Cell Biology International ISSN 1065-6995
doi: 10.1002/cbin.11274

RESEARCH ARTICLE

Melatonin reverses the oxidative stress and mitochondrial dysfunction caused by *LETM1* silencing

Cenk Aral*, Seyma Demirkesen, Rıfat Bircan and Duygu Yasar Sirin

Department of Molecular Biology and Genetics, Faculty of Science and Arts, Namık Kemal University, 59030, Tekirdağ, Turkey

Abstract

LETM1 is a mitochondrial inner-membrane protein, which is encoded by a gene present in a locus of 4p, which, in turn, is deleted in the Wolf–Hirschhorn Syndrome, and is assumed to be related to its pathogenesis. The cellular damage caused by the deletion is presumably related to oxidative stress. Melatonin has many beneficial roles in protecting mitochondria by scavenging reactive oxygen species, maintaining membrane potential, and improving functions. The aim of this study was to investigate the effects of melatonin administration to *LETM1*-silenced mouse embryonic fibroblast cells as a cellular model for *LETM1* deficiency. We transfected mouse embryonic fibroblast cells with a pair of siRNA against *LETM1* and monitored the oxidative stress and mitochondrial functions with or without melatonin addition. MnSOD expression and aconitase activity decreased and oxidized protein levels increased in *LETM1*-silenced cells. *LETM1* suppression did not alter the expression of OXPHOS complexes, but the oxygen consumption rates decreased significantly; however, this change was not related to complex I but instead involved complex IV and complex II. Melatonin supplementation effectively normalized the parameters studied, including the oxygen consumption rate. Our findings identified a novel effect of *LETM1* deficiency on cellular respiration via complex II as well as a potential beneficial role of melatonin treatment. On the other hand, these effects may be specific to the cell line used and need to be verified in other cell lines.

Keywords: *LETM1*; melatonin; MnSOD; oxidative phosphorylation complexes; oxidative stress; Wolf–Hirschhorn Syndrome

Introduction

Wolf–Hirschhorn Syndrome (WHS, OMIM#194190) is a congenital malformation syndrome that is characterized by pre- and postnatal growth retardation, mental defects, microcephaly, characteristic craniofacial features, and seizures (Hirschhorn et al., 1965; Wolf et al., 1965). It is caused by the partial deletion of the distal arm of chromosome 4p and the frequency is estimated at 1/20,000 to 1/50,000 births (Battaglia et al., 1999). The leucine zipper EF-hand-containing transmembrane protein-1 (*LETM1*) gene is present in one of the disease contributor loci of 4p, namely the WHS critical region-2 (*WHSCR-2*) and it has been suggested that the loss of *LETM1* protein is responsible for seizures in WHS patients (Dimmer et al., 2008; Tamai et al., 2008).

LETM1 protein is located in the inner membrane of mitochondria. *LETM1* has a prominent function in mitochondrial K^+ and Ca^{2+} homeostasis (Austin and Nowikovsky, 2019). Although studies assessing the effects of *LETM1* deficiency are limited, some studies have reported that the deficiency of *LETM1* results in changes in mitochondrial morphology and membrane potential, the disruption of ion homeostasis, defects in the formation of oxidative phosphorylation (OXPHOS) complexes, altered oxygen consumption rate (OCR), cell death, and oxidative stress; and that these changes may play a role in the pathogenesis of WHS (Dimmer et al., 2008; McQuibban et al., 2010; Hart et al., 2014; Nowikovsky and Bernardi, 2014). Doonan et al. (2014) reported that mitochondrial function was normalized with an increase in the antioxidant defense by gene transfer (MnSOD/GPX), and that it is therefore functional in the pathogenesis of the disease. Dimmer et al. (2008) reported mitochondrial swelling in

*Corresponding author: e-mail: caral@nku.edu.tr

Abbreviations: *LETM1*, leucine zipper EF-hand-containing transmembrane protein-1; MEF, mouse embryonic fibroblast; OCR, oxygen consumption rate; OXPHOS, oxidative phosphorylation; RCR, respiratory control ratio; ROS, reactive oxygen species; WHS, Wolf–Hirschhorn syndrome; WHSCR-2, WHS critical region-2

LETM1-deficient cells without a change in OXPHOS complexes and mitochondrial OCR. In contrast, Tamai et al. (2008) showed a significant reduction in the formation of complex I, III, and IV, and Doonan et al. (2014) showed reduced mitochondrial respiration. Similarly, Quan et al. (2015) also showed a reduction in the number of OXPHOS complexes and a disruption of the mitochondrial membrane potential. It has been suggested that oxidative stress may be an important contributor to these abovementioned cellular dysfunctions (Doonan et al., 2014; Hart et al., 2014; Quan et al., 2015).

Many studies have reported that melatonin plays a role in detoxification mechanisms of free radicals and the stimulation of other cellular anti-oxidant mechanisms (Acuna Castroviejo et al., 2002; Acuna Castroviejo et al., 2011; Escames et al., 2010; Paradies et al., 2015). Melatonin is a hormone, primarily secreted by the pineal gland and other organs and tissues such as retina, bone marrow cells, skin, and is effective in many intracellular compartments and in mitochondria. Furthermore, the protective effect of melatonin has been demonstrated to be more effective than vitamin E and MitoQ (mitochondria-targeted coenzyme Q), and it has been shown to be functional in increasing the activity of mitochondrial OXPHOS complexes, normalizing membrane potential, and normalizing OCR as well as detoxifying the reactive oxygen species (ROS) (Martin et al., 2000, 2002; Jou et al., 2007; Tapias et al., 2009; Paradies et al., 2015). Although previous studies have focused on the effects of *LETM1* deficiency on mitochondrial physiology, to date there has been no study on the effect of melatonin administration in *LETM1* deficiency, which may mimic the effects of melatonin in WHS.

We, therefore, hypothesized that melatonin may effectively enhance the mitochondrial function by reducing the oxidative stress in *LETM1*-silenced cells. We aimed to assess this by (1) monitoring the effects of *LETM1* suppression on mitochondrial dysfunction and ROS formation in a non-cancerous mouse embryonic fibroblast (MEF) cell line and (2) demonstrating the beneficial effects of melatonin on reducing *LETM1*-deficiency induced oxidative stress and mitochondrial dysfunction.

Materials and Methods

Reagents

All cell culture media and additives were purchased from Gibco (USA). Melatonin and all other reagents were obtained from Sigma-Aldrich (Germany) unless otherwise indicated. All buffers were prepared with MilliQ (USA) ddH_2O.

Antibodies

Antibodies used were as follows: polyclonal anti-*LETM1* (1:1,000, cat.no.NBP1–33433; Novus Biologicals, USA), polyclonal anti-GRP75 (1:1,000, cat.no.sc-13967; Santa Cruz Biotechnology, USA), polyclonal anti-MnSOD (1:500, cat.no.NB100–1992; Novus Biologicals), and monoclonal anti-Actin (1:1,000; Novus Biologicals). To determine the OXPHOS complexes, monoclonal anti-NDUFA9 (cat.no.ab14713), anti-core I (cat.no.ab110252), and anti-subunit IV (cat.no.ab14744) antibodies were used in a 1:1,000 final concentration (Abcam, USA). In addition, a cocktail of antibodies against OXPHOS complexes were used at 1:1000 dilution (cat.no.457999; Life Technologies, USA). Secondary horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG antibodies were obtained from Santa Cruz Biotechnology.

Cell culture, transfection, and melatonin treatment

The MEF cells were kindly provided by Dr. L. Scorrano (Padova University, Italy). The cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL uridine and kept at 37°C in a 5% CO_2 atmosphere. The cells were regularly examined for any microbiological contamination and all cell culture experiments were conducted on cells up to a maximum of 20 passages. For the transfection experiments, the cells were trypsinized, stained with trypan blue, and counted. A total of 9×10^4 cells and 90×10^4 cells were seeded in six-well plates and 10 cm tissue culture dishes, respectively. Two siRNAs against *LETM1* (i.e., *LETM1* mRNA) were synthesized from the following sequences 5′-auuccgcugccuguaguutt-3′ and 5′-ccacuuaauaauucuuuaatt-3′ and were used in combination, each at a final concentration of 100 nM. *LETM1*-silenced group is hereafter referred to as siRNA^{Letm1}. As a control group, a negative silencer siRNA was used in the same final concentration. All siRNAs were purchased from Ambion (UK). All transfections were performed with Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. To investigate the effect of melatonin, the cells were incubated with 100 or 200 μM final concentrations of melatonin for 24 or 48 h before the samples were collected (Jou et al., 2007). For these experiments, melatonin was solubilized in the cell culture grade, dimethyl sulfoxide (DMSO), and diluted in the culture medium before use. Thus, the final concentrations of DMSO were 0.002% and 0.004% for 100 and 200 micromolar treatments, respectively. For all experiments, control cells were treated with the same dose of DMSO to eliminate potential confounding effects of this compound.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

The cells were washed with 1× phosphate-buffered saline (PBS) and lyzed with radioimmunoprecipitation assay cell lysis buffer containing protease inhibitors (Santa Cruz Biotechnology) to determine the abundance of *LETM1*, TOMM20, GRP75, and Actin. The amount of total protein was determined using Bradford's reagent (Bio-Rad, USA). The proteins were electrophoresed on a 10% SDS-PAGE using pre-cast Tris-Glycine polyacrylamide gels (Life Technologies) and were blotted on polyvinylidene difluoride membranes (PVDF; Santa Cruz Biotechnology). The membranes were blocked with 5% non-fat dry milk dissolved in Dulbecco's PBS containing 0.05% Tween 20 (PBS-T) for 1 h and probed using the corresponding antibodies overnight at 4°C. After three washes with PBS-T, the blots were incubated for 1 h at 25°C with HRP-conjugated secondary antibodies followed by detection by chemiluminescence. Images were quantified using ImageJ (https://imagej.nih.gov/ij/index.html). Sample chemiluminescent exposures were shown for clarity but horizontally cropped to include all lanes.

Fluorescence microscopy

The siRNA^{Letm1} transfected and control cells with or without melatonin treatment were co-stained with MitoTracker Green (Life Technologies) and MitoSOX (Life Technologies) according to the manufacturer's instructions and examined under a fluorescence microscope. Images were merged using ImageJ.

Aconitase activity

Aconitase (aconitate hydratase; EC 4.2.1.3) is an iron–sulfur protein that catalyzes the reversible interconversion of citrate and isocitrate, via a cis-aconitate intermediate, in both the TCA and glyoxylate cycles. The active form of the enzyme is inhibited by citrate analogs, fluoracetate, and oxidative stress agents such as peroxynitrite, hydrogen peroxide, and superoxide. Thus, it is considered as a good marker of mitochondrial and cellular oxidative stress. In this study, aconitase activity was determined using an aconitase assay kit (Abnova, Taiwan) according to manufacturer's recommendations. The aconitase activity was expressed as mU/mL/mg protein.

Detection of oxidized proteins

Oxidation of proteins is an important consequence of oxidative stress. The changes in oxidized protein levels were determined using an Oxyblot kit (Chemicon/Millipore, USA) according to the manufacturer's recommendations. In brief, proteins were derivatized with 4-dinitrophenylhydrazine (DNP) for 15 min and then incubated at 25°C with a neutralization buffer. The derivatized proteins were electrophoresed on a 10% SDS-PAGE and immunoblotted, as previously described, using an anti-DNP primary antibody provided in the kit. In the positive control experiment, untransfected cells were incubated with H_2O_2 for 24 h prior to protein extraction.

Blue native gel electrophoresis (BN-PAGE)

BN-PAGE is a simple and reliable method for the determination of formation and expression of mitochondrial respiratory supercomplexes as a whole. To monitor the difference in expression or formation of the supercomplexes in *LETM1*-supressed cells, we performed BN-PAGE. For this purpose, mitochondria from transfected and control cells were isolated as previously described (Frezza et al., 2007). The cells were homogenized using a teflon pestle operated at 1,600 rpm in a glass potter after being collected by scraping and centrifugation. The homogenate was centrifuged at 600*g* for 10 min at 4°C. The supernatant was collected and centrifuged at 7,000*g* for 10 min at 4°C to collect mitochondria. The supernatant was discarded and the pellet was suspended in ice-cold Tris/MOPS-EGTA/Tris buffer. The samples were prepared with the NativePAGE Sample Prep Kit and electrophoresis was conducted using the NativePAGE Novex Bis-Tris Gel System according to the manufacturer's instructions. The proteins were solubilized by digitonin (Thermo Fisher Scientific, USA) (1:6 g/g) and a total of 20 μg mitochondrial proteins were separated on 4–16% gradient gel. The proteins were then transferred to a PVDF membrane and immunoblotted as previously stipulated using the appropriate antibodies. To eliminate the influence of the antibody cocktail, immunoblotting was performed using either an antibody cocktail for complex I, III, and IV or antibodies for each complex separately. Due to discrepancy in the literature, the experiments were replicated 10 times to increase the sensitivity of the assessment.

Oxygen consumption assay

The OCR is a valuable indicator of mitochondrial function under typical cell culture conditions (Fernandez-Gil et al., 2019). The mitochondrial respiration of digitonin-permeabilized cells was measured polarographically using a Clark oxygen electrode (Hansatech Instruments, UK) at 37°C with constant stirring (60 rpm), as previously described (Frezza et al., 2007; Divakaruni et al., 2014). The cells ($\times 10^7$) were added to 0.5 mL of a respiration medium composed of 0.02% digitonin, 50 mM KCl, 10 mM Tris, 1.5 mM Pi, 1 mM EGTA, 3 mM $MgCl_2$, and 1 mg/mL fatty acid-free BSA. Following 2 min incubation, respiration was initiated by the addition of

NADH substrates (i.e., 5 mM glutamate and 2.5 mM malate) to energize the mitochondria via complex I. ADP (200 μM) was then added and state 3_{ADP} respiration was recorded. State 4_{oligo} respiration was recorded after the addition of oligomycin (concentration: 2 μM), an inhibitor of ATPase. Finally, 0.4 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was added to record uncoupled respiration. To assess respiration via complex II, succinate (10 mM) was used as the respiration substrate. Complex I was inhibited by the addition of rotenone (2 μM) and state 3 and 4 OCR were recorded as previously stipulated. Respiratory control ratio (RCR) was defined as state 3/state 4. Lastly, we used ascorbate (10 mM) plus *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD, 0.2 mM), which donate electrons directly to cytochrome *c* in complex IV, as the respiratory substrates for the estimation of oxidation in complex IV. After 5 min of recording, 700 μM potassium cyanide (KCN) was added to block respiration and the oxygen consumption was then recorded for another 5 min. The final respiratory rate was obtained by subtracting the KCN insensitive respiration rate. The measurements for different substrates were conducted as separate experiments and all experimental groups were evaluated on the same day.

Statistical analyses

Values were presented as means ± standard deviation and *N* is the number of independent experiments. *P* values were obtained by randomized block-analysis of variances with Dunnett's multiple comparisons test (Lew, 2007) and were considered significant at $P < 0.05$.

Results

Detection of hemizygous *LETM1* silencing in MEF cells

To address the effect of *LETM1* knockdown on oxidative stress, MEF cells were transfected with siRNA targeted to *LETM1* or negative silencer siRNA. The transfection efficiency was checked by immunoblotting for every experiment. As shown in Figure 1, the transfection of MEF cells with siRNA^{Letm1} revealed that the protein level decreased by ~50% compared with the control group ($P < 0.001$). To exclude the possibility that siRNA transfection led to reduced mitochondrial mass, we checked the GRP75 and TOMM20 levels, and found no significant differences ($P > 0.05$). GRP75 is primarily localized in mitochondria but is also found in other parts of the cell such as the endoplasmic reticulum and cytoplasmic vesicles. TOMM20 is a member of translocase of the outer mitochondrial membrane. Our findings suggested that the decrease in *LETM1* protein levels was not a result of a reduction in the number of mitochondria.

Detection of the *LETM1*-knockdown effects on oxidative stress and mitochondrial anti-oxidant defense in MEF cells

MitoSOX, a derivative of hydroethidine, has been widely used to detect mitochondrial ROS. In this study, we found that MitoSOX staining showed an increased oxidative stress with *LETM1* suppression (Figure 2). Co-localization of mitoSOX signal with mitochondria-specific MitoTracker

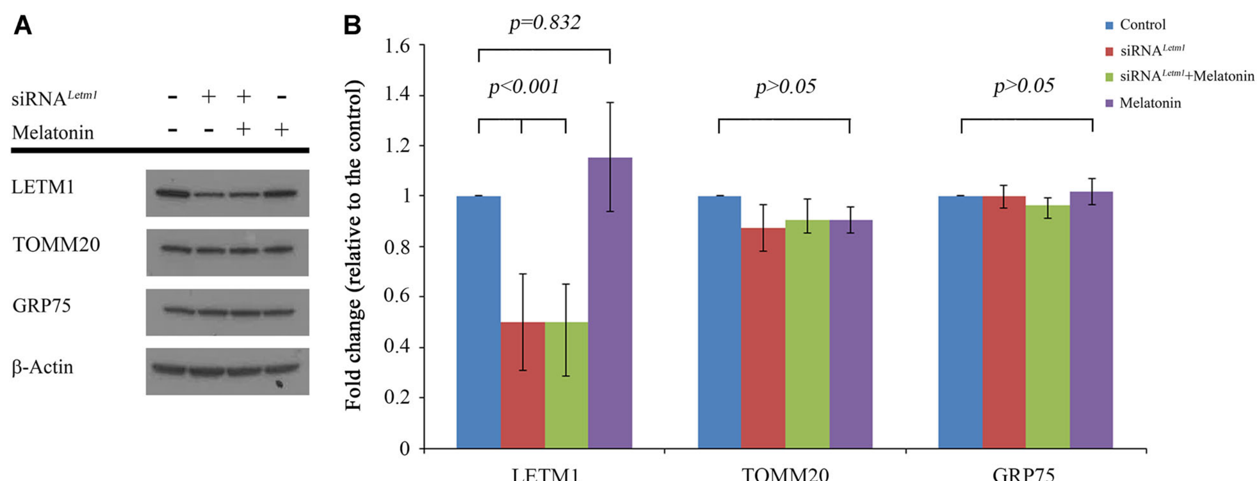

Figure 1 siRNALetm1 transfection reduced LETM1 expression ~50% without affecting the mitochondrial mass. LETM1, GRP75, and TOMM20 levels in negative silencer siRNA-transfected control cells, siRNALetm1 cells, and melatonin-treated cells were determined by immunoblotting. (A) An immunoblot sample of target proteins. (B) Densitometric quantification of 10 independent immunoblots. Data are presented as mean ± standard deviation of 10 independent experiments. Randomized block-analysis of variance with Dunnett's multiple comparisons test was used.

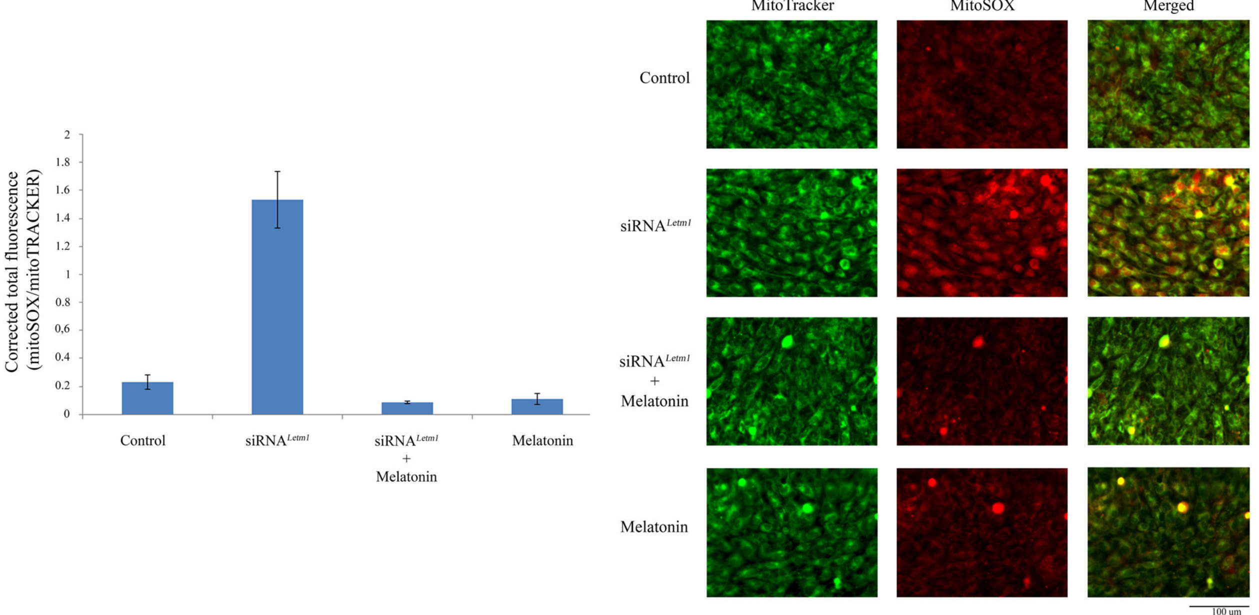

Figure 2 Right: Generation of reactive oxygen species among the experimental groups. The cells were co-stained with Mitotracker green (left panel) and MitoSOX (middle panel). Yellow dots indicate co-localized signal in the merged images (right panel). Left: Representative quantification of Mitosox fluorescence (mean ± standard deviation) calculated by ImageJ analyzer as described ($N = 3$) (https://theolb.readthedocs.io/en/latest/imaging/measuring-cell-fluorescence-using-imagej.html).

Green indicates that mitochondria is the primary source of ROS in *LETM1*-silenced cells.

Aconitase activity, a biomarker of the oxidative stress, decreased fivefold by *LETM1* suppression compared with the control group ($P < 0.001$, 1.45 ± 0.34 vs. 9.79 ± 1.3, respectively) (Figure 3). MnSOD is localized in mitochondria and is an anti-oxidant defense enzyme that plays an important role against the oxidative stress. In this study, surprisingly, we found that the MnSOD level significantly decreased after *LETM1* suppression with siRNA^{Letm1} transfection ($P < 0.001$) (Figure 4). This observed decrease in the MnSOD level might indicate that the cellular defense against oxidative stress was reduced in *LETM1*-suppressed cells. Then, the overall oxidation of cellular proteins was analyzed and a notable increase in carbonylated protein levels was observed in siRNA^{Letm1}-transfected cells but not in control cells (Figure 5). Collectively, our findings showed that a 50% reduction in *LETM1* caused an increase in ROS production in mitochondria, oxidation of cellular proteins, inhibition of aconitase activity, and a decrease in mitochondrial anti-oxidant defense.

Effects of *LETM1* silencing on OXPHOS complexes and OCR

The presence of OXPHOS complexes is shown in Figure 6. To confirm the presence of protein complexes, mitochondrial membrane proteins were solubilized with digitonin and separated by BN-PAGE. An antibody mix against OXPHOS complexes was used and no difference was found between siRNA^{Letm1} and control group (data not shown). As this finding was contradictory to some findings in the literature (Tamai et al., 2008), we used antibodies against complexes I, III, and IV separately. These complexes are responsible for proton pumping to the inner membrane space via the process of OXPHOS and it has been

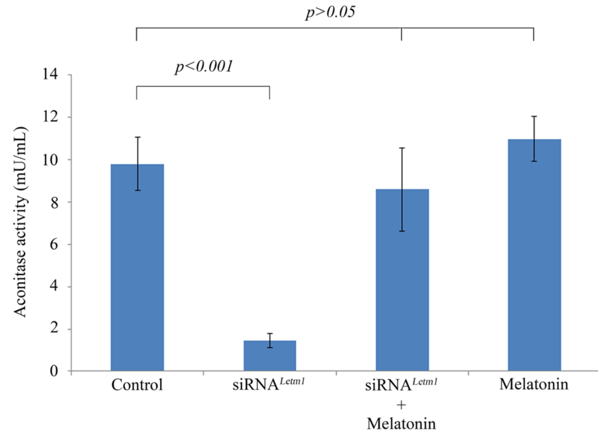

Figure 3 Change in aconitase activity among the experimental groups was determined colorimetrically. Data are presented as mean ± standard deviation ($N = 12$). Randomized block-analysis of variance with Dunnett's multiple comparisons test was used.

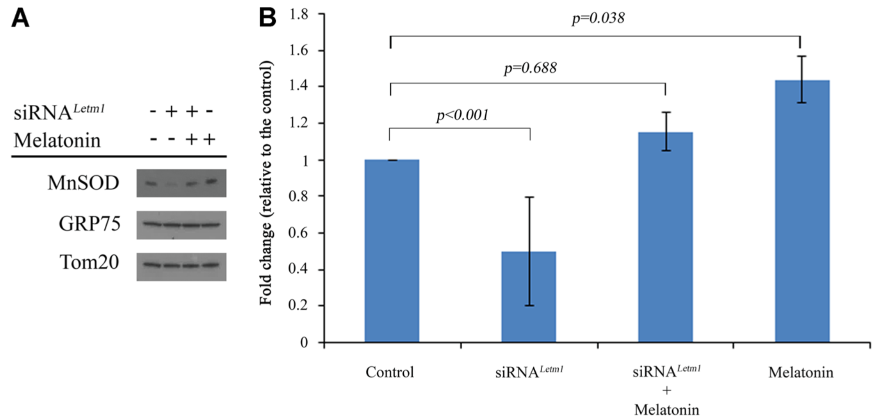

Figure 4 Melatonin promoted diminished MnSOD levels in LETM1-silenced cells. (A) Sample immunoblot image. (B) Densitometric quantification of five independent blots. Band intensity and fold change (relative to the control group) were calculated as described in Figure 1, however, GRP75 was used instead of actin. Data are presented as mean ± standard deviation ($N = 10$). Randomized block-analysis of variance with Dunnett's multiple comparisons test was used.

determined that *LETM1* suppression has no effect on the assembly of the complexes (Figure 6). No difference in the formation of complexes was detected in any of the replicated experiments.

In the final step of our study, we investigated the efficiency of the mitochondrial OXPHOS system using real-time oxygen measurements. Glutamate and malate were used as the substrates in the OCR for complex I. No change was detected between the control and *LETM1*-suppressed groups in state 3 respiration after addition of ADP to the system (Figure 7A) ($P > 0.05$). Also, there was no difference between the control and *LETM1*-suppressed groups in state 4 respiration after oligomycin addition (Figure 7B). As expected from previous findings, the RCR was similar in the *LETM1*-suppressed and control groups ($P > 0.05$) (Figure 7D). In addition, FCCP-mediated uncoupled respiration was similar between the experimental groups ($P > 0.05$) (Figure 7C). To estimate the efficiency of complex II-dependent respiration, complex I activity was inhibited by rotenone and the measurements performed with the addition of succinate. As expected, the OCR increased after succinate addition in the presence of rotenone in the system, however, the state 3 respiration, which was sustained with ADP addition, was significantly lower in the *LETM1*-suppressed group than in the control group ($P = 0.002$) (Figure 8A). There was no significant difference between the control and *LETM1*-suppressed groups in state 4 respiration, which was sustained with oligomycin addition ($P > 0.05$) (Figure 8B). RCR was also significantly lower in the *LETM1*-suppressed group, which was consistent with the results from the state 3 respiration assessments ($P = 0.029$) (Figure 8C). The oxygen consumption was examined in the presence of TMPD and ascorbate, and OCR was found to be lower in *LETM1*-suppressed cells than the control ($P = 0.05$) (Figure 9). However, this finding was not as statistically significant as that of complex II. In summary, our study results indicated that *LETM1* suppression led to impaired function in the OXPHOS complexes.

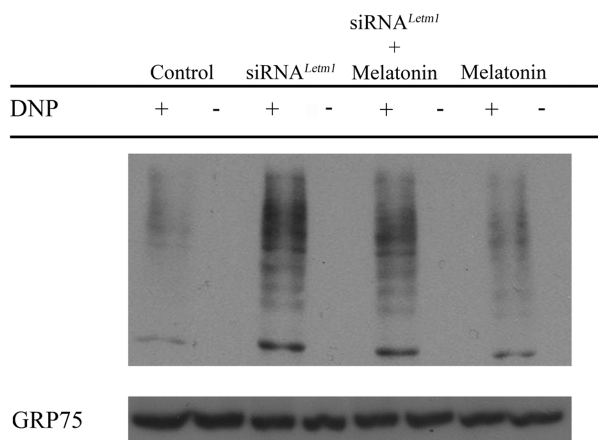

Figure 5 Total protein isolated from the cells in each experimental group and 4-dinitrophenylhydrazine (DNP)-derivatized and underivatized samples were loaded to sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels. After blotting, oxidatively damaged proteins were visualized using DNP-specific antibody (top blot). In this blot, empty lanes represent underivatized samples. After stripping, membranes were incubated with anti-GRP75 antibody as loading control (bottom blot). Although the carbonylated proteins in the *LETM1*-silenced cells increased, melatonin treatment reduced this effect ($N = 3$).

Melatonin administration restored the mitochondrial anti-oxidant defense, decreased ROS production, and improved impaired OCR in *LETM1*-knockdown cells

We tested different incubation times (24 and 48 h) and doses (100–200 μM) of melatonin and found that 24 h

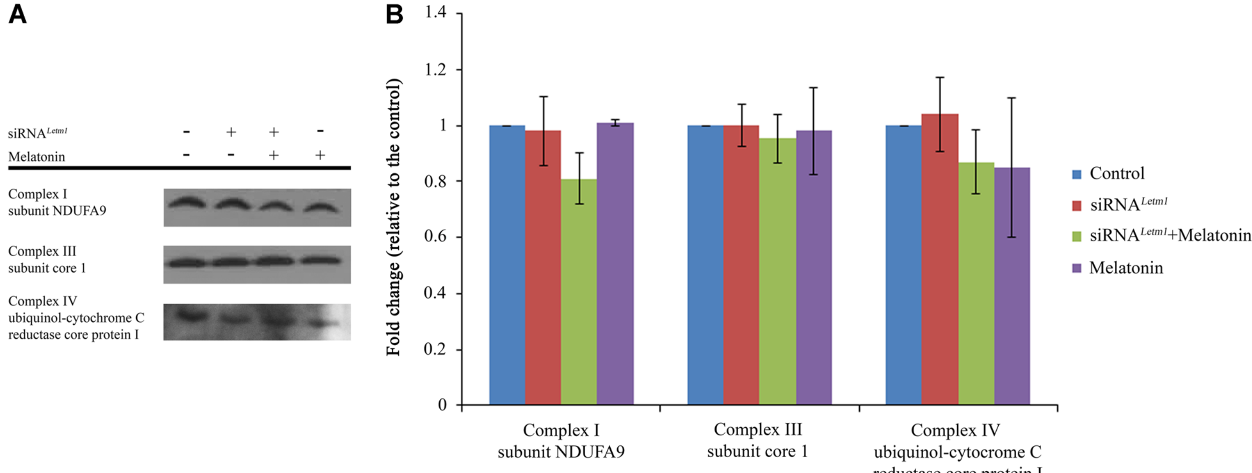

Figure 6 Neither *LETM1* silencing nor melatonin supplementation affected the formation of oxidative phosphorylation complexes. (A) Sample immunoblot image. (B) Densitometric quantification of 10 independent blots. Data are presented as mean ± standard deviation. Randomized block-analysis of variance with Dunnett's multiple comparisons test was used.

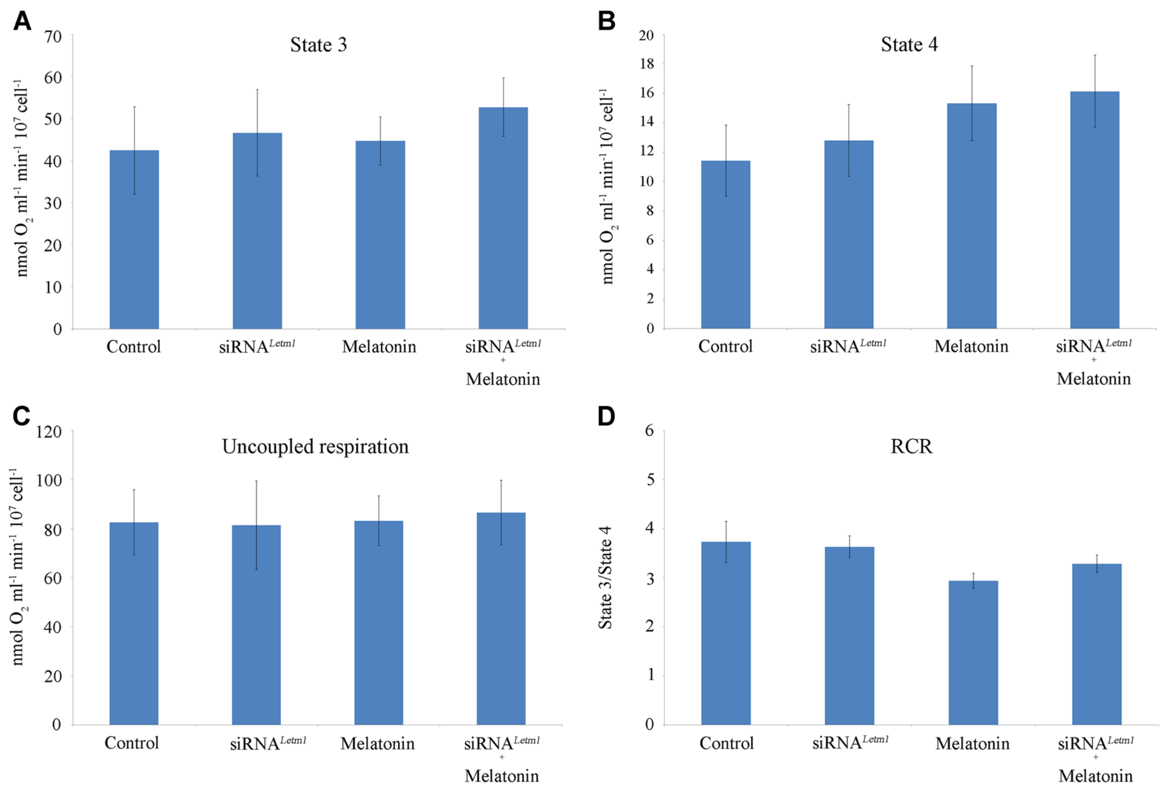

Figure 7 Oxygen consumption rate did not differ in digitonin-permeabilized cells in each experimental group supplied with glutamate and malate as complex I substrates. State 4 respiration was higher only in the presence of melatonin. (A) State 3 respiration obtained by the addition of ADP. (B) State 4 respiration determined after addition of oligomycin. (C) Uncoupled respiration in the presence of *p*-trifluoromethoxyphenylhydrazone (FCCP). (D) Respiration control rate (RCR) among groups was calculated as state 3/state 4 ratio. Data are presented as mean ± standard deviation ($N = 10$). Randomized block-analysis of variance with Dunnett's multiple comparisons test was used.

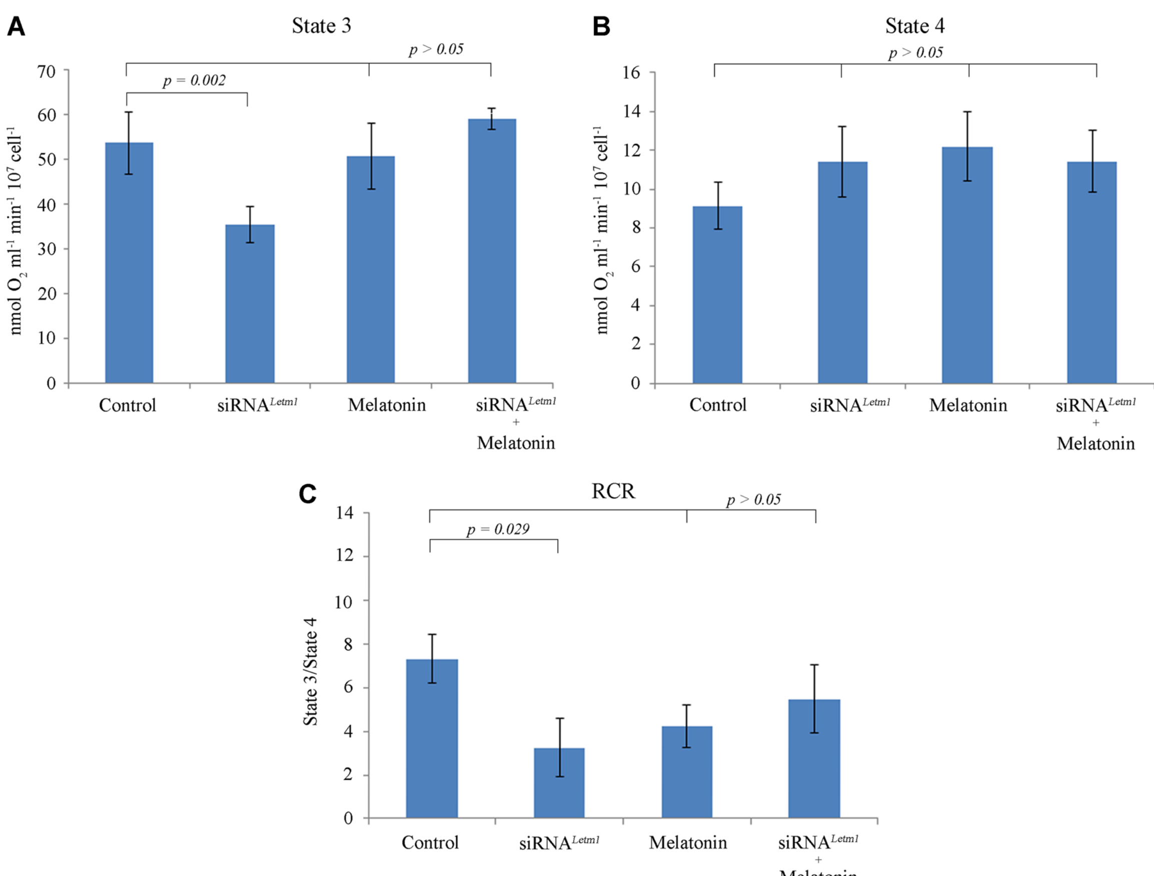

Figure 8 *LETM1* silencing interfered with state 3 respiration in permeabilized mouse embryonic fibroblast (MEF) cells supplied with succinate as complex II substrate and rotenone as complex I inhibitor. Melatonin supplementation was beneficial in reversing this effect. (A) State 3 respiration driven by the addition of ADP. (B) State 4 respiration obtained with the addition of oligomycin. (C) Respiration control rate calculated as state 3/state 4. Data are presented as mean ± standard deviation ($N = 10$). Randomized block-analysis of variance with Dunnett's multiple comparisons test was used.

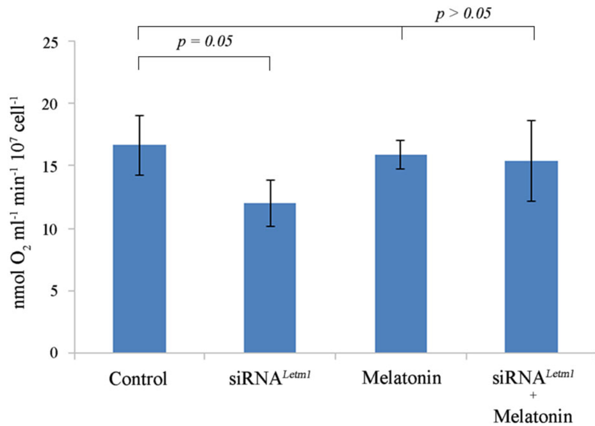

Figure 9 Reduced respiration driven by *N,N,N′,N′*-tetramethyl-p-phenylenediamine (TMPD)/ascorbate in the *LETM1*-silenced cells restored with melatonin supplementation. Data are presented as mean ± standard deviation ($N = 10$). Randomized block-analysis of variance with Dunnett's multiple comparisons test was used.

incubation with 100 μM melatonin had no effect on cell viability, as determined by trypan blue staining (Data not shown). We also found no differences in the effects of melatonin treatment on *LETM1*, TOMM20, and GRP75 levels ($P > 0.05$) (Figure 1).

On the other hand, *LETM1* knockdown reduced the MnSOD level by 50%, however, it was determined that the MnSOD levels were the same as the control group in the *LETM1*-knockdown cells supplemented with 100 μM melatonin ($P = 0.688$) (Figure 4). The aconitase activity was also restored in parallel to MnSOD in the *LETM1*-knockdown MEF cells after melatonin administration. The aconitase activity in these cells were found to be similar as in those of the control group after 100 μM melatonin supplementation ($P > 0.05$) (Figure 3). As seen in Figure 5, there was also a decrease in the oxidized protein levels in melatonin-supplemented *LETM1*-silenced cells. As previously indicated, MitoSOX staining showed increased oxidative stress with *LETM1* suppression but the oxidative

stress was seen to have been normalized in MEF cells after melatonin supplementation (Figure 2).

When the effects of melatonin on cellular respiration were examined, the OCRs for glutamate and malate-energized mitochondria state 3 respiration did not differ between the experimental groups ($P > 0.05$) (Figure 7A). Interestingly, state 4 respiration, which was sustained with oligomycin addition, was found to have been higher in the siRNA^{Letm1} + melatonin group than in the control group ($P = 0.039$) (Figure 7B). A slight increase in state 4 respiration was observed in the cells treated with melatonin, however, this change was not statistically significant ($P = 0.189$). RCR was, however, significantly lower in the melatonin-supplemented group compared with the control ($P = 0.001$). Finally, uncoupled respiration, which was driven by the addition of FCCP, did not differ between the experimental groups ($P > 0.05$) (Figure 7C).

When succinate was used as a respiration substrate in the presence of the complex I inhibitor rotenone, the state 3 respiration rate dramatically decreased in *LETM1*-suppressed cells and the impaired state 3 respiration was normalized with melatonin supplementation (siRNA^{Letm1} + melatonin). In addition, there was no difference between the control and melatonin-supplemented groups ($P > 0.05$) (Figure 8A). No significant difference was observed in state 4 respiration after supplementation with melatonin, with or without siRNA^{Letm1} transfection for complex II ($P > 0.05$) (Figure 8B). These results demonstrate that melatonin effectively ameliorated state 3 respiration via complex II. As expected, RCR was also normalized in melatonin-treated *LETM1*-silenced cells (Figure 8C).

With regard to complex IV, melatonin supplementation effectively increased OCR, which was consistent with the previous data in the *LETM1*-silenced cells (control vs. siRNA^{Letm1} + melatonin, $P > 0.05$) (Figure 9). In summary, these findings demonstrated that melatonin was an effective agent in improving mitochondrial function in *LETM1*-silenced MEF cells.

Discussion

We evaluated the increase in oxidative stress and alterations in mitochondrial function that resulted from *LETM1* suppression and assessed the potential improvement effect of melatonin on these emergent mitochondrial changes.

The protein expression was reduced by 50% in the siRNA^{Letm1}-transfected MEF cells relative to the control group, which is consistent with previous reports that the hemizygous deletion of the *LETM1* gene is associated with WHS and such a reduction in protein levels. It is emphasized that a 50% reduction in *LETM1* protein levels is important to exhibit WHS symptoms (Hart et al., 2014; Austin et al., 2017).

In our study, melatonin administration to the cells was shown to protect viability (and reduce mitochondrial ROS production) at 100 μM concentration, which was consistent with the findings of Lopez et al. (2009). In addition, Lopez et al. (2009) reported that the effect of melatonin administration on mitochondrial RCR was minimal. In another study, it was found that melatonin administration at the same dose enhanced complex I and IV activity and ATP synthesis in mitochondrial preparations obtained from the brain and liver (Martin et al., 2000).

In the present study, surprisingly, we found that the MnSOD level in the total protein extracts was significantly decreased in *LETM1*-suppressed cells compared with the control group. This is because *MnSOD* encoded in a different chromosome than *LETM1* and a decrease in MnSOD levels was unexpected. It may be due to diminished mitochondrial function with *LETM1* reduction and/or a signal transduction event. In this study, we were not able to explain this reduction and further studies are needed to clarify this issue in different cell lines and experimental approaches. Doonan et al (2014) suggested that *LETM1* suppression increased the oxidative stress and reconstitution of *LETM1* or that the transfection of cells with the *MnSOD*-encoding plasmid was effective in improving mitochondrial Ca^{2+} transport and bioenergetics. We could not, however, find any study that reveals an association between WHS and MnSOD in the literature. In the present study, we showed that there was increase in the impaired MnSOD level in melatonin-treated samples. In accordance with our results, it is suggested that melatonin administration is effective in raising the MnSOD levels, which play a role in the genetic regulation of enzymes responsible for anti-oxidant defense and, furthermore, in the reduction of epileptic seizures (Kotler et al., 1998; Acuna Castroviejo et al., 2002; Atanasova et al., 2013).

We also observed that aconitase activity decreased in *LETM1*-suppressed cells together with MnSOD, which was consistent with reports in the literature. Aconitase belongs to the family of iron–sulfur-containing dehydratases and it is very susceptible to O_2^-. Thus, the aconitase activity is widely used as a biomarker for oxidative stress and has been suggested to serve as an intramitochondrial sensor of redox status. Anti-oxidant enzyme systems [Cu/Zn SOD (SOD1) and MnSOD (SOD2)] capture O_2^- with their critical thiol groups and protect aconitase activity against $O_2^{\cdot -}$ mediated inactivation (Inarrea et al., 2011; Lushchak et al., 2014). It may, therefore, be suggested that the free O_2^- level increases in *LETM1*-suppressed cells, and that the increased O_2^- level reversibly inactivates aconitase. Furthermore, it was observed that the administration of melatonin to *LETM1*-suppressed cells re-normalized aconitase activity, similar to the MnSOD in this study. Another finding of the present study is the increase in oxidized

protein levels with decreased *LETM1* expression. As a free-radical scavenger, melatonin treatment also decreased oxidized protein levels. On the other hand, we were not able to identify protein oxidation after mitochondria isolation to determine if OXPHOS complexes were oxidized. This was possible due to the procedure we applied to isolate mitochondria because we identified unexplainable levels of protein oxidation in mitochondrial preparations (data not shown). Regardless, however, a decrease in carbonylated protein levels from the whole-cell extracts treated with melatonin further supported our hypothesis on the beneficial role of melatonin for WHS.

Using the BN-PAGE method, we also investigated if there is an alteration in the formation of mitochondrial OXPHOS complexes following the determination of oxidative stress after *LETM1* suppression. The findings on the association between *LETM1* and formation of mitochondrial OXPHOS complexes are still debated in the literature. Frazier et al. (2006) showed that lack of MDM38, a *LETM1* yeast homolog, results in decreased levels of complex III and IV and an accumulation of ATP6 without any complex formation. Similarly, Tamai et al. (2008) suggested that *LETM1* suppression in HeLa cells results in impaired formation of complex I, III, and IV. In contrast, Dimmer et al. (2008) did not detect any alteration in the formation of OXPHOS complexes in response to *LETM1* suppression in HeLa cells. We also did not observe any alteration in the formation of OXPHOS complexes. It might be possible that the difference in results reported by different studies may be attributed to differences in the quality of the mitochondria-isolation techniques, solubilization approach of membrane proteins, detergent:protein ratio, and experimental approaches. Also, considering that even the results of two studies in the same cell line (HeLa) differ, it may be postulated that the differences in our results may be related to the cell line that we used.

In the present study, we assessed the effects of *LETM1* suppression on mitochondrial respiration using electron-donor substrates and appropriate inhibitors for complex I, II, and IV. No significant difference was observed between the control group and *LETM1*-suppressed cells in the presence of glutamate and malate for complex I. Whereas, the respiration rate for complex II and complex IV decreased significantly and that the decrease was normalized by melatonin administration. A study conducted on $LETM1^{+/-}$ mice reported that *LETM1* deficiency does not significantly affect the function of electron transport system (ETS), however, the cells in the medium with low-glucose content had a lower oxygen uptake rate. The authors concluded that glucose metabolism was impaired in *LETM1* deficiency and as a consequence, ATP production decreased (Jiang et al., 2013). In this study, OXPHOS was found to be defective at least through complex II and IV in cells cultured in a high-glucose medium, similar to that of the previous study. Similar to findings of Jiang et al. (2013), Dimmer et al. (2008) reported that *LETM1* suppression in HeLa cells do not affect the glutamate/malate-mediated basal respiration and uncoupled respiration. It was also suggested that residual *LETM1* may be sufficient for normal mitochondrial function, although the *LETM1* level decreased ~60%. In accordance with the previous studies, we did not detect any alteration in glutamate/malate-mediated basal respiration and uncoupled respiration. On the other hand, Dimmer et al. (2008) did not include substrates, such as succinate or TMPD, which feed complex II or IV directly. Doonan et al. (2014) reported that the basal OCR decreased in HeLa and 293T cells, but that OCR did not vary with complex I and complex II substrates. The authors suggested that OCR decreases significantly in the presence of complex IV substrates, also that the main reason for the basal oxygen consumption and low ATP production is complex IV. Zhang et al. (2012) investigated the effects of *LETM1* and *LETM2* on mitochondrial respiration in *Arabidopsis thaliana.* The authors observed that the OCR decreased significantly in *LETM1* (−/−) *LETM2* (+/−) and *LETM1* (−/−) *LETM2* (−/−) plants through complex II and this decrease was accelerated with ADP addition (state 3); similar to our findings. In addition, succinate dehydrogenase activity was also examined and no change was observed. Zhang et al. (2012) also investigated the effect of homozygous and heterozygous suppression of *LETM2* on mitochondrial respiration in a condition that presented the homozygote loss of *LETM1*. The present study is, therefore, the first to demonstrate that *LETM1* affected OXPHOS through complex II. In terms of total oxygen consumption, electron feeding via complex I or complex II depends on complex IV. Considering that the reduction of oxygen to water mainly takes place in complex IV and that we observed a decline in complex IV efficacy, the impaired respiration through complex II may be considered as a function of this complex. On the other hand, the regular mitochondrial function when complex I substrates were used indicates that complex II is the primary structure responsible for ATP synthesis deficiency or reduced basal respiration, as previously mentioned in the literature.

Complex II differs from other respiratory complexes in ETS with some unique features. First, all four subunits (SDHA, SDHB, SDHC, and SDHD) that form the structure of complex II are encoded only by the nuclear genome. In contrast, the subunits of the other respiratory complexes are encoded by both the nuclear and mitochondrial genomes. Besides, this complex does not perform the proton pumping, which is the main driving force for ATP production. Nevertheless, complex II reduces the coenzyme Q_{10} and indirectly contributes to the proton pumping mediated by the Q cycle of OXPHOS (Rustin et al., 2002).

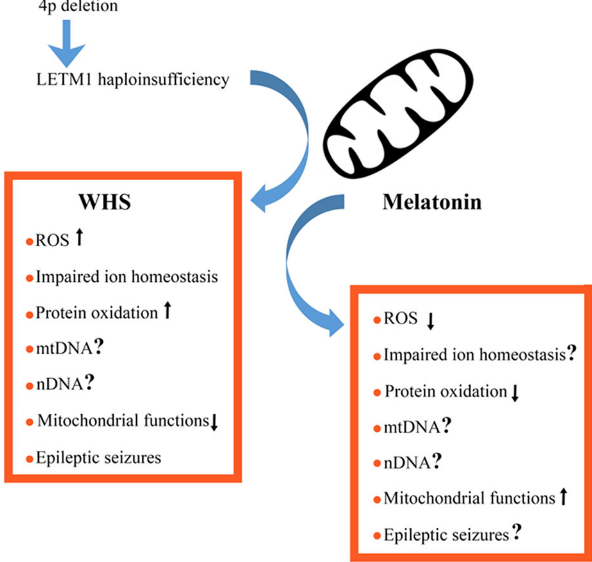

Figure 10 Summary of the effects of *LETM1* haploinsufficiency and melatonin.

One of the most important factors that play a role in the dysfunction of this complex is increased ROS production. The findings of our study elucidate the role of increased ROS in complex II dysfunction. In the literature, it is reported that mutations or loss of function of complex II subunits are associated with a number of neurodegenerative diseases such as Leigh's syndrome and Huntington's disease (Browne et al., 1997; Benchoua et al., 2006; Pandey et al., 2010). Considering that WHS is a neurodegenerative disorder, complex II may play an important role in WHS etiology, but further studies are necessary to elucidate the role of complex II dysfunction in WHS pathophysiology.

The present study demonstrated, as expected, that melatonin is a valuable agent in recovering defective respiration in *LETM1*-suppressed MEF cells. In addition, we confirmed that melatonin had its essential effect in OXPHOS process through complex I and IV, as previously suggested (Martin et al., 2000). It is postulated that this effect is a consequence of the direct interaction of these complexes with melatonin. Although several studies have reported that the activities of other complexes are also affected by melatonin, our findings suggest that the effect of melatonin is mainly due to the reduction of oxidative stress.

Conclusion

Our preliminary study had shown some beneficial effects of melatonin in vitro on MEFs but confirmation of these data in other cells lines and cells obtained from WHS patients is needed. Also, lack of some experimental approaches such as determination of membrane potential, determination of mRNA levels of some related genes such as *MnSOD*, *aconitase*, and determination of ATP levels are the main limitations of our study. On the other hand, the present study not only reports new findings but also poses new questions (Figure 10). We successfully represented *LETM1* haploinsufficiency and that an obvious (50%) reduction in *LETM1* levels increased the oxidative stress, which resulted in physiological consequences, such as diminished aconitase activity and increased protein oxidation. The melatonin administration was effective in decreasing these effects. On the other hand, a growing body of data in the literature postulates that *LETM1* loss causes impaired ion homeostasis. It is, therefore, unclear whether melatonin is a beneficial agent to repair this damage or not. Also, there is no evidence that mtDNA or nDNA (other than the deleted chromosome 4) are affected by the loss of *LETM1*. The consequences of oxidative DNA damage are important to consider in future research. We also demonstrated that especially complex II was affected by *LETM1* suppression in MEF cells. It is, therefore, suggested that complex II might play a pivotal role in *LETM1* deficiency.

Funding

This study was supported by a grant (Grant no: 115S455) from The Scientific and Technological Research Council of Turkey (TUBITAK).

Conflict of interest

The authors have no conflicts of interest to declare.

References

Acuna Castroviejo D, Escames G, Carazo A, Leon J, Khaldy H, Reiter RJ (2002) Melatonin, mitochondrial homeostasis and mitochondrial-related diseases. Curr Top Med Chem 2: 133–51.

Acuna Castroviejo D, Lopez LC, Escames G, Lopez A, Garcia JA, Reiter RJ (2011) Melatonin-mitochondria interplay in health and disease. Curr Top Med Chem 11: 221–40.

Atanasova M, Petkova Z, Pechlivanova D, Dragomirova P, Blazhev A, Tchekalarova J (2013) Strain-dependent effects of long-term treatment with melatonin on kainic acid-induced status epilepticus, oxidative stress and the expression of heat shock proteins. Pharmacol Biochem Behav 111: 44–50.

Austin S, Nowikovsky K (2019) *LETM1*: essential for mitochondrial biology and cation homeostasis? Trends Biochem Sci 44: 648–58.

Austin S, Tavakoli M, Pfeiffer C, Seifert J, Mattarei A, De Stefani D, Zoratti M, Nowikovsky K (2017) *LETM1*-Mediated K(+)

and Na(+) Homeostasis Regulates Mitochondrial Ca(2+) Efflux. Front Physiol 8: 839.

Battaglia A, Carey JC, Cederholm P, Viskochil DH, Brothman AR, Galasso C (1999) Natural history of Wolf-Hirschhorn syndrome: experience with 15 cases. Pediatrics 103: 830–6.

Benchoua A, Trioulier Y, Zala D, Gaillard MC, Lefort N, Dufour N, Saudou F, Elalouf JM, Hirsch E, Hantraye P, Déglon N, Brouillet E (2006) Involvement of mitochondrial complex II defects in neuronal death produced by N-terminus fragment of mutated huntingtin. Mol Biol Cell 17: 1652–63.

Browne SE, Bowling AC, MacGarvey U, Baik MJ, Berger SC, Muquit MMK, Bird ED, Beal MF (1997) Oxidative damage and metabolic dysfunction in Huntington's disease: selective vulnerability of the basal ganglia. Ann Neurol 41: 646–53.

Dimmer KS, Navoni F, Casarin A, Trevisson E, Endele S, Winterpacht A, Salviati L, Scorrano L (2008) *LETM1*, deleted in Wolf-Hirschhorn syndrome is required for normal mitochondrial morphology and cellular viability. Hum Mol Genet 17: 201–14.

Divakaruni AS, Rogers GW, Murphy AN (2014) Measuring mitochondrial function in permeabilized cells using the seahorse XF analyzer or a clark-type oxygen electrode. Curr Protoc Toxicol 60: 25.2.1–16.

Doonan PJ, Chandramoorthy HC, Hoffman NE, Zhang X, Cárdenas C, Shanmughapriya S, Rajan S, Vallem S, Chen X, Foskett JK, Cheung JY, Houser SR, Madesh M (2014) *LETM1*-dependent mitochondrial Ca2+ flux modulates cellular bioenergetics and proliferation. FASEB J 28: 4936–49.

Escames G, Lopez A, Antonio Garcia J, Garcia L, Acuna-Castroviejo D, Joaquin Garcia J, Carlos Lopez L (2010) The role of mitochondria in brain aging and the effects of melatonin. Curr Neuropharmacol 8: 182–93.

Fernandez-Gil BI, Guerra-Librero A, Shen YQ, Florido J, Martínez-Ruiz L, García-López S, Adan C, Rodríguez-Santana C, Acuña-Castroviejo D, Quiñones-Hinojosa A, Fernández-Martínez J, Abdel Moneim AE, López LC, Rodríguez Ferrer JM, Escames G (2019) Melatonin enhances cisplatin and radiation cytotoxicity in head and neck squamous cell carcinoma by stimulating mitochondrial ROS generation, apoptosis, and autophagy. Oxid Med Cell Longev 2019: 7187128.

Frazier AE, Taylor RD, Mick DU, Warscheid B, Stoepel N, Meyer HE, Ryan MT, Guiard B, Rehling P (2006) Mdm38 interacts with ribosomes and is a component of the mitochondrial protein export machinery. J Cell Biol 172: 553–64.

Frezza C, Cipolat S, Scorrano L (2007) Organelle isolation: functional mitochondria from mouse liver, muscle and cultured fibroblasts. Nat Protoc 2: 287–95.

Hart L, Rauch A, Carr AM, Vermeesch JR, O'Driscoll M (2014) *LETM1* haploinsufficiency causes mitochondrial defects in cells from humans with Wolf-Hirschhorn syndrome: implications for dissecting the underlying pathomechanisms in this condition. Dis Model Mech 7: 535–45.

Hirschhorn K, Cooper HL, Firschein IL (1965) Deletion of short arms of chromosome 4-5 in a child with defects of midline fusion. Humangenetik 1: 479–82.

Inarrea P, Casanova A, Alava MA, Iturralde M, Cadenas E (2011) Melatonin and steroid hormones activate intermembrane Cu,Zn-superoxide dismutase by means of mitochondrial cytochrome P450. Free Radic Biol Med 50: 1575–81.

Jiang D, Zhao L, Clish CB, Clapham DE (2013) Letm1, the mitochondrial Ca2+/H+ antiporter, is essential for normal glucose metabolism and alters brain function in Wolf-Hirschhorn syndrome. Proc Natl Acad Sci U S A 110: E2249–54.

Jou MJ, Peng TI, Yu PZ, Jou SB, Reiter RJ, Chen JY, Wu HY, Chen CC, Hsu LF (2007) Melatonin protects against common deletion of mitochondrial DNA-augmented mitochondrial oxidative stress and apoptosis. J Pineal Res 43: 389–403.

Kotler M, Rodriguez C, Sainz RM, Antolin I, Menendez-Pelaez A (1998) Melatonin increases gene expression for antioxidant enzymes in rat brain cortex. J Pineal Res 24: 83–9.

Lew M (2007) Good statistical practice in pharmacology. Problem 1. Br J Pharmacol 152: 295–8.

Lushchak OV, Piroddi M, Galli F, Lushchak VI (2014) Aconitase post-translational modification as a key in linkage between Krebs cycle, iron homeostasis, redox signaling, and metabolism of reactive oxygen species. Redox Rep 19: 8–15.

López A, García JA, Escames G, Venegas C, Ortiz F, López LC, Acuña-Castroviejo D (2009) Melatonin protects the mitochondria from oxidative damage reducing oxygen consumption, membrane potential, and superoxide anion production. J Pineal Res 46: 188–98.

Martin M, Macias M, Escames G, Reiter RJ, Agapito MT, Ortiz GG, Acuna-Castroviejo D (2000) Melatonin-induced increased activity of the respiratory chain complexes I and IV can prevent mitochondrial damage induced by ruthenium red in vivo. J Pineal Res 28: 242–8.

Martin M, Macias M, Leon J, Escames G, Khaldy H, Acuna-Castroviejo D (2002) Melatonin increases the activity of the oxidative phosphorylation enzymes and the production of ATP in rat brain and liver mitochondria. Int J Biochem Cell Biol 34: 348–57.

McQuibban AG, Joza N, Megighian A, Scorzeto M, Zanini D, Reipert S, Richter C, Schweyen RJ, Nowikovsky K (2010) A Drosophila mutant of *LETM1*, a candidate gene for seizures in Wolf-Hirschhorn syndrome. Hum Mol Genet 19: 987–1000.

Nowikovsky K, Bernardi P (2014) *LETM1* in mitochondrial cation transport. Front Physiol 5: 83.

Pandey M, Mohanakumar KP, Usha R (2010) Mitochondrial functional alterations in relation to pathophysiology of Huntington's disease. J Bioenerg Biomembr 42: 217–26.

Paradies G, Paradies V, Ruggiero FM, Petrosillo G (2015) Protective role of melatonin in mitochondrial dysfunction and related disorders. Arch Toxicol 89: 923–39.

Quan X, Nguyen TT, Choi SK, Xu S, Das R, Cha SK, Kim N, Han J, Wiederkehr A, Wollheim CB, Park KS (2015) Essential role of mitochondrial Ca2+ uniporter in the generation of mitochondrial pH gradient and metabolism-secretion coupling in insulin-releasing cells. J Biol Chem 290: 4086–96.

Rustin P, Munnich A, Rotig A (2002) Succinate dehydrogenase and human diseases: new insights into a well-known enzyme. Eur J Hum Genet 10: 289–91.

Tamai S, Iida H, Yokota S, Sayano T, Kiguchiya S, Ishihara N, Hayashi JI, Mihara K, Oka T (2008) Characterization of the mitochondrial protein *LETM1*, which maintains the mitochondrial tubular shapes and interacts with the AAA-ATPase BCS1L. J Cell Sci 121: 2588–600.

Tapias V, Escames G, López LC, López A, Camacho E, Carrión MD, Entrena A, Gallo MA, Espinosa A, Acuña-Castroviejo D (2009) Melatonin and its brain metabolite N(1)-acetyl-5-methoxykynuramine prevent mitochondrial nitric oxide synthase induction in Parkinsonian mice. J Neurosci Res 87: 3002–10.

Wolf U, Reinwein H, Porsch R, Schroter R, Baitsch H (1965) [Deficiency on the short arms of a chromosome No. 4]. Humangenetik 1: 397–413.

Zhang B, Carrie C, Ivanova A, Narsai R, Murcha MW, Duncan O, Wang Y, Law SR, Albrecht V, Pogson B, Giraud E, Van Aken O, Whelan J (2012) LETM proteins play a role in the accumulation of mitochondrially encoded proteins in Arabidopsis thaliana and AtLETM2 displays parent of origin effects. J Biol Chem 287: 41757–73.

Received 24 May 2019; accepted 25 November 2019.
Final version published online 12 December 2019.