

# Overexpression of tumor necrosis factor receptor-associated protein 1 (TRAP1), leads to mitochondrial aberrations in mouse fibroblast NIH/3T3 cells

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**Cancer cells undergo uncontrolled proliferation, and aberrant mitochondrial alterations. Tumor necrosis factor receptor-associated protein 1 (TRAP1) is a mitochondrial heat shock protein. TRAP1 mRNA is highly expressed in some cancer cell lines and tumor tissues. However, the effects of its overexpression on mitochondria are unclear. In this study, we assessed mitochondrial changes accompanying TRAP1 overexpression, in a mouse cell line, NIH/3T3. We found that overexpression of TRAP1 leads to a series of mitochondrial aberrations, including increase in basal ROS levels, and decrease in mitochondrial biogenesis, together with a decrease in peroxisome proliferator-activated receptor gamma coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) mRNA levels. We also observed increased extracellular signal-regulated kinase (ERK) phosphorylation, and enhanced proliferation of TRAP1 overexpressing cells. This study suggests that overexpression of TRAP1 might be a critical link between mitochondrial disturbances and carcinogenesis. [BMB Reports 2014; 47(5): 280-285]**

## INTRODUCTION

Mitochondria are vitally important organelles, in which the majority of the ATP is synthesized via oxidative phosphorylation. Mitochondria are composed of a double-membrane system. Mitochondrial matrix contains approximately 16.5 kb genome, encoding complexes I, III, IV and V (1, 2). The mitochondrial respiratory apparatus is the product of nuclear and mitochondrial genes. Peroxisome proliferator-activated receptor gamma coactivator-1 $\alpha$  (PGC-1 $\alpha$ ), an important transcriptional regulator, was shown to be involved in mitochon-

drial biogenesis (3, 4). Mitochondrial biogenesis is adversely affected, when mitochondrial DNA is exposed to ROS produced during oxidative phosphorylation, resulting in irreversible alteration and cancer (5-7). Among the cellular changes occurring in most cancers are abnormal cell proliferation, with aberrations, such as hyper-activation of extracellular signal-regulated kinase (ERK) (8), mtDNA mutations, and mitochondrial dysfunctions (9-15).

Tumor necrosis factor-associated protein 1 (TRAP1) is a mitochondrial heat shock protein (HSP), belonging to the HSP90 family (16). A recent study, mostly focused on the role of TRAP1 during stress condition, showed that TRAP1 protects cells from ROS-induced apoptosis and senescence (17-20). Although both TRAP1 mRNA and protein are highly expressed in cancer cell lines and tumors (21, 22), little is known of the effect of TRAP1 overexpression on mitochondria under physiological conditions. We therefore investigated the effect of TRAP1 overexpression on mitochondria, in a mouse fibroblast cell line, NIH/3T3. We found that overexpression of TRAP1 caused a series of mitochondrial aberrations, including increase in basal ROS levels, and decrease in mitochondrial biogenesis, together with a decrease in PGC-1 $\alpha$  mRNA levels. We also observed increased pERK, and enhanced proliferation of TRAP1 overexpressing cells. These findings suggest that highly expressed TRAP1 may play an important role in carcinogenesis, through disturbing mitochondria, and accelerating cell proliferation.

## RESULTS

### **TRAP1 is highly expressed and mitochondrial mass is decreased in lung carcinoma cell line A549, compared with a normal lung fibroblast, WI-38**

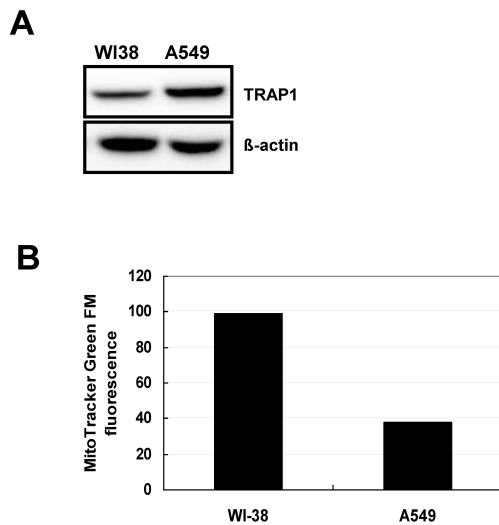
We compared the expression of TRAP1 at the protein level and mitochondrial mass (determined using MitoTracker Green FM probe), in the lung carcinoma cell line, A549, with those in a normal lung cell line, WI-38. Expression of TRAP1 protein was higher in A549 cells, than in WI-38 cells (Fig. 1A), while the mitochondrial mass was less in A549, than in WI-38 (Fig. 1B).

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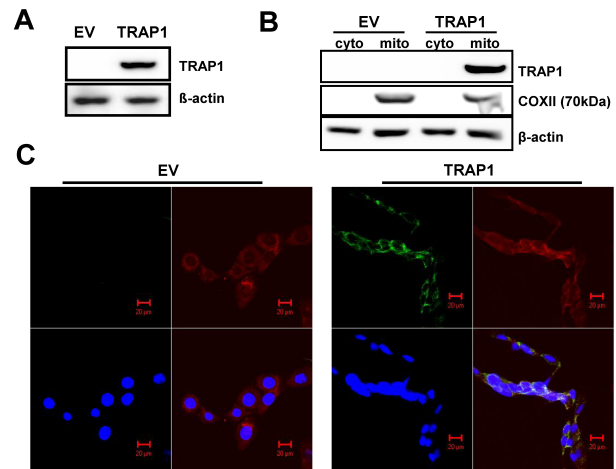
**Keywords:** ERK, Mitochondria, PGC-1 $\alpha$ , ROS, TRAP1



**Fig. 1.** Overexpression of TRAP1 protein, and the decrease of mitochondrial mass in A549 cancer cell line. (A) TRAP1 protein levels in WI38 and A549 cells. TRAP1 protein levels were analyzed by Western blotting in WI-38 and A549 cell lines. (B) Mitochondrial mass in WI-38 and A549 cell lines. After incubating cells with MitoTracker Green FM, cells were analyzed, using flow cytometry.

### Overexpressed TRAP1 is targeted to mitochondria in NIH/3T3 cells

Based on the apparent reciprocal relationship between TRAP1 overexpression and mitochondrial mass seen in Fig. 1, we postulated that TRAP1 overexpression might affect mitochondria. In order to verify this, we established TRAP1 overexpressing NIH/3T3 cells, after transfection of human TRAP1 full-length cDNA into these cells, and selecting cells using G418. There were three reasons that we utilized a mouse NIH/3T3 cell line for the TRAP1 stable cell line. First, we wanted to distinguish exogenous TRAP1 expression, using the antibody specific for human TRAP1 protein, through transfection of human full-length TRAP1 cDNA. Second, we speculated that it might be hard to establish normal cells (WI-38), using general transfection technique with plasmid, compared with NIH/3T3 cells. This NIH/3T3 cell line is known as a suitable cell line for transfection. Third, we would like to gather any insights into the TRAP1-NIH/3T3 cell line, and to establish TRAP1 transgenic mice, using the same human TRAP cDNA. TRAP1-overexpressing cells were morphologically distinct from empty vector (EV) transfected cells. Since we transfected human full-length cDNA of TRAP1 to establish the TRAP1 stable cell line, we used anti-human TRAP1 antibody to detect exogenous TRAP1 protein in mouse NIH/3T3 cells. Western blotting was then performed, using a specific antibody, which detects only human TRAP1 protein. TRAP1 protein was detected in TRAP1 cDNA transfected cells, but not in EV cells (Fig. 2A). To determine whether

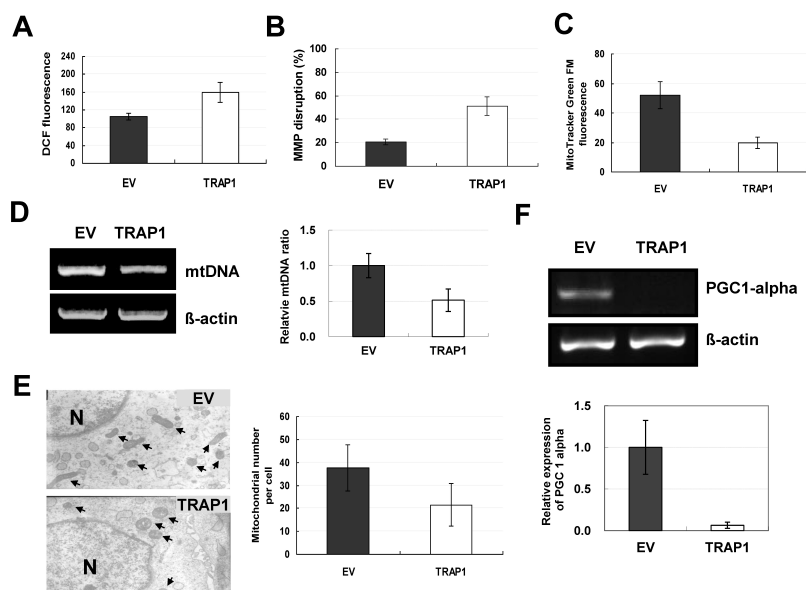


**Fig. 2.** Establishment of human TRAP1 overexpressing NIH/3T3 cells. (A) Western blot analysis for TRAP1, in empty vector (EV) and TRAP1 cells. After selecting TRAP1 and EV cells, as described in Materials and Methods, TRAP1 protein levels were analyzed, via Western blotting. (B) TRAP1 expression in mitochondrial fraction. Cells were fractionated into cytosolic (cyto) and mitochondrial (mito) fractions, and subjected to Western blotting against TRAP1. COXII and  $\beta$ -actin were used as a mitochondrial marker and a loading control, respectively. (C) Immunofluorescence analysis for TRAP1 (green, TRAP1; red, MitoTracker; blue, DAPI).

TRAP1 is targeted into mitochondria, we examined subcellular localization of TRAP1, after subcellular fractionation (Fig. 2B) and confocal microscopy (Fig. 2C), using Cox I and MitoTracker probe, two mitochondrial specific markers. It can be seen that the TRAP1 that was expressed was successfully targeted into mitochondria in TRAP1 cells.

### Overexpression of TRAP1 leads to mitochondrial aberrations, and decrease of PGC1- $\alpha$ mRNA levels

Since TRAP1 was previously shown to be associated with ROS regulation under stress conditions (17, 19, 23), we examined how the basal ROS levels are altered under physiological conditions, in TRAP1 cDNA transfected and empty vector (EV) transfected cells. As shown in Fig. 3A, basal ROS levels were higher in TRAP1 cells ( $159.20 \pm 21.96$ ,  $P < 0.001$ ), than in EV cells ( $105.39 \pm 7.52$ ). Next, using JC-1 staining, we examined whether MMP changed, concurrently with the increase of basal ROS levels in the two cell types. Fig. 3B shows that MMP disruption significantly increased in TRAP1 cells ( $51.13 \pm 7.92\%$ ,  $P < 0.05$ ), compared to EV cells ( $20.70 \pm 2.51\%$ ). We then examined whether the increases in ROS levels and MMP disruption might continuously affect mitochondrial mass, using Green FM probe, and mtDNA content via PCR, using specific primers against  $\beta$ -actin as a genomic DNA (gDNA), and cox-I as a mitochondrial DNA. Green FM fluorescence significantly decreased in TRAP1 cells, compared to EV cells, confirming the decrease in mi-

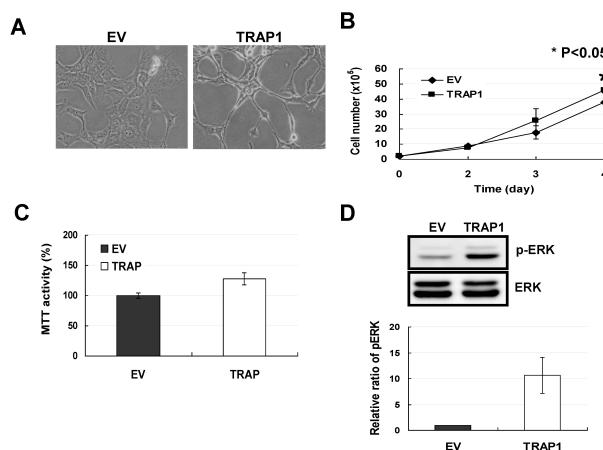


**Fig. 3.** Alterations of mitochondria in TRAP1 cells. (A) ROS measurement, (B) MMP disruption, and (C) Mitochondrial mass in EV and TRAP1 cells. Cells were analyzed using flow cytometry, and expressed as mean  $\pm$  s.d. (D) mtDNA content and relative mtDNA ratio in EV and TRAP1 cells. After each PCR against mtDNA or  $\beta$ -actin was conducted, products were analyzed. Intensities were calculated as mtDNA divided by gDNA, and then were expressed as relative ratio to EV (mean  $\pm$  s.d.). These data were obtained from three independent experiments. (E) Mitochondrial morphology and number in EV and TRAP1 stable cells. Mitochondria were taken under transmission electron microscope ( $\times 10,000$ ). Arrows indicate mitochondria. Mitochondrial number per cell was counted, and expressed as mean  $\pm$  s.d. (F) Decrease in PGC-1 $\alpha$  mRNA levels in TRAP1 cells. Total RNA was isolated, and subjected to RT-PCR against PGC-1 $\alpha$ . Mouse TRAP1 (mTRAP1) mRNA was utilized as an internal control. Intensities were measured, and were expressed as relative ratio to EV (mean  $\pm$  s.d.). These data were obtained from three independent experiments.

tochondrial mass in TRAP1 cells (Fig. 3C). As shown in Fig. 3D, the ratio of mtDNA to gDNA also significantly decreased in TRAP1 cells ( $P < 0.05$ ), again confirming a decrease in mitochondrial mass. In addition, electron microscopic studies showed that mitochondrial number per cell significantly decreased in TRAP1 cells, compared with EV cells ( $P < 0.01$ , Fig. 3E). Because PGC1- $\alpha$  is thought to be an important regulator in mitochondrial biogenesis [3], we next determined PGC1- $\alpha$  mRNA levels in these cells, using RT-PCR. Fig. 3F shows the dramatic decrease of PGC1- $\alpha$  levels in TRAP1 cells, compared with EV cells ( $P < 0.05$ ).

#### Overexpression of TRAP1 alters cell morphology, and increases cell proliferation and MTT activity

While maintaining these cells, we observed significant differences between TRAP1 cells and EV cells. Under the inverted phase contrast microscope and confocal microscope, EV cells were more flat and round, while TRAP1 cells were more tubule-like (Fig. 2C and Fig. 4A). In addition, TRAP1 cells proliferated more rapidly, in comparison with EV cells, counting cell numbers at 2, 3, and 4 days. We found, as shown in Fig. 4B, that the number of TRAP1 cells moderately but significantly increased by day 4 (mean  $\pm$  s.d,  $44.89 \pm 3.02 \times 10^5$  cells,  $P < 0.05$ ), compared to EV cells (mean  $\pm$  s.d, EV;  $32.69 \pm 5.86 \times 10^5$  cells). Fig. 4C shows that the changes in MTT activity were consistent with the cell counting results (mean  $\pm$  s.d, EV;  $100 \pm 3.9\%$  vs. TRAP1;  $127.7 \pm 9.6\%$ ,  $P < 0.05$ ). Phosphorylated ERK (pERK) is an important component in the signal transduction pathway, and is believed to be associated with cell growth and cancer (8). We therefore examined whether pERK is altered in EV and TRAP1 cells, by



**Fig. 4.** Alteration of morphology and enhancement of proliferation in TRAP1 cells. (A) Morphology in EV and TRAP1 cells. Cellular morphology was taken under inverted phase contrast microscope ( $\times 400$ ). (B) Cell number in EV and TRAP1 cells. After seeding cells ( $2 \times 10^5$ ) into 100 mm dish, cell number was counted at 2, 3, and 4 days, respectively, and expressed as Mean  $\pm$  S.D. (C) MTT activity in EV and TRAP1 stable cells. MTT activity was measured at 3 days, and expressed as mean  $\pm$  s.d. These data were obtained from three independent experiments. (D) Increased ERK activation in TRAP1 cells. Western blotting against p-ERK and ERK was conducted. Intensities of three independent images were measured using Image J program, normalized by ERK, and represented as relative ratio to EV.

Western blotting against ERK phosphorylation. ERK phosphorylation significantly increased in TRAP1 cells, compared with EV cells (Fig. 4D).

## DISCUSSION

Both the dysregulation of HSPs (24-26), and dysfunction of mitochondria (27-31), have been implicated in carcinogenesis. The mRNA of TRAP1, a mitochondrial HSP, has been shown to be highly expressed in some cancer cells (21). TRAP1 is expressed at very high levels in breast, colon, and lung tumors, in contrast to normal mouse tissues (22). TRAP1 overexpression confers resistance against toxic agents, such as H<sub>2</sub>O<sub>2</sub> and granzyme M, by regulating ROS accumulation during apoptosis (17, 18, 20). However, the effects of TRAP1 overexpression on mitochondria under normal conditions have not been hitherto clarified. Our study shows that TRAP1 protein levels were higher in A549 cancer cells, than in normal WI38 cells, in agreement with previous findings (21, 22). In addition, A549 cells had less mitochondrial mass than WI38. These results suggest a linkage between TRAP1 overexpression and abnormal mitochondria in cancer cells. In this study of TRAP1 overexpressing NIH/3T3 cells, we demonstrate, in agreement with the previous findings (16, 19, 20), that TRAP1 was targeted into mitochondria, and basal ROS levels increased in TRAP1 cells. Why ROS levels are high in these cells is not clear, and requires further studies. Our observation that MMP disruption increased in TRAP1 cells is consistent with previous reports that the intrinsic MMP of colonic carcinoma cells is an important factor in determining the probability of tumorigenesis (30, 31). Eukaryotes containing mitochondria produce ROS in the process of OXPHOS, for most of ATP synthesis. Mitochondrial DNA located in the matrix is thought to be particularly vulnerable to ROS. Furthermore, mtDNA alterations by ROS have been implicated in most cancers under constitutive oxidative stress (6, 7, 32, 33). In addition, intracellular signals, such as ROS, might be associated with the expression of PGC-1 $\alpha$ , which regulates respiratory genes (3). A decrease of PGC-1 $\alpha$  in TRAP1 cells may cause, at least partially, the observed decreases in mtDNA, mitochondrial number and mitochondrial mass. We could not detect any significant differences in the expression of representative respiratory proteins, such as complex I 17kDa subunit, complex II 70kDa subunit, complex III core 1, 2, and Fe-S protein, and complex IV subunit 1 (data not shown), although it is possible that other respiratory proteins are altered in TRAP1 cells. Taken together, these results suggest that ROS might possibly be an important player in the mitochondrial alterations. ROS may also function as a second messenger in the process of signal transduction, and moderate concentrations of ROS can enhance cellular proliferation. We found moderately increased cell proliferation, with increase in pERK in TRAP1 cells. The activation of ERK as a component of Ras-Raf-MEK-ERK pathway has been implicated in many cancers, and ERK is considered a good candidate target for cancer therapeutics (8, 34, 35). Therefore, it might be inferred that ERK activation may partly result from increased ROS production in TRAP1 cells. Mitochondrial HSP70 might play a role in mitochondrial bio-

genesis, including mtDNA replication (36, 37). TRAP1 might be included in the list of molecules regulating mitochondrial biogenesis, together with mitochondrial HSP70.

On the other hand, Marco *et al.* (38) recently showed TRAP1 did not affect the rate of cell growth. We postulated some possibilities of discrepancy between our results, and the data from Marco *et al.* We focused on the effect of human TRAP1 overexpression on mitochondrial functions (mitochondrial mass and morphology, ROS etc.) in a mouse cell line, while Marco *et al.* mainly described relationships between TRAP1 and succinate dehydrogenase via HIF1 $\alpha$ , using knockdown of TRAP1 in human cancer cell lines (SAOS-2, HCT116 and HeLa). Although they showed that growth of colonies in soft agar was also assessed in TRAP1 stable mouse embryonic fibroblasts, they did not determine the effects of TRAP1 knockdown or TRAP1 overexpression on the levels of ROS, but described that the down-regulation of TRAP1 did not affect mitochondrial mass (Fig. S2E). Based on recent suggestions that there is an important link between TRAP1 and ROS, our data suggest that overexpression of TRAP1 leads to mitochondrial alterations and leaks to ROS, and results in activation of ERK. At this moment, we guess that this discrepancy may arise from different cellular context and methodology. Further study is required to elucidate whether our TRAP1 overexpressing cells form foci, and grow in soft agar.

In summary, we found TRAP1 overexpressing cells are characterized by mitochondrial aberrations, such as the increases of ROS, MMP disruption, decreases in mtDNA, mitochondrial mass, and mitochondrial number, as well as decreases in PGC-1 $\alpha$  mRNA levels, and increases in pERK and cellular proliferation. This study suggests that the overexpression of TRAP1, a mitochondrial HSP, might be a crucial link between mitochondrial disturbances and carcinogenesis. The molecular mechanisms underlying this hypothesized connection between TRAP1 overexpression and mitochondrial disturbances require further investigation.

## MATERIALS AND METHODS

### Cell culture

Normal lung fibroblast cell line, WI-38, and carcinoma cell line, A549 (ATCC), were maintained in RPMI(1640), supplemented with 10% (v/v) fetal bovine serum, 100 units/ml of penicillin, and 100  $\mu$ g/ml of streptomycin, in an incubator, in a 5% CO<sub>2</sub> atmosphere, at 37°C. A mouse fibroblast cell line, NIH/3T3 (ATCC) was cultured in Dulbecco's Modified Eagle's Medium (DMEM; WelGENE Inc.) with the same supplements, under the same incubation conditions described above.

### Stable transfection

Plasmid cDNA3.1-TRAP1 was prepared, by subcloning into the pcDNA3.1 plasmid, under the control of the EF1 $\alpha$ -promoter (a gift from Macrogen, Inc., Korea), with the original pOTB7-TRAP1 vector (obtained from Korea Research Institute of Bioscience and Biotechnology, Korea). Two  $\mu$ g plasmids

were transfected into NIH/3T3 cells ( $1 \times 10^6$  cells), with Lipofectamine Plus reagent (Invitrogen). After 48 hours of incubation, G418 (1 mg/ml, A.G. Scientific Inc.) was added to the culture medium. The selected clones were pooled after 3 weeks. The morphology of these cells was examined under the inverted phase contrast microscope ( $\times 400$ ).

### Western blotting

Cells were lysed in a buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and complete protease inhibitor cocktail (Roche); and proteins were separated by 10% SDS-PAGE, transferred to nitrocellulose membranes (S&S), and incubated with specific antibodies: anti-human TRAP1 antibody (NeoMarkers), anti-mouse  $\beta$ -actin and ERK antibodies (Santa Cruz Biotechnology), and anti-rabbit phospho-ERK (Thr202/Tyr204) antibody (Signaling). Images were obtained on LAS 3000 (Fujifilm), and image intensities were measured, using Image J program (National Institutes of Health, USA).

### Immunofluorescence analysis

Cells were incubated with MitoTracker Orange CMTMRos or Green FM (Molecular Probes) (19).

### Subcellular fractionation

The cytosolic and the mitochondrial fractions were isolated with digitonin (Sigma) (19).

### Cell counts and MTT assay

Cell numbers were counted after 2, 3, and 4 days, under an inverted phase contrast microscope. For MTT assay, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) reagent was added to each well. After 4 hours, 200  $\mu$ l of dimethyl sulfoxide (Sigma) was added, and absorption was measured, at a wavelength of 570 nm.

### Measurements of ROS, mitochondrial membrane potential (MMP), and mitochondrial mass

Intracellular ROS levels were measured, using CM-H<sub>2</sub>-DCF-DA (Molecular Probes), and the MMP was estimated, using 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazole carbocyanide iodide (JC-1; Molecular Probes) (19).

### Mitochondrial DNA (mtDNA)

Mitochondrial DNA was measured, using polymerase chain reaction (PCR). The total DNA was purified, using PUREGENE DNA purification kit (Gentra systems), in accordance with the manufacturer's instructions. Conditions for PCR against mitochondrial cox I as mitochondrial gene were as follows: forward primer; 5'-gcaacacctacacggaggtaa-3', reverse primer; 5'-agggtgttcctcgaatgtg-3' from accession no. NC\_005089, 17 cycles of PCR; denaturation for 30 seconds at 94°C, annealing for 30 seconds at 60°C, and a final extension for 30 seconds at 72°C. Conditions for PCR against  $\beta$ -actin as genomic DNA

control were as follows: forward primer; 5'-cgggtgctaagaaggc tggtc-3', reverse primer; 5'-gctgtggtggtgaagctgta-3' from accession no. NC\_000071, 35 cycles of PCR: denaturation for 30 seconds at 94°C, annealing for 30 seconds at 65°C, and a final extension for 30 seconds at 72°C. Specific PCR products were resolved on 1% agarose gels, and visualized by ethidium bromide staining.

### Electron microscopy

Cells were fixed with 2.5% glutaraldehyde, and post-fixed in 1% buffered osmium tetroxide (19).

### Reverse Transcription-PCR

Total RNA was purified with TRI<sup>®</sup> reagent (Sigma), in accordance with the manufacturer's instructions (19). Each 1  $\mu$ l cDNA in the sample was amplified via 40 cycles of PCR, as follows: denaturation for 30 seconds at 94°C, 30 seconds of annealing at 60°C, and a final 30 seconds of extension at 72°C, using specific primers against mouse  $\beta$ -actin (sense: 5'-tgttaccactgggacgaca-3' and antisense: 5'-ttgatgtcacgcacgattt-3' from accession no. NM\_007393), PGC1- $\alpha$  (sense: 5'-atgtgtcgcttctgctct-3' and antisense: 5'-tgattctcaattcacca-3' from accession no. NM\_008904).

### Statistical analysis

Student's *t*-tests were employed, in order to compare the differences between two different groups. *P* value of  $< 0.05$  was considered statistically significant.

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