DNA, and then the mixture was incubated at 37°C for 30 min.

Enzymatic digests of DNA were analyzed by competitive ELISA using a monoclonal antibody to 8-OHdG (8-OHdG Check, Genox Corp, Baltimore, MD). Fifty microliters of DNA digest or standard 8-OHdG solution was added to the wells of a 96-well plate pre-coated with 8-OHdG, then 50 μ l of the primary antibody (mouse anti-8-OHdG) was added, and the plate was incubated at 37°C for 1 h. After incubation, the wells were washed with 200 μ l/well of 0.05% (v/v) Tween 20 in 0.01 M phosphate-buffered saline (PBS; pH 7.4); 100 μ l/well of the secondary antibody was added (peroxidase conjugated antimouse IgG), and the plate was incubated for 1 h at 37°C. Following incubation, the peroxidase substrate (*o*-phenylenediamine/hydrogen peroxide/PBS) was added, following 30 min later by 2 N sulfuric acid, and the absorbance was read at 450 nm.

Western Blot Analysis

Total protein was extracted from harvested cells using lysis buffer [25 mM Tris-phosphate (pH 7.8), 2 mM DTT, 10% glycerol, and 1% Triton X-100] with a protease inhibitor. The cell lysate was cleared of cell debris by low-speed centrifugation at 10,000*g* for 5 min. The protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, IL). Proteins were resolved on polyacrylamide gels, and then proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia, Piscataway, NJ). Membranes were blocked for 1 h at 4°C with 10% skim milk in TBST buffer (1 M Tris-HCl, 100 mM NaCl, and 1% Tween-20). Blots were probed with the following primary antibodies: mAbs against COX I, COX II, succinate-ubiquinone oxidoreductase (SDHA), and ubiquinone oxidoreductase α subcomplex 9 (NDUFA) (Molecular Probes, Eugene, OR), or a polyclonal antibody against GAPDH. Blots were then incubated with the appropriate horseradish peroxidase-conjugated anti-IgG antibody. Antibody-bound

protein was detected using enhanced chemiluminescence and exposure to film.

Adenosine Triphosphate (ATP) Determination

Cells were collected and lysed in 200 μ l lysis buffer [25 mM Tris-phosphate (pH 7.8), 20 mM DTT, 2 mM 1,2 diaminocyclohexane-*N,N,N,N*-tetraacetic acid, 10% glycerol, and 1% triton X-100]. ATP was measured using an ATP assay kit (Molecular Probes); 100 μ l of each sample or ATP standard was mixed with an equal amount of ATP assay mix, containing luciferase, luciferin, dithiothreitol, reaction buffer including tricine buffer (pH 7.8), MgSO₄, ethylenediaminetetraacetic acid (EDTA), and sodium azide. The light emitted at 560 nm was measured in a luminometer and was proportional to the ATP present.

Measurement of Respiratory Enzyme Activities

Mitochondria were isolated from cells, and submitochondrial particles were used to assay various enzyme activities of the respiratory chain. Activities of respiratory enzyme complexes I, III, and IV were respectively assayed according to a published method [Hsieh et al., 2001]. All respiratory enzyme activities were assayed on a Hitachi spectrophotometer using a 100 μ l sample cuvette thermostatically maintained at 30°C.

RESULTS

Knockdown of *Tfam* Expression by siRNA in 143B Cells

To downregulate mitochondrial transcription and replication, siRNA specifically designed for the *Tfam* gene was constructed. *Tfam* gene expressions from different stable clones knocked down by siRNA were determined by semiquantitative RT-PCR. There were significant reductions in the expressions in TFA 102, 103, 108, and 207 clones (Fig. 1a). Our previous studies showed a compensatory effect of increased gene expression after transient siRNA interruption. To prevent unstable *Tfam* gene expression, a further 3 weeks of culture was carried out to confirm the knockdown effect. Clones 102, 103, and 108 respectively presented 65%, 95%, and 95% reductions and maintained their knockdown effect on the *Tfam* gene. Clone 106 had a stronger diminished effect at 60% reduction, while clones 201 and 207 showed the

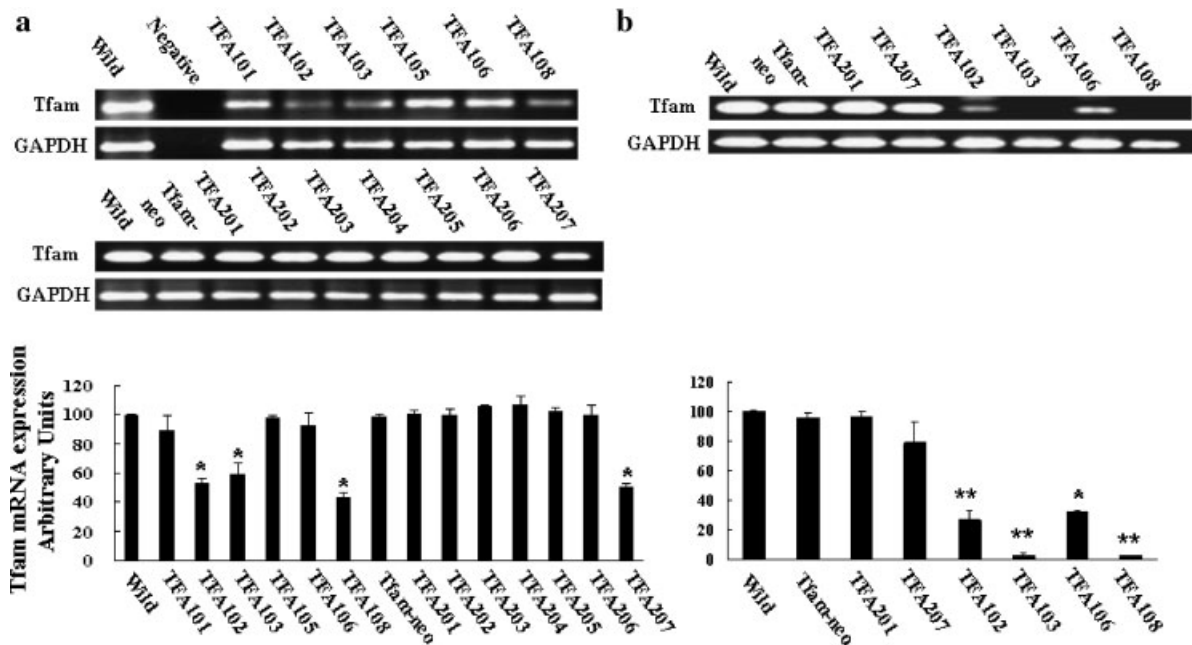


Fig. 1. RT-PCR analysis of *Tfam* gene expression in cells transfected with *Tfam*-siRNA. **a:** Two constructs with siRNA to inhibit the *Tfam* genes, TFA1 and TFA2, were transfected into 143B cells. Six TFA1- and seven TFA2-transfected G-418-resistant cells were selected. **b:** Stable knockdown of *Tfam* expression clones as shown in (a) selected for a further 3 weeks of culture. RNA was extracted and reverse-transcribed into cDNA,

and then PCR amplification was carried out with primers for human *Tfam* cDNA. Wild-type, 143B cells without treatment; *Tfam*-neo, 143B cells stably transfected with an empty vector control. Data are expressed as the mean \pm SD, and the ratio of analyzed genes divided by GAPDH of three separate experiments is shown (* $P < 0.05$; ** $P < 0.01$).

same levels of *Tfam* expression as before (Fig. 1b).

Decreased Levels of the mtDNA Expression and Mitochondrial Respiratory Enzyme Subunit in Clones With Downregulated *Tfam* Expression

In order to estimate the effect of decreased *Tfam* gene expression on mtDNA gene expression, the RNA expression levels of mitochondrial NADH dehydrogenase subunit 1 (ND1), Cyt b, and COX I genes from each stable clone were determined. Different levels of gene expressions were determined in various clones. Clones 102, 103, 106, and 108 presented significantly reduced expressions of the analyzed genes (Fig. 2a). In particular, expressions of both the Cyt b and COX I genes by clone 108 mRNA were nearly completely abolished after the *Tfam* gene was disrupted. Clones 201 and 207 showed moderate decreases in mitochondrial RNA expression (Fig. 2a). Mitochondrial oxidative phosphorylation enzyme complexes are composed of mtDNA- and nDNA-encoded subunits. To determine whether interference with *Tfam* gene expression resulted in abnormal mtDNA-encoded and/or nDNA-

encoded protein expressions, mtDNA-encoded COX I and COX II and nDNA-encoded SDHA and NDUFA levels in various clones were determined by Western blotting. Clones 102, 103, and 108 also presented decreased protein expressions of both mtDNA-encoded COX I and COX II. However, there were no significant differences in nDNA encoded SDHA or NDUFA (Fig. 2b).

Cell Characteristics in Various Clones Harboring Decreased mtDNA and mtRNA Levels

The cell characteristics and mitochondrial functions of five clones (TFA 207, 102, 103, 106, and 108) and one EtBr-treated clone were determined. The growth rate, cell morphology, and mtDNA mutations were determined in clones harboring a decreased mtDNA copy number and mtRNA expression (Table I). From 100% to <1% of the mtDNA copy numbers in various clones were determined by real-time PCR analysis. Clones with a mtDNA copy number of <21% showed significantly slower growth rates compared with wild-type cells. The cell morphology was observed to have changed from a rhombus-like to a spindle-like shape in

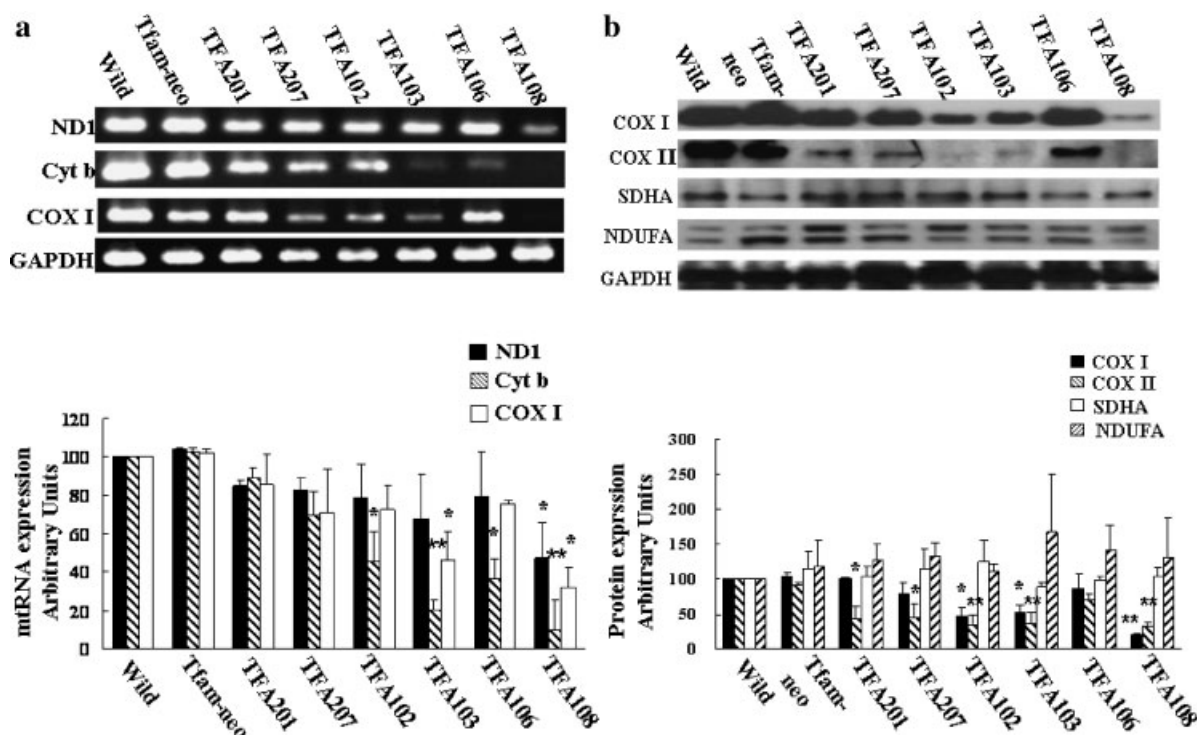


Fig. 2. a: Decreased mtDNA expression in cells with effects of siRNA knockdown of Tfam expression. RNA was extracted and reverse-transcribed into cDNA, and then PCR amplification was carried out with primers for the mitochondrial NADH dehydrogenase subunit 1 (ND1), cytochrome b (Cyt b), and cytochrome c oxidase subunit I (COX I) genes. b: Western blot analysis of nuclear- and mtDNA-encoded proteins in siRNA stably transduced cells. mtDNA-encoded COX I, COX II, nDNA-encoded

succinate-ubiquinone oxidoreductase (SDHA), and the ubiquinone oxidoreductase α subcomplex 9 (NDUFA) were determined. Wild-type, 143B cells without treatment; Tfam-neo, 143B cells stably transduced with an empty vector control. Data are expressed as the mean \pm SD, and the ratio of analyzed genes divided by GAPDH of three separate experiments is shown (* $P < 0.05$; ** $P < 0.01$).

clones harboring significantly decreased mtDNA levels (Fig. 3 and Table I).

To determine whether interruption of mitochondrial functions resulted in elevated mtDNA

mutations, 8-OHdG in mtDNA and nDNA, the 4,977-bp mtDNA deletion, and the 3,243-point mutation were examined. Contents of 8-OHdG in nDNA significantly differed in the two clones

TABLE I. Cell Characteristics and Mutated DNA of the Studied Clones

Clone ^a	mtDNA ^{b,c} (%)	Growth ^d rate	Morphology ^e	8-OHdG (1/10 ⁵ dG) ^c			
				mtDNA	nDNA	4977 ^c (%)	3243 ^c (%)
Wild-type	100	+++	R	2.13 \pm 0.73	0.17 \pm 0.04	0.54 \pm 0.47	1.94 \pm 0.45
TFA201	82 \pm 5	+++	R	3.43 \pm 1.18	0.41 \pm 0.54	0.87 \pm 0.36	2.36 \pm 0.71
TFA207	66 \pm 12	+++	R	2.84 \pm 0.69	0.29 \pm 0.37	1.33 \pm 0.62	1.66 \pm 0.56
TFA103	21 \pm 6*	++	S	1.67 \pm 0.48	0.69 \pm 0.25*	0.63 \pm 0.51	1.74 \pm 0.47
Re-103	113 \pm 19	+++	R	2.89 \pm 0.78	0.82 \pm 0.49	2.14 \pm 0.91	3.43 \pm 1.23
TFA102	9 \pm 5*	+	S	ND	ND	ND	ND
Re-102	121 \pm 23	+++	R	2.19 \pm 0.71	1.07 \pm 0.55	2.56 \pm 0.89*	2.87 \pm 0.93
TFA108	<1*	+	S	ND	ND	ND	ND
EtBr	24 \pm 8*	+	S	2.64 \pm 1.33	0.88 \pm 0.42*	0.58 \pm 0.49	0.84 \pm 0.21
Re-EtBr	67 \pm 11*	++	S	3.81 \pm 1.59	1.71 \pm 1.08*	1.77 \pm 0.76	1.64 \pm 0.93

^aRe-, cells were examined after removal of interrupting factors; EtBr, cells after ethidium bromide treatment for 3 months.

^bThe mtDNA copy number was determined by real-time PCR analysis.

^c8-OHdG, 8-hydroxy-2'-deoxyguanosine; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; 4977, 4,977-bp mtDNA deletion; 3243, 3,243-point mutation.

^d+++ The growth rate the same as the wild-type; ++, <60% of the wild-type; +, <30% of the wild-type.

^eR, rhombus-like; S, spindle-like.

*Data are expressed as the mean \pm SD of three independent experiments. Statistically significant difference ($P < 0.05$).

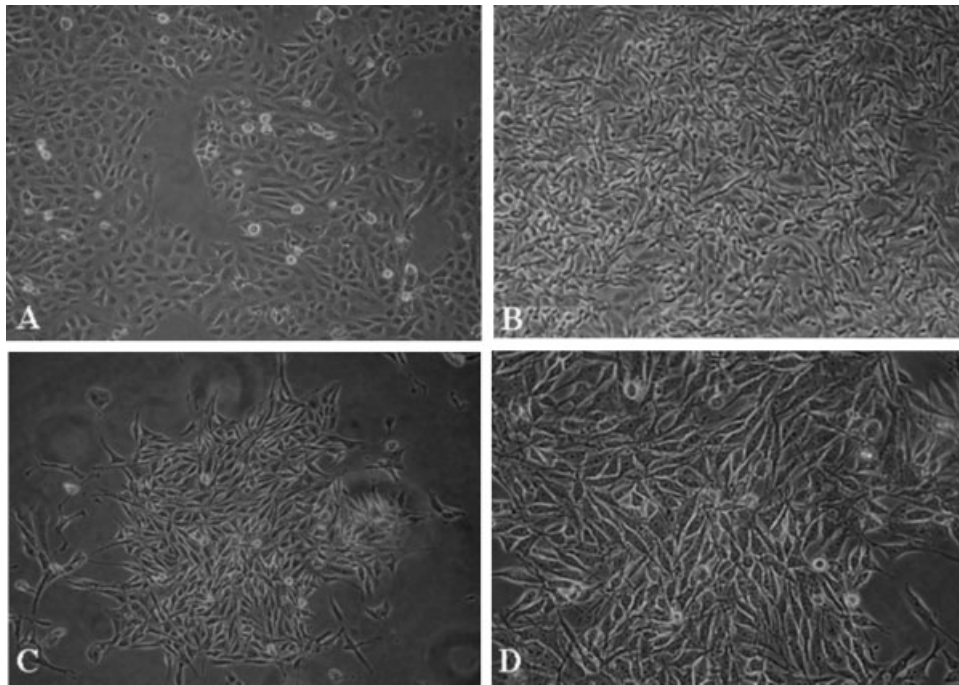


Fig. 3. Downregulation of Tfam and mitochondrial transcription expressions resulting in changes in cell morphology. **A:** Rhombus-like morphology of wild-type 143B cells (100 \times); **B:** spindle-like morphology of EtBr-treated 143B rho-negative cells (100 \times); **C:** spindle-like morphology of Tfam-siRNA transfected cells (40 \times); **D:** spindle-like morphology of Tfam-siRNA transfected cells (200 \times).

harboring <21% mtDNA and the EtBr-treated clone. Differences in the proportions of the 4,977-bp deletion and the 3,243-point mutation were not observed in the studied clones (Table I).

Mitochondrial enzyme complexes I, III, and IV, and ATP production exhibited significantly inhibition in clones with <21% mtDNA as well as the EtBr-treated clone. However, there were no significant differences in clones TFA201 and 207 with 82% and 66% mtDNA (Fig. 4a).

Effect of Mitochondrial Functional Recovery After an Increase in the mtDNA Copy Number

In order to determine whether mitochondrial function can recover after removal of interrupting factors, siRNA and EtBr were removed, and mitochondrial functions and mutated DNA were examined. Clones TFA103 and TFA102 were examined after removal of the selected drug (G418) for 1 week, and both clones recouped their losses in terms of the mtDNA copy number, which exceeded that of the wild-type clone. The morphology and growth rates of both clones were also the same as those of previously untreated cells (Table I). The enzyme activities and ATP production significantly

increased compared with treated cells in clones TFA103 and TFA102 but not in the clone from which EtBr was removed, compared with treated cells (Fig. 4b). The 8-OHdG content in the clone from which EtBr was removed and the proportion of the 4,977-bp deletion in the clone from which G418 was removed significantly differed from levels of previously treated clones, but no differences were observed among the other cohorts (Table I).

DISCUSSION

The current results show that using siRNA, respectively targeting exons 1 and 2 of the *Tfam* gene, resulted in downregulation of targeted gene expression in various cell clones including 102, 103, 108, and 207 (Fig. 1). The development of vectors for siRNA production and gene silencing for transfecting cells with the expression construct have been reported [Sui et al., 2002; Yu et al., 2002]. Constructs harboring siRNA can be stably integrated into the nuclear genome and sustain a simultaneous long-term knockdown effect of gene transcripts in cell culture [Chen et al., 2004]. Although the gene-

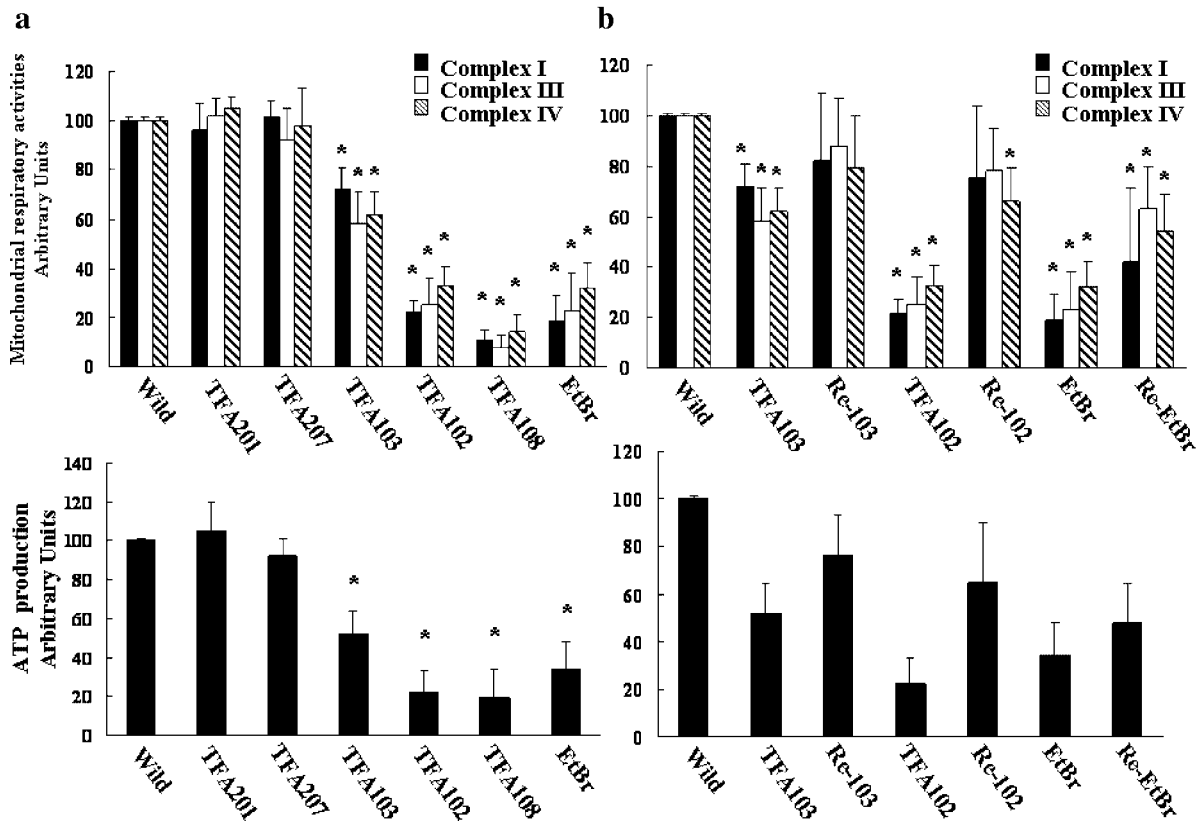


Fig. 4. a: Mitochondrial respiratory enzyme activities and ATP production by clones with downregulated *Tfam*. b: Recovery of mitochondrial respiratory enzyme activities and ATP production in clones after an increase in the mtDNA copy number. Clones TFA103 and 102, and the EtBr-treated clone were examined after the removal of gentamycin or EtBr for 1 week. Re-, cells were examined after removal of interrupting factors. Mitochondrial respiratory enzyme complexes I, III, and IV, and ATP production were determined. Data are expressed as the mean \pm SD of three separate experiments (* $P < 0.05$).

silencing effect was significant in early selected clones, whether the silencing effect can be stably maintained was determined at 3 weeks after the earlier analysis. *Tfam* gene expression did not recover in these selected clones, suggesting that TFA1 siRNA construct transfection targeted exon 1 of the *Tfam* gene which contributed to the knockdown effect. Moreover, the effect on the TFA1 construct was more pronounced than what was expected with the TFA2 construct (Fig. 1).

Biogenesis of the mitochondrial oxidative phosphorylation enzyme complex requires the concordant expression of mtDNA and nDNA genes which both encode mitochondrial proteins and their regulatory factors. One of these control factors is *Tfam*, which plays a major role in regulating mtDNA transcription and replication [Virbasius and Scarpulla, 1994; Montoya et al., 1997; Wang et al., 1999]. Other factors such as Ras and p66^{Shc} were also reported

contributed in the regulation of mtDNA copy number [Trinei et al., 2006]. In this study, four stable clones, that is, 102, 103, 106, and 108, showed significantly downregulated *Tfam* expression by siRNA, which may have resulted in interference with mtDNA gene expression. Decreased expressions of the ND1, Cyt b, and COX I genes were observed in these reported clones (Fig. 2). Recent studies presented the downregulation of *Tfam* expression or knockout of the *Tfam* gene, which may have resulted in mtDNA depletion [Inagaki et al., 1997, 1998; Larsson et al., 1998; Pohjoismaki et al., 2006]. The marked reduction in mRNA encoding *Tfam* and mitochondrial gene transcription was suppressed after HeLa cells were cultured with interferons, thereby showing interferon suppression of mtDNA transcription by depletion of *Tfam* [Inagaki et al., 1997]. The expressions of COX I and COX III genes encoded by mtDNA were inhibited as reported using antisense RNA

in a *Tfam*-transformation experiment [Inagaki et al., 1998]. Moreover, mice with heterozygous knockout of *Tfam* exhibited reduced mtDNA copy numbers and respiratory chain deficiencies in the heart, while homozygous knockout embryos exhibited severe mtDNA depletion with abolition of oxidative phosphorylation. These *Tfam* knockout data also provide direct evidence that the expression of mtDNA and maintenance of mtDNA are under the control of *Tfam* [Larsson et al., 1998]. On the other hand, Montoya et al. [1997] reported that *Tfam* was overexpressed in HeLa cells, resulting in increases in mitochondrial transcripts of COX I and 12S rRNA. Importation of recombinant *Tfam* into rat liver mitochondria may stimulate transcription of mtDNA [Garstka et al., 2003]. Overexpression of *Tfam* after severe redox stress was also reported to contribute to regeneration of the mtDNA pool loss caused by H₂O₂ [Noack et al., 2006]. In these experiments, downregulation of *Tfam* decreased mtDNA expression, and in contrast, upregulation of *Tfam* increased mtDNA expression, indicating that *Tfam* indeed regulates mitochondrial transcription.

The mitochondrial oxidative phosphorylation enzyme complex is composed of mitochondrial- and nuclear-encoded subunits. The relationship between the relative amounts of nuclear and mitochondrial genes for subunits of the oxidative phosphorylation enzyme complex was investigated in several human tissues and cell lines [Taanman et al., 1996; Marusich et al., 1997; Vijayasarathy et al., 2003]. In this study, protein expressions of mtDNA-encoded COX I and COX II and nDNA-encoded SDHA and NDUFA were determined, and both mtDNA-encoded proteins showed significantly decreased levels. However, no reduced protein expression of nDNA-encoded SDHA or NDUFA was found (Fig. 2b). In the current study, stable expression of siRNA significantly inhibited *Tfam* expression and consequentially interrupted mitochondrial transcription and translation. Although siRNA could knock-down *Tfam* gene expression by up to 90%, there was a residual low level of target gene expression. These results indicate that stable transfection of siRNA to reduce *Tfam* gene expression is effective but insufficient to completely eliminate the target gene expression. In a chemically and genetically derived Rho0 cell experiment, mtDNA-encoded subunits of

COX were not expressed in Rho0 cells, and other nDNA-encoded mitochondrial proteins were maintained in the same cells [Marusich et al., 1997]. These data are consistent with our findings, in that interruption of mitochondrial gene expression may downregulate mitochondrial-encoded proteins but not nuclear-encoded proteins.

In this study, we established a method to deplete mtDNA and decrease mitochondrial oxidative phosphorylation enzymes in human cells with the siRNA technique. In fact, 9-cis retinoic acid treatment for 24 h may significantly enhanced expression of mtDNA encoded RNA and protein in the cell clones described in this manuscript (data not shown). We have also created a mtDNA-depleted cell model for future studies.

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