# Human Blood-Derived Lymphoblastoid Cybrids for Assessment of Mitochondrial tRNAs Deficiency by MTO1 Silence

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**Research Article**

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# Abstract

**Background:** Mutations in the mitochondrial translation optimization 1 (MTO1) gene can cause hypertrophic cardiomyopathy. Although the functional role of MTO1 deficiency in certain cells is gradually confirmed, the phenotype of MTO1 deficiency in a lymphoblastoid cybrid line is not yet reported. In this study, we characterized changes of mitochondrial function in MTO1 silenced cybrid cells derived from human lymphoblastoids, immature white blood cells that give rise to lymphocytes.

**Results:** We showed that MTO1 silence decreased the levels of 2-thiourylation of mitochondrial $tRNA^{Lys}$, $tRNA^{Glu}$, and $tRNA^{Gln}$, nevertheless, the aminoacylation efficiency of $tRNA^{Lys}$ and the steady state of mitochondrial tRNAs were elevated. These aberrant tRNA changes caused a significant decrease in protein levels of oxidative phosphorylation complex subunits including complex I, III, IV and V. Furthermore, dysfunctional mitochondria promoted apoptosis in stress, evidenced by elevated ratios of apoptotic cells and increased levels of apoptosis-activated proteins in the MTO1 knockdown cell lines, as compared to the controls.

**Conclusions:** Our data provide new insights into the important functional role of MTO1 in lymphoblastoid mitochondria. We envision the cybrid cell line approach we have established provides an alternative model for the cardiac tissue of high-energy demands characteristics, and they hold promises for the diagnosis and drug screening for the therapeutic agents of hypertrophic cardiomyopathy caused by MTO1 dysfunction.


# 1. Introduction

Mitochondrial diseases are usually caused by defects in oxidative phosphorylation and have a strong tissue-specific phenotype [1, 2]. MTO1 is an evolutionarily conserved protein expressed in tissues with high-energy requirements, such as the myocardial tissue, and has been associated with human early-onset hypertrophic cardiomyopathy [3]. In patients with mutations of MTO1, the most prominent signs and symptoms were cardiovascular and included bradycardia and cardiomyopathy. By whole exome sequencing and direct sequencing of the gene MTO1, Ghezzi D *et al.* first identified missense mutations, which were all predicted to cause deleterious effect of MTO1, and are related to cardiomyopathy [4]. Later, the same group applied the next-generation sequencing to identify additional mutations in MTO1 in two individuals from a family of inherited cardiomyopathy, one frameshift mutation and one missense mutation [5]. They have found that mutant muscle and fibroblasts carrying these MTO1 mutations showed variable reductions in mtDNA-dependent respiratory chain activities. In 2014, Becker L. *et al.* have successfully generated MTO1-deficient mouse model, which showed typical symptoms of cardiomyopathy. In addition, the mutant mice showed a marked worsening of arrhythmias during induction and reversal of anaesthesia [6].

Human mitochondrial translation machinery is composed of 22 mt-tRNAs and 2 rRNAs encoded by mtDNA, and more than 150 proteins encoded by nuclear genome, including ribosomal proteins, ribosomal

assembly proteins, aminoacyl-tRNA synthetases, tRNA modifying enzymes and several initiation, elongation and termination factors [1, 2]. The impairments of mitochondrial translation have been associated with a wide spectrum of human diseases, including diabetes, neurological disorders, heart failure, and hearing loss [7–11]. Most of these diseases were caused by the mutations in mitochondrial genes inducing changes of mt-tRNAs, which affect the processing of the tRNAs from the primary transcripts, stability of the folded secondary and tertiary tRNA structures, the aminoacylation of the tRNA, and the codon-anticodon interaction in the process of translation [10].

Previously, we have identified and cloned the mice and human gene *MTO1* encoding the mitochondrial protein MTO1, which is a tRNA modifying enzyme highly conserved from prokaryotes to eukaryotes [12, 13]. Others and we have shown that the human MTO1, like its yeast counterpart, is an evolutionarily conserved protein that implicates a role in the modifications of 5-taurinomethyl-2-thiouridine ($\tau m^5 s^2 U$) at positions 34 (wobble position) of mt-$tRNA^{Lys}$ [14–16]. We found that MTO1 played a synergistic role in the pathogenesis of deafness-associated A1555G mutation in 12S rRNA gene. The A1555G mutation produced a clinical phenotype that ranges from severe congenital to normal hearing [17]. The human MTO1 could contribute to the phenotypic variability of A1555G mutation by enhancing or suppressing the phenotypic manifestation of A1555G mutation. In addition, we proposed that the most two common mutations in tRNAs were related to the lack of modification provided by MTO1 [17]. These two mutations are: 1) the A3243G mutation in the $tRNA^{Leu(UUR)}$ gene, which was associated with mitochondrial encephalopathy lactic acidosis and stroke-like episodes (MELAS), and 2) the A8344G mutation in the $tRNA^{Lys}$ gene, which is the main cause for myoclonus epilepsy associated with ragged red fibers (MERRF) [18–20]. Indeed, Suzuki and colleagues have demonstrated elegantly using mass-spectrometry that MTO1 was responsible for the initial $\tau m^5 s^2 U$ modification of $tRNA^{Lys}$ [15].

To further determine the phenotypic role of MTO1, we examined the MTO1 null mutants in yeast [13]. However, MTO1 null mutant expressed the respiratory deficient phenotype only when coupled with the C1409G mutation of mitochondrial 15S rRNA. In another study, we found that MTO1 in complex with MTO2, and MSS1 genes, which were all encoded by the mtDNA as highly conserved tRNA modifying enzymes, were jointly responsible for the biosynthesis of $\tau m^5 s^2 U$ in mitochondrial $tRNA^{Lys}$, $tRNA^{Glu}$, and $tRNA^{Gln}$ [16]. Therefore, the phenotypic role of the human MTO1 and the consequence of mt-tRNA modification changes remain elusive. We speculated that silencing of MTO1 alone may cause mild mitochondrial defect and can be compensated by other modifier enzymes. Because the mitochondrial function of the cells is related to their metabolic rates, we thought it would be feasible to analyze an amplified phenotypic deficiency of MTO1 in a cellular system with a high metabolic rate. Lymphoblasts derived from the human blood are preferred for many diagnoses of diseases. Compared to other cells such as fibroblasts, lymphoblasts have much higher energy requirement due to a faster growth rate and a higher metabolic rate [21–23].

In this study, by applying MTO1 silencing in cybrid cell lines derived from lymphoblasts of a healthy individual, we determined the phenotypic role of MTO1 on mitochondrial function, aming to fill in a

knowledge gap in the understanding of the cellular consequences of MTO1 mutations which are associated with cardiomyopathy. Specifically, we generated stable short-hairpin (sh) RNA-mediated knockdown of MTO1 in cybrid cells. The resultant stable MTO silenced cell lines demonstrated that the effects of defective MTO1 led to decreased mitochondrial tRNA metabolism, translation, enzymatic activities of electron transport chain complexes, the rate of $O_2$ consumption, ATP as well as mitochondrial membrane potential. Furthermore, dysfunctional mitochondria promoted apoptosis in stress, evidenced by elevated ratios of apoptotic cells and increased levels of apoptosis-activated proteins in the MTO1 knockdown cell lines, as compared to the controls.

# 2. Materials And Methods

**Experimental human participants.** For cell line generation involving blood samples taken from human participants, informed consent, blood samples, and clinical evaluations were obtained under protocols approved by the Ethic Committee of Zhejiang University Schoolof Medicine and Institutional Review Board of Cincinnati Children's Hospital Medical Center, which is in accordance with the Declaration of Helsinki. We confirm that all research was performed in accordance with relevant guidelines/regulations, and include in their manuscript a statement confirming that informed consent was obtained from all participants and/or their legal guardians.

**Generation of MTO1 knocking down cell lines**. lymphoblastoid cell lines were derived from a healthy individual directly following previously described procedures [24]. In brief, cell lines were grown in RPMI 1640 medium with 10% fetal bovine serum (FBS, Thermo Fisher Scientific). The mtDNA-less $\rho^o$206 cell line, derived from 143.TK$^-$ cell line was grown under the same conditions as the parental line, except for the addition of 50 ug of uridine/ml. Two cyrbid cell lines 143B-1 and 143B-2 were constructed with enucleated lymphoblastoid cell lines and were maintained in the same medium as the 143.TK$^-$ cell line.

The shRNA oligo primers targeting to silence MTO1 were:

Forward, 5'CCGGCCAGGGAGTTCAGCAAGATGACTCGAGTCATCTTGCTGAACTCCCTGGTTTTTG3',

Reverse, 5'AATTCAAAAACCAGGGAGTTCAGCAAGATGACTCGAGTCATCTTGCTGAACTCCCTGG3'.

Lentiviral vectors, pLKO.1 with MTO1 shRNA or scramble shRNA, were co-transfected into HEK293T cells with psPAX2 and pMD2.G for lentivirus production. Complete medium was changed after 4-6 hours transfection. Virus was collected 48 hours after transfection and centrifuged at 12,000 g. Cell lines were cultured in medium containing virus for 48 hours. The stable shMTO1 and scramble clones were isolated by culturing cells in DMEM medium supplemented with 10% FBS and 1 μg/ml of puromycin for another 48 hours. The resultant clones were examined for the expression of MTO1 by Western blot analysis as detailed below.

**Mitochondrial tRNA analysis**. Total RNAs were generated using Totally RNATM kits (Ambion) from mitochondria isolated from knocking down and scramble control cell lines (~$2.0 \times 10^8$ cells), following

manufacturer's instructions. The levels of the thiouridine modification in the tRNAs were detected by the retardation of electrophoretic mobility in a polyacrylamide gel that contained 0.05 mg/ml ((N-acryloylamino)phenyl)mercuric chloride (APM) [20]. Ten μg of total RNAs were loaded in a 10% polyacrylamide-8 M urea gel electrophoresis in Tris–borate–EDTA buffer (TBE) and subsequently blotted onto positively charged membrane (Roche). To further discriminate unthiouridylated tRNA from thiouridylated tRNA, the samples were dethiolated through 1 hour incubation in EDTA-PBS buffer with $H_2O_2$ at room temperature and run in parallel. The membrane was incubated with specific non-radioactive DIG oligodeoxynucleoside probes at the 3' termini according to the DIG Northern Starter Kit (Roche). Oligonucleotide probes for tRNALys, tRNAGlu, and tRNAGln were 5'-AAAGAGGTGTTGGTTCTCTTAATCTTTAAC-3',

5'- ATTCTCGCACGGACTACAACCACGACCAAT-3', 5'-AGGACTATGAGAATCGAACCCATCCCTGAG-3', respectively. DIG-labeled oligodeoxynucleosides were generated by using the DIG oligonucleoside tailing kit (Roche). The bands were visualized by a CLINX ChemiScope bioimaging analyzer.

The aminoacylation assays was carried out as described elsewhere [25]. Briefly, total RNAs were isolated under acid conditions, and two μg of total RNAs was electrophoresed at 4°C through an acid (pH 5.2) 10% polyacrylamide–8 M urea gel to separate the uncharged and charged tRNA. The steady state tRNA Northern blot analysis was performed similar to thiouridine modification detection without APM. DIG-labeled oligodeoxynucleotide probes for tRNALeu(UUR), tRNATyr and 5S rRNA were: 5'-AGAAGAGGAATTGAACCTCTGACTGTAAAG-3',

5'- GGTAAAAAGAGGCCTAACCCCTGTCTTTAG-3',

5'- GGGTGGTATGGCCGTAGAC-3', respectively. The hybridization and quantification of density in each band were performed according to the DIG Northern Starter Kit (Roche)

**Western blot analysis**. Western blotting analysis was performed as detailed previously [24]. Twenty micrograms of total proteins obtained from cells were denatured and loaded on sodium dodecyl sulfate (SDS) polyacrylamide gels. The gels were electroblotted onto a polyvinylidene difluoride (PVDF) membrane for hybridization. The antibodies used for this investigation were from Abcam [YARS2 (ab68725), ND3 (ab170681), and CO2 (ab110258), TRMU (ab50895), TOM20 (ab56783), GAPDH (ab8245), Cytochrome c (ab13575), total OXPHOS human WB antibody cocktail (ab110411)], Santa Cruz Biotechnology [ND4 (sc-20499-R), ND6 (sc-20667)), MTO1 (sc-398760)], Proteintech [TFAM (19998-1-AP), TFB2M (24411-1-AP), TUFM (26730-1-AP), KARS2 (14951-1-AP), LARS2 (17097-1-AP), CYTB (55090-1-AP), and ATP8 (26723-1-AP)], and Cell Signaling Technology [Caspase-3 (#9664), Caspase-7 (#8438), Caspase-9 (#7237) and PARP (#5625)]. Peroxidase Affini Pure goat anti-mouse IgG and goat anti-rabbit IgG (Jackson) were used as a secondary antibody and protein signals were detected using the ECL system (CWBIO). Quantification of density in each band was performed as detailed previously [26].

**Enzymatic Assays**. The enzymatic activities of complex I, II, III, IV and V were measured as detailed elsewhere [27]. In brief, citrate synthase activity was analyzed by the reduction of 5,5'-dithiobis-2-

nitrobenzoic acid at 412 nm in the assay buffer containing 0.1 mM DTNB, 50 μM acetyl coenzyme A, and 250 μM oxaloacetate. Complex I activity was determined with 10 μg/ml antimycin A and 2 mM KCN by following the decrease in the absorbance due to the NADH oxidation at 340 nm in assay buffer. The activity of complex II was analyzed by tracking the secondary reduction of 2,6-dichlorophenolindophenol (DCPIP) by DB at 600 nm in the assay buffer. Complex III activity was determined in the presence of 2 μg/ml antimycin A and 2 mM KCN by measuring the reduction of cytochrome c at 550 nm with reduced decylubiquinone in the assay buffer. Complex IV activity was measured by monitoring the oxidation of reduced cytochrome c as a decrease of absorbance at 550 nm in the assay buffer. Complex V activity was monitored by the oxidation of NADH at 340 nm in the assay buffer. All assays were performed by using Synergy H1 (Biotek, Winooski, VT, United States). Complex I-V activities were normalized by citrate synthase activity.

**Measurements of oxygen consumption.** The rates of oxygen consumption in mutant and wild type cell lines were assayed with a Seahorse Bioscience XF-96 extracellular flux analyzer (Seahorse Bioscience), as detailed previously [24]. Non-mitochondrial OCR was analyzed as the OCR after rotenone/antimycinA treatment. Basal OCR was determined as OCR before oligomycin addition minus OCR after rotenone/antimycin A treatment. ATP-linked OCR was measured as OCR before minus after oligomycin addition. Proton leak was calculated as Basal OCR minus ATP-linked OCR. Maximal OCR was determined as the OCR after FCCP addition minus non-mitochondrial OCR. Reserve Capacity was defined as the difference between Maximal OCR minus Basal OCR. The protein content of each well was then measured to normalize OCR values.

**ATP measurements.** The Cell Titer-Glo® Luminescent Cell Viability Assay kit (Promega) was used for the measurement of cellular and mitochondrial ATP levels, following the modified manufacturer's instructions.

**Assessment of mitochondrial membrane potential.** Mitochondrial membrane potential was assessed with JC-10 Assay Kit - Flow cytometry (Abcam) according to general manufacturer's recommendations with some modifications as detailed elsewhere [24].

**Flow cytometry for apoptosis analysis.** For discrimination of apoptotic and non-apoptotic cells by Annexin V/ PI staining, cells were treated with or without 1 mM $H_2O_2$ (Sigma) for 4 hours. Cells were harvested and stained with Annexin V and 1 μl propidium iodide (PI) (V13242, ThemoFisher Scientific) according to the manufacturer’s instruction. Each sample was detected by FACSCalibur C6 (BD Biosciences) and analyzed using FlowJo software (Treestar).

**Computer analysis.** Statistical analysis was carried out using the unpaired, two-tailed Student’s t-test contained in the Microsoft-Excel 2013 or GraphPad Prism 7. Differences were considered significant at a $p<0.05$.

# 3. Results

# 3.1. MTO1 depletion caused aberrant mitochondrial tRNA metabolism

To determine the role of MTO1 in lymphoblastoid cells and how it is affecting the mitochondrial tRNA metabolism, we first generated the cybrid cell lines by shRNA-mediated MTO1 knockdown. These cell lines were lymphoblast cells isolated from a healthy individual and we generated cybrid cell lines following previously described procedures [16]. Then, we designed a 22-mer targeting in MTO1 or scramble sequence without a target and cloned into pLKO.1 vectors for lentivirus production. After lentiviral infection for 48 hours, stable knocking down cell lines were generated in the presence of 1 μg/ml puromycin for 48 hours. The 143B cell line was further selected and utilized for the subsequent shRNA-mediated MTO1-knockdown experiment. The levels of MTO1 in 143B-1-shMTO1 and 143B-2-shMTO1 cell lines were 36.6% and 35.8% of those in the scramble cell lines, respectively, while TRMU and GTPBP3 were comparable in these cells (Fig. 1G). These results confirmed the specific silencing of the MTO1 gene in both shMTO1 cell lines, which were used for the biochemical characterizations described below.

To investigate whether MTO1 knockdown affected the 2-thiouridine modification at U34 in tRNAs, we examined the thiouridylation levels of mt-tRNAs in polyacrylaminde gel containing 0.05 mg/ml ((N-acryloylamino)phenyl) mercuric chloride (APM). In our test, we detected the sulfur modification of the mt-tRNAs that appear as a single band (labeled as 2-thio tRNA) appeared at a higher molecular weight of the unmodified tRNA. We observed that three tRNAs ($tRNA^{Lys}$, $tRNA^{Glu}$, and $tRNA^{Gln}$) which were known substrate of MTO1 to contained thiouridylation indeed showed 2-thio tRNA bands, whereas the $tRNA^{Leu(UUR)}$ control (substrate of MTO1 and unthiouridylated) does not contain sulfur modification and therefore did not show such band. Comparing shMTO1 to the scrambled conditions, we noticed that the absolute intensity of thiouridylation for $tRNA^{Lys}$, $tRNA^{Glu}$, and $tRNA^{Gln}$ do not seem to follow an obvious trend, especially there is a seemingly discrepancy between data obtained from 143B-1 and 143B-2 shMTO1 cells (Fig. 1A). However, we noticed that the ratio of thiouridylation (2-thio tRNA/(2-thio tRNA + unthiouridylated tRNA)) demonstrated a consistent decrease in the shMTO1 cell lines. We further distinguished unthiouridylated tRNAs from thiouridylated tRNAs by using dethiolated tRNAs samples by incubation in EDTA-PBS buffer with $H_2O_2$. These samples were loaded in parallel with untreated sample. The proportions of the thiouridylation levels of the tRNAs in shMTO1 cell lines were 45%, 49%, 46% in $tRNA^{Lys}$, $tRNA^{Glu}$, $tRNA^{Gln}$, respectively, all significantly decreased compared to scramble cell lines which were 55%, 62%, 56%, respectively (Fig. 1B).

To evaluate whether the MTO1 knockdown altered the aminoacylation of tRNA, we examined the aminoacylation capacities of $tRNA^{Lys}$ in detail. To make a systematic comparison, we have used $tRNA^{Leu(UUR)}$ (substrate of MTO1 and unthiouridylated) and $tRNA^{Tyr}$ (not a substrate of MTO1 and unthiouridylated) as two controls (Fig. 1C). The ratios of aminoacylated $tRNA^{Lys}$ in the 143B-1-shMTO1 and 143B-2-shMTO1 cell lines were 66% and 52%, significantly increased compared to those in scramble cell lines, which were measured as 41% and 34%, respectively. As expected for the controls, the levels of aminoacylation in $tRNA^{Leu(UUR)}$ and $tRNA^{Tyr}$ in mutant cell lines were unaffected compared to the control

cell lines (Fig. 1D). These results revealed that the increase of $tRNA^{Lys}$ aminoacylation is a specific consequence of MTO1 knockdown. In addition, we observed significant increase in the steady-state level of mitochondrial $tRNA^{Leu(UUR)}$ and $tRNA^{Tyr}$, but not in $tRNA^{Lys}$, $tRNA^{Glu}$, and $tRNA^{Gln}$ (Fig. 1E and 1F). These results confirmed MTO1 depletion caused aberrant mitochondrial tRNA metabolism.

# 3.2 Alterations in mitochondrial tRNA led to reduced levels of mitochondrial proteins

To investigate whether mitochondrial translation was affected, we analyzed the expression of mtDNA-encoded proteins in mutant and control cell lines with TOM20 as a loading control. Six kinds of proteins were analyzed in the mutant cell lines and compared to those in the scramble cell lines (Fig. 2A). We have found that the overall level of mitochondrial protein expression were 37% ($P < 0.001$) (Fig. 2B), significantly decreased to the mean value measured in the scramble cell lines. The average levels of ND3, ND4, ND6, CO2, ATP8, and CYTB in these mutant cell lines were 37.8% ($P = 0.003$), 27.5% ($P < 0.001$), 67.7% ($P = 0.104$), 21.7% ($P < 0.001$), 35.2% ($P = 0.002$), and 32.6% ($P < 0.001$) of the average values of scramble cell lines, respectively (Fig. 2B). Overall, ND6 demonstrated the smallest reduction, whereas CO2 had the highest reduction.

We then examined the levels of five subunits of OXPHOS complexes in scramble and shMTO1 cell lines (Fig. 2C). These included the CO2 encoded by mtDNA, and NDUFB8, UQCRC2, ATP5A and SDHB, encoded by nuclear genes. The average levels of CO2, NDUFB8, UQCRC2, ATP5A and SDHB in the mutant cell lines were 37% ($P = 0.005$), 50% ($P = 0.014$), 38% ($P < 0.001$), 67% ($P = 0.017$) and 94% ($P = 0.406$) of those in the scramble cell lines, respectively (Fig. 2D).

# 3.3 Defects in mitochondrial translation gave rise to respiration deficiency

To assess if the MTO1 knockdown altered cellular bioenergetics, we examined the oxygen consumption rates (OCR) of mutant and scramble cell lines (Fig. 3A). The average basal OCRs in shMTO1 cell lines were 30.7% ($P =0.002$) of the mean values of the scramble cell lines. To investigate which of the enzyme complexes of the respiratory chain was affected in shMTO1 cell lines, various OCRs were measured after the sequential addition of oligomycin, FCCP, rotenone and antimycin. The ATP-linked OCR, proton leak OCR, maximal OCR, reserve capacity, and non-mitochondrial OCR in shMTO1 cell lines were 25.1% ($P < 0.001$) 51.5% ($P = 0.104$), 32.7% ($P < 0.001$), 36.9% ($P = 0.040$), and 89.6% ($P = 0.776$), relative to the mean value measured in the scramble cell lines, respectively (Fig. 3B).

We then measured the activities of respiratory complexes using isolated mitochondria in various assays (Fig. 3C). The activities of complexes I, II, III, IV and V in shMTO1 cell lines were 56.4% ($P < 0.001$), 92.8% ($P = 0.041$), 53.4% ($P < 0.001$), 43.8% ($P < 0.001$) and 54.6% ($P < 0.001$) of the mean value measured in the scramble cell lines, respectively. The levels of ATP production were further measured using a luciferin/luciferase assay. The relative ratio of mitochondrial ATP production in shMTO1 cell lines was 65% ($P < 0.001$) of the average value measured in the scramble cell lines (Fig. 3D). To examine whether

the MTO1 knockdown affected mitochondrial membrane potential (ΔΨm), a fluorescence probe JC-10 assay was utilized (Fig. 3E). The relative levels of ΔΨm in the 143B-1-shMTO1 and 143B-2-shMTO1 cell lines were significantly decreased, with 25.8% ($P < 0.001$) and 33.9% ($P = 0.007$) of the mean values measured in scramble cell lines, respectively (Fig. 3F).

## 3.4 Dysfunctional mitochondria altered apoptotic state in stress

To assess whether MTO1 knockdown affected apoptotic process, we examined the apoptotic state of shMTO1 and scramble cell lines using Annexin-V/PI based flowcytometry and western blotting analysis. After cells were treated with $H_2O_2$ as an oxidative stress, the average ratio of Annexin-V positive cells of shMTO1 cell lines were 134% ($P < 0.001$) of the mean value measured in the scramble cell lines (Fig. 4A and 4B). We further measured the levels of apoptosis-related proteins: cytochrome C, cleaved caspases 3, 7 and 9, as well as PARP in $H_2O_2$-treated shMTO1 and scramble cell lines by western blot analysis (Fig. 4C). The average levels of cytochrome C, caspase 3, 7, 9 and PARP in shMTO1 cell lines were 166%, 211%, 183%, 191%, and 177% of the average values measured in the scramble cell lines ($P = 0.001$, 0.002, 0.001, 0.005, 0.001), respectively (Fig. 4D).

# 4. Discussion

In the present study, we investigated the consequences of MTO1 silencing on mitochondrial metabolism and function using a lymphoblastoid cybrid line as an alternative model for the high energy demands characteristic of cardiac tissue. This is the first time the human lymphoblastoid being used to study MTO1 function has been reported. Lymphoblasts have several advantages as a clinical diagnostic cellular tool of cardiomyopathy: 1) they grow faster and are easier to culture than other cell types such as fibroblasts, 2) they are immortalized cell lines which can be passaged infinite times, 3) blood sample collection is less traumatic than skin biopsies needed for fibroblast culture.

Overall, we found that the lymphoblastoid derived cybrid response by the MTO1 deficiency was more pronounced than previous studies using other cellular systems. As a highly conserved tRNA modifying enzyme, MTO1 mutations or silencing were known to cause the deficient nucleotide modification and affected both structure and function of tRNAs, including the stability, turnover, aminoacylation, and codon recognition [10, 18, 28]. In *Escherichia coli*, *gidA* (the bacterial *MTO1* homologue) mutants exhibited the deficient biosynthesis of mnm5s2U34 of $tRNA^{Glu}$ and translational frameshift [13, 20, 29]. In *S. cerevisiae*, the unmodified tRNAs due to *MTO1* null mutation caused the instability and inefficient aminoacylation of mitochondrial tRNAs and impairment of mitochondrial translation [13, 20]. However, the deletion of *MTO1* in *S. cerevisiae* led to the respiratory deficient phenotype, only when coexisting with the mitochondrial 15S rRNA C1409G mutation [20]. Moreover, although the siRNA-induced knocking down of human MTO1 in fibroblasts resulted in a 2-thio modification defect $tRNA^{Lys}$, the overall phenotypic effect was rather mild compared to the MTO1 silence in the mitochondrial Arg464Cys mutated cell [30, 31].

The specific repression of the human *MTO1* gene resulted in significant reduction in the thiouridylation modification of $tRNA^{Lys}$, $tRNA^{Glu}$ and $tRNA^{Gln}$, as well as abnormal tRNA steady state and aminoacylation level, which led to the reduced oxygen consumption, defective mitochondrial membrane potentials and increased apoptosis rate. This nicely explains others and our previous observation that the modification deficiency of mt-tRNAs results in poor decoding of cognate codons for mitochondrial protein synthesis [12, 17–19, 20]. However, we noticed several discrepancies. Previously, we found that when silencing MTO1 alone in yeast, the $\tau m^5s^2U$ level in mitochondrial $tRNA^{Lys}$, $tRNA^{Glu}$, and $tRNA^{Gln}$ were relatively unchanged compared to the level detected in the wild-type strain, whereas the levels of several mt-tRNAs (Lys, Glu, Gln, Leu, Gly, Arg, and Met) were all decreased. Also, in yeast, the levels of aminoacylated mt-tRNAs (Lys, and Arg) were decreased. Our observation in this study demonstrated a significant reduction in the thiolation level in mitochondrial $tRNA^{Lys}$, $tRNA^{Glu}$, and $tRNA^{Gln}$. Also, levels of the tRNAs increase in the steady-state level of mitochondrial $tRNA^{Leu(UUR)}$ and $tRNA^{Tyr}$, but not in $tRNA^{Lys}$, $tRNA^{Glu}$, and $tRNA^{Gln}$. These results suggest that MTO1 might affect the mitochondrial tRNA metabolism in human blood cells through a distinct mechanism, in contrast with those observed in the yeast cells. We speculate that such changes could be due to a compensatory effect in the mammalian system, which might be absent in the yeast, since elevated levels of aminoacylation and steady-state could provide certain levels of stabilization for unmodified tRNAs [15, 32]. These unmodified tRNAs may also mischarge with a noncognate amino acid, as anticodon modifications acts as anti-determinants [32], evidenced by the overall decrease of mitochondrial protein level, the up-regulation of mitochondrial proteases in MTO1 mutant mice [31] and MTO1 silenced fibroblasts [30]. Moreover, alterations in mitochondrial $tRNA^{Leu(UUR)}$ and $tRNA^{Tyr}$ indicated an unconventional role of MTO1 in regulating mitochondrial RNA metabolism.

We further demonstrated that the disruption due to the MTO1 knockdown leads to increased induction of apoptosis, which has not been reported previously. Although MTO1 deficiency has been proposed to perturb mitochondrial protein translation and OXPHOS function, nevertheless, direct evidence in mammalian systems for this was rather limited due to the mild-responses from MTO1 silencing. For example, Boutoual *et al.* have reported the alterations of OXPHOS complexes in fibroblasts carrying MTO1 mutation c.1392 C>T (p.Arg464Cys) in homozygosis were accompanied with a reduction of the steady-state levels of several nuclear- and mitochondrial-encoded OXPHOS subunits, however, only complexes I and IV reduction was observed in MTO1-depleted fibroblasts [30]. In addition, effect of MTO1 deficiency on membrane potential, ROS production and antioxidant response were not significant.

It is remarkable to observe 63% reductions in the overall levels of six mitochondrial proteins due to MTO1 silence. Furthermore, the marked decreases in the levels of NDUFB8, UQCRC2and ATP5A in the mutant cell lines clearly confirmed that MTO1 is involved in biogenesis of the entire OXPHOS system. Such respiratory deficiency then led to the alterations on ATP synthesis, oxidative stress and subsequent failure of cellular energetic process, which was not clearly shown previously. In addition, the marked reduction of mitochondrial ATP production due to MTO1 silencing is similar to the cell lines bearing TRMU A10S mutation [20]. Furthermore, the mitochondrial membrane potentials that is a major indicator of cellular viability, was decreased [11, 24, 33]. All these reductions due to MTO1 silence indicated the impaired

proton pumping and more electron leakage across the mitochondrial membrane [32]. We attributed the amplified MTO1 knockdown associated mitochondrial dysfunction to the metabolic rate of the cells.

# 5. Conclusions

Our study attempted to fill in a knowledge gap in the understanding of the cellular consequences of MTO1 malfunction, which is associated with cardiomyopathy. Our results demonstrate the potential of the blood-cell derived lymphoblastoid cybrids as a diagnostic tool for evaluation of MTO1 related diseases. We can imagine using blood cells extracted from the body of patients with MTO1 mutation related myocardial hypertrophy as a cellular tool for evaluating mitochondrial function. These evaluation indicators include tRNA modifications, oxidative phosphorylation complex protein levels, and apoptosis level as described herein. In recent years, there is a significant development of new drugs for the treatment of myocardial hypertrophy [34]. We can envision the use of such cellular tools to evaluate the therapeutic compounds and quantitatively measure whether they improve or restore mitochondrial function, so to achieve a better precision treatment of hypertrophic cardiomyopathy caused by defects in the MTO1 gene. The system described can be a convenient tool for the analysis of various mitochondrial parameters, albeit with the limitation that aside from the shared feature of high metabolic demand, the tissues are very different in most other ways, which can have additional effects on disease progression. Overall, we propose that this approach of using blood-derived lymphoblasts is a useful non-invasive approach for analyzing mitochondrial dysfunction and evaluation of therapeutic interventions.

# Declarations

**Ethics approval and consent:** Blood samples taken from human participants, informed consent, blood samples, and clinical evaluations were obtained under protocols approved by the Ethic Committee of Zhejiang University Schoolof Medicine and Institutional Review Board of Cincinnati Children's Hospital Medical Center, which is in accordance with the Declaration of Helsinki. We confirm that all research was performed in accordance with relevant guidelines/regulations, and include in their manuscript a statement confirming that informed consent was obtained from all participants and/or their legal guardians.

**Consent for publication:** all authors have agreed for publication.

**Availability of dat and materials**: All data and cell lines are available in the main and supplementary files or available from authors upon reasonable request.

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# References

1. Attardi G, Schatz G (1988) Biogenesis of mitochondria. Annu Rev Cell Biol 4:289–333. doi:10.1146/annurev.cb.04.110188.001445
2. Wallace DC (1999) Mitochondrial diseases in man and mouse. Science 283:1482–1488. doi:10.1126/science.283.5407.1482
3. O'Byrne JJ, Tarailo-Graovac M, Ghani A, Champion M, Deshpande C, Dursun A, Ozgul RK, Freisinger P, Garber I, Haack TB, Horvath R, Baric I, Husain RA, Kluijtmans LAJ, Kotzaeridou U, Morris AA, Ross CJ, Santra S, Smeitink J, Tarnopolsky M, Wortmann SB, Mayr JA, Brunner-Krainz M, Prokisch H, Wasserman WW, Wevers RA, Engelke UF, Rodenburg RJ, Ting TW, McFarland R, Taylor RW, Salvarinova R, van Karnebeek CDM (2018) The genotypic and phenotypic spectrum of MTO1 deficiency. Mol Genet Metab 123:28–42. doi:10.1016/j.ymgme.2017.11.003
4. Ghezzi D, Baruffini E, Haack TB, Invernizzi F, Melchionda L, Dallabona C, Strom TM, Parini R, Burlina AB, Meitinger T, Prokisch H, Ferrero I, Zeviani M (2012) Mutations of the mitochondrial-tRNA modifier MTO1 cause hypertrophic cardiomyopathy and lactic acidosis. Am J Hum Genet 90:1079–1087. doi:10.1016/j.ajhg.2012.04.011
5. Baruffini E, Dallabona C, Invernizzi F, Yarham JW, Melchionda L, Blakely EL, Lamantea E, Donnini C, Santra S, Vijayaraghavan S, Roper HP, Burlina A, Kopajtich R, Walther A, Strom TM, Haack TB, Prokisch H, Taylor RW, Ferrero I, Zeviani M, Ghezzi D (2013) MTO1 mutations are associated with hypertrophic cardiomyopathy and lactic acidosis and cause respiratory chain deficiency in humans and yeast. Hum Mutat 34:1501–1509. doi:10.1002/humu.22393
6. Becker L, Kling E, Schiller E, Zeh R, Schrewe A, Holter SM, Mossbrugger I, Calzada-Wack J, Strecker V, Wittig I, Dumitru I, Wenz T, Bender A, Aichler M, Janik D, Neff F, Walch A, Quintanilla-Fend L, Floss T, Bekeredjian R, Gailus-Durner V, Fuchs H, Wurst W, Meitinger T, Prokisch H, de Angelis MH, Klopstock T (2014) MTO1-deficient mouse model mirrors the human phenotype showing complex I defect and cardiomyopathy. PLoS ONE 9:e114918. doi:10.1371/journal.pone.0114918
7. Boczonadi V, Horvath R (2014) Mitochondria: impaired mitochondrial translation in human disease. Int J Biochem Cell Biol 48:77–84. doi:10.1016/j.biocel.2013.12.011

8. Chen C, Chen Y, Guan MX (2015) A peep into mitochondrial disorder: multifaceted from mitochondrial DNA mutations to nuclear gene modulation. Protein Cell 6:862–870. doi:10.1007/s13238-015-0175-z
9. Rotig A (2011) Human diseases with impaired mitochondrial protein synthesis. Biochim Biophys Acta 1807:1198–1205. doi:10.1016/j.bbabio.2011.06.010
10. Suzuki T, Nagao A, Suzuki T (2011) Human mitochondrial tRNAs: biogenesis, function, structural aspects, and diseases. Annu Rev Genet 45:299–329. doi:10.1146/annurev-genet-110410-132531
11. Wallace DC (2005) A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. Annu Rev Genet 39:359–407. doi:10.1146/annurev.genet.39.110304.095751
12. Li R, Li X, Yan Q, Qin Mo J, Guan MX (2003) Identification and characterization of mouse MTO1 gene related to mitochondrial tRNA modification. Biochim Biophys Acta 1629:53–59. doi:10.1016/s0167-4781(03)00160-x
13. Wang X, Yan Q, Guan MX (2009) Mutation in MTO1 involved in tRNA modification impairs mitochondrial RNA metabolism in the yeast Saccharomyces cerevisiae. Mitochondrion 9:180–185. doi:10.1016/j.mito.2009.01.010
14. Colby G, Wu M, Tzagoloff A (1998) MTO1 codes for a mitochondrial protein required for respiration in paromomycin-resistant mutants of Saccharomyces cerevisiae. J Biol Chem 273:27945–27952. doi:10.1074/jbc.273.43.27945
15. Fakruddin M, Wei FY, Suzuki T, Asano K, Kaieda T, Omori A, Izumi R, Fujimura A, Kaitsuka T, Miyata K, Araki K, Oike Y, Scorrano L, Suzuki T, Tomizawa K (2018) Defective Mitochondrial tRNA Taurine Modification Activates Global Proteostress and Leads to Mitochondrial Disease. Cell Rep 22:482–496. doi:10.1016/j.celrep.2017.12.051
16. Wang X, Yan Q, Guan MX (2010) Combination of the loss of cmnm5U34 with the lack of s2U34 modifications of tRNALys, tRNAGlu, and tRNAGln altered mitochondrial biogenesis and respiration. J Mol Biol 395:1038–1048. doi:10.1016/j.jmb.2009.12.002
17. Li X, Li R, Lin X, Guan MX (2002) Isolation and characterization of the putative nuclear modifier gene MTO1 involved in the pathogenesis of deafness-associated mitochondrial 12 S rRNA A1555G mutation. J Biol Chem 277:27256–27264. doi:10.1074/jbc.M203267200
18. Yasukawa T, Suzuki T, Ueda T, Ohta S, Watanabe K (2000) Modification defect at anticodon wobble nucleotide of mitochondrial tRNAs(Leu)(UUR) with pathogenic mutations of mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes. J Biol Chem 275:4251–4257. doi:10.1074/jbc.275.6.4251
19. Suzuki T, Suzuki T (2014) A complete landscape of post-transcriptional modifications in mammalian mitochondrial tRNAs. Nucleic Acids Res 42:7346–7357. doi:10.1093/nar/gku390
20. Umeda N, Suzuki T, Yukawa M, Ohya Y, Shindo H, Watanabe K, Suzuki T (2005) Mitochondria-specific RNA-modifying enzymes responsible for the biosynthesis of the wobble base in

mitochondrial tRNAs. Implications for the molecular pathogenesis of human mitochondrial diseases. J Biol Chem 280:1613–1624. doi:10.1074/jbc.M409306200

21. Chuang JL, Chuang DT (2000) Diagnosis and mutational analysis of maple syrup urine disease using cell cultures. Methods Enzymol 324:413–423. doi:10.1016/s0076-6879(00)24250-x
22. Guan MX, Fischel-Ghodsian N, Attardi G (2001) Nuclear background determines biochemical phenotype in the deafness-associated mitochondrial 12S rRNA mutation. Hum Mol Genet 10:573–580. doi:10.1093/hmg/10.6.573
23. Bornstein B, Enriquez JA, Montoya J, Garesse R (1998) [Studies of pathogenicity and characterization of molecular phenotype caused by mutations in human mitochondrial DNA]. Rev Neurol 26(Suppl 1):S36–43
24. Gong S, Peng Y, Jiang P, Wang M, Fan M, Wang X, Zhou H, Li H, Yan Q, Huang T, Guan MX (2014) A deafness-associated tRNAHis mutation alters the mitochondrial function, ROS production and membrane potential. Nucleic Acids Res 42:8039–8048. doi:10.1093/nar/gku466
25. Enriquez JA, Attardi G (1996) Analysis of aminoacylation of human mitochondrial tRNAs. Methods Enzymol 264:183–196
26. Suzuki T, Suzuki T, Wada T, Saigo K, Watanabe K (2002) Taurine as a constituent of mitochondrial tRNAs: new insights into the functions of taurine and human mitochondrial diseases. EMBO J 21:6581–6589
27. Thorburn DR, Chow CW, Kirby DM (2004) Respiratory chain enzyme analysis in muscle and liver. Mitochondrion 4:363–375
28. Bjork GR, Hagervall TG (2014) Transfer RNA Modification: Presence, Synthesis, and Function. EcoSal Plus 6. doi:10.1128/ecosalplus.ESP-0007-2013
29. Bregeon D, Colot V, Radman M, Taddei F (2001) Translational misreading: a tRNA modification counteracts a +2 ribosomal frameshift. Genes Dev 15:2295–2306. doi:10.1101/gad.207701
30. Boutoual R, Meseguer S, Villarroya M, Martin-Hernandez E, Errami M, Martin MA, Casado M, Armengod ME (2018) Defects in the mitochondrial-tRNA modification enzymes MTO1 and GTPBP3 promote different metabolic reprogramming through a HIF-PPARgamma-UCP2-AMPK axis. Sci Rep 8:1163. doi:10.1038/s41598-018-19587-5
31. Meseguer S, Boix O, Navarro-Gonzalez C, Villarroya M, Boutoual R, Emperador S, Garcia-Arumi E, Montoya J, Armengod ME (2017) microRNA-mediated differential expression of TRMU, GTPBP3 and MTO1 in cell models of mitochondrial-DNA diseases. Sci Rep 7:6209. doi:10.1038/s41598-017-06553-w
32. Giege R, Sissler M, Florentz C (1998) Universal rules and idiosyncratic features in tRNA identity. Nucleic Acids Res 26:5017–5035. doi:10.1093/nar/26.22.5017
33. Meng F, Cang X, Peng Y, Li R, Zhang Z, Li F, Fan Q, Guan AS, Fischel-Ghosian N, Zhao X, Guan MX (2017) Biochemical Evidence for a Nuclear Modifier Allele (A10S) in TRMU (Methylaminomethyl-2-thiouridylate-methyltransferase) Related to Mitochondrial tRNA Modification in the Phenotypic

Manifestation of Deafness-associated 12S rRNA Mutation. J Biol Chem 292:2881–2892. doi:10.1074/jbc.M116.74937

34. Andries G, Yandrapalli S, Naidu SS, Panza JA (2018) Novel Pharmacotherapy in Hypertrophic Cardiomyopathy. Cardiol Rev 26:239–244. doi:10.1097/CRD.0000000000000211

# Figures

### Figure 1

MTO1 depletion caused aberrant mitochondrial tRNA metabolism. (A) 2-thiouridylation (thio) level analysis of mt-tRNAs. Equal amounts (10 μg) of total RNAs were separated by polyacrylamide gel electrophoresis that contains 0.05 mg/ml APM and electroblotted onto a positively charged membrane and hybridized with DIG-labeled oligonucleotide probes specific for the $tRNA^{Lys}$. The blots were then stripped and rehybridized with DIG-labeled probes for $tRNA^{Glu}$ and $tRNA^{Gln}$, respectively. The retarded bands of 2-thiolated tRNAs and non-retarded bands of tRNA without thiolation are marked. (B). Decrease in 2-thiouridylation levels of mt-tRNAs. The values are expressed as ratio of 2-thiolated tRNA/ (2-thiolated tRNA + unthiolated tRNA). (C) Aminoacylation level analysis of mt-tRNAs. Total RNAs were specifically probed for the $tRNA^{Lys}$, $tRNA^{Leu(UUR)}$, and $tRNA^{Tyr}$, respectively. (D) Quantification of aminoacylated proportions of $tRNA^{Lys}$, $tRNA^{Leu(UUR)}$, and $tRNA^{Tyr}$ in the mutant and controls. The values are expressed as ratio of aminoacylated tRNA/ (aminoacylated tRNA + unaminoacylated tRNA). (E) Steady state analysis of mt-tRNAs. Ten μg of total RNA from various cell lines were electrophoresed through a denaturing polyacrylamide gel, electroblotted and hybridized with DIG-labeled oligonucleotide probes specific for the $tRNA^{Lys}$, $tRNA^{Glu}$, $tRNA^{Gln}$, $tRNA^{Leu(UUR)}$, and $tRNA^{Tyr}$ as well as 5S rRNA, respectively. (F) Average relative level of each tRNA. The values for the shMTO1 cell lines are expressed as percentages of the average values for the scramble cell lines. (G) Western blot analysis of two shMTO1 and two scramble cell lines. Twenty μg of total cellular proteins from various cell lines were electrophoresed through a denaturing polyacrylamide gel, electroblotted and hybridized with antibodies against MTO1, TRMU, GTPBP3, respectively, and with TOM20 as a loading control. The calculations were based on three independent determinations of each tRNA in each cell line. The error bars indicate standard deviation (unidirectional labeling to avoid confusion with the column pattern); P indicates the significance, according to Student's t test, of the difference between mutant shMTO1 and control scramble for each tRNA.

### Figure 2

Alterations in mitochondrial tRNA led to reduced levels of mitochondrial proteins. (A) Twenty micrograms of total cellular proteins from various cell lines were electrophoresed through a denaturing

polyacrylamide gel, electroblotted and hybridized with antibodies specific for ND3, ND4, ND6, CO2, ATP8, and CYTB and with TOM20 as a loading control. (B) Quantification of total mitochondrial protein levels. Average relative ND3, ND4, ND6, CO2, ATP8, and CYTB proteins, normalized to the average content per condition of TOM20 in two scramble cells. (C) Western blotting analysis for subunits of each OXPHOS complex including ATP5A, UQCRC2, SDHB, CO2 and NDUFB8 in mutant and control cell lines, with TOM20 as a loading control. (D) Quantification of the subunits. Average relative OXPHOS proteins, normalized to the average content per condition of TOM20 in two scramble cells. Calculations were based on three independent determinations. The error bars indicate standard deviation (unidirectional labeling to avoid confusion with the column pattern); P indicates the significance, according to Student's t test, of the difference between shMTO1 and scramble for each protein.

## Figure 3

Defects in mitochondrial translation gave rise to respiration deficiency. (A) Analysis of the rates of $O_2$ (OCR) in cell lines using different inhibitors. OCR was first measured on $2\times10^4$ cells of each cell line under basal condition and then sequentially added oligomycin (1.5 μM), carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP) (0.5 μM), antimycin A (1 μM) and rotenone (1 μM) at indicated times to determine different parameters of mitochondrial functions. (B) Graphs presented the ATP-linked OCR, proton leak OCR, maximal OCR, reserve capacity and non-mitochondrial OCR in mutant and control cell lines. Non-mitochondrial OCR was determined as the OCR after rotenone/antimycinA treatment. Basal OCR was determined as OCR before oligomycin minus OCR after rotenone/antimycin A. ATP-linked OCR was determined as OCR before oligomycin minus OCR after oligomycin. Proton leak was determined as Basal OCR minus ATP-linked OCR. Maximal was determined as the OCR after FCCP minus non-mitochondrial OCR. Reserve Capacity was defined as the difference between Maximal OCR after FCCP minus Basal OCR. OCR values were expressed in picomoles of oxygen/minute/microgram of protein. (C) The activities of respiratory complexes were investigated by enzymatic assay on complexes I, II, III, IV and V in mitochondria isolated from various cell lines. (D) Measurement of mitochondrial ATP levels. shMTO1 and control cell lines were incubated with 5 mM 2-deoxy-D-glucose plus 5 mM pyruvate to determine ATP generation under mitochondrial ATP synthesis. Average rates of ATP level per cell line in mitochondria are shown. (E) Representative flow cytometry images of two shMTO1 cell lines and two scramble cell lines using a fluorescence probe JC-10 assay system. (F) The mitochondrial membrane potential (ΔΨm) was measured in various lines as relative ratio of JC-10 fluorescence intensities. The ratio of fluorescence intensities at Ex/Em=490/590 nm and 490/530 nm (FL590/FL530) were recorded to delineate the ΔΨm level of each sample. The relative ratios of cell in gate P3 were calculated to reflect the level of ΔΨm. The calculations were based on 3-5 independent experiments.

## Figure 4

Dysfunctional mitochondria altered apoptotic state in stress. (A) Representative Annexin-V/PI staining for various cell lines incubated with $H_2O_2$. (B) Quantitation of Annexin-V staining data. (C) Western blotting analysis of five apoptosis-related proteins. Twenty micrograms of total proteins from various cell lines were electrophoresed, electroblotted and hybridized with Cytochrome C and cleaved Caspases 3, 7, 9 and PARP antibodies and with GAPDH as a loading control. (D) Quantification of five apoptosis-related proteins. Average relative levels of Cytochrome C and cleaved Caspases 3, 7, 9 and PARP proteins, normalized to the average content per condition of GADPH in two scramble and two shMTO1 cells. Three independent determinations were done in each cell line.

## Supplementary Files

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