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Triiodothyronine induces UCP-1 expression and mitochondrial biogenesis in human adipocytes

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Lee JY, Takahashi N, Yasubuchi M, Kim YI, Hashizaki H, Kim MJ, Sakamoto T, Goto T, Kawada T. Triiodothyronine induces UCP-1 expression and mitochondrial biogenesis in human adipocytes. *Am J Physiol Cell Physiol* 302: C463–C472, 2012. First published November 9, 2011; doi:10.1152/ajpcell.00010.2011.—Uncoupling protein (UCP)-1 expressed in brown adipose tissue plays an important role in thermogenesis. Recent data suggest that brown-like adipocytes in white adipose tissue (WAT) and skeletal muscle play a crucial role in the regulation of body weight. Understanding of the mechanism underlying the increase in UCP-1 expression level in these organs should, therefore, provide an approach to managing obesity. The thyroid hormone (TH) has profound effects on mitochondrial biogenesis and promotes the mRNA expression of UCP in skeletal muscle and brown adipose tissue. However, the action of TH on the induction of brown-like adipocytes in WAT has not been elucidated. Thus we investigate whether TH could regulate UCP-1 expression in WAT using multipotent cells isolated from human adipose tissue. In this study, triiodothyronine (T_3) treatment induced UCP-1 expression and mitochondrial biogenesis, accompanied by the induction of the CCAAT/enhancer binding protein, peroxisome proliferator-activated receptor-γ coactivator-1α, and nuclear respiratory factor-1 in differentiated human multipotent adipose-derived stem cells. The effects of T_3 on UCP-1 induction were dependent on TH receptor-β. Moreover, T_3 treatment increased oxygen consumption rate. These findings indicate that T_3 is an active modulator, which induces energy utilization in white adipocytes through the regulation of UCP-1 expression and mitochondrial biogenesis. Our findings provide evidence that T_3 serves as a bipotential mediator of mitochondrial biogenesis.

human multipotent adipose-derived stem cells

IN BOTH RODENTS AND humans, brown adipose tissue (BAT) is specialized for energy expenditure through thermogenesis (7, 11). Uncoupling protein (UCP)-1 is considered to play an important role in thermogenesis in BAT. UCP-1 generates heat by leaking proton across the mitochondrial inner membrane, thus uncoupling oxidative phosphorylations without ATP production (32). Ablating UCP-1 causes cold sensitivity (10) and obesity in mice (11). Thus UCP-1 in BAT is essential for thermogenesis (12). However, Nagase et al. (26) reported that chronic stimulation of β-adrenergic receptor induces the ectopic expression of UCP-1 in white adipose tissue (WAT) and skeletal muscle. Indeed, some brown adipocytes arise from progenitor cells in WAT under certain physiological and pharmacological conditions (27). The increase in UCP-1 expression level in WAT has been suggested as the mechanism that prevents obesity (13). These brown-like adipocytes show an increase in oxygen consumption rate (OCR) in response to noradrenalin stimulation (27) and are abundant in an obesity-resistant strain of mice (1), suggesting a crucial role of these cells in the regulation of body weight. However, the mechanism underlying UCP-1 induction in white adipocytes is poorly known. Human multipotent adipose-derived stem (hMADS) cells are a suitable human fat cell model for investigating the mechanism because they are able to differentiate into various lineages, including adipocytes with molecular and functional characteristics of human adipocytes (30, 31). In addition, chronic peroxisome proliferator-activated receptor (PPAR)-γ activation leads to the conversion of these cells to functional brown adipocytes (9). Thus hMADS cells are suitable for investigating the mechanism underlying the regulation of UCP-1 expression in WAT.

The thyroid hormone (TH) is an important physiological modulator for energy homeostasis in the entire body (21). The physiological effects of TH are exerted at the level of transcription through interaction with specific TH receptors (TRs), TR-α and TR-β (3). TH induces mitochondrial biogenesis by modulating multiple mitochondrial respiratory gene expression, thereby enhancing coupled oxidative phosphorylations in a tissue-specific manner (34). In adipose tissue, TH regulates multiple aspects of lipid metabolism, including lipogenesis, lipolysis, and thermogenesis (2, 19, 43). TH treatment induces *UCP-1* expression in brown adipocytes (14) by interaction with a specific TR in the nucleus (28). TR-β mediates triiodothyronine (T_3)-induced *UCP-1* expression, whereas the TR-α isoform regulates adaptive thermogenesis in BAT (29). The effect is also dependent on the induction of type 2 iodothyronine deiodinase (D2), which increases the amount of active TH (T_3) in brown adipocytes (8). Furthermore, activating this enzyme increases energy expenditure in BAT (45), suggesting a crucial role of TH in thermogenesis in the tissue. These observations suggest that TH plays a pivotal role in the regulation of energy homeostasis through direct interaction with adipocytes. However, the action of TH on UCP-1 expression in white adipocytes has not been elucidated.

In this study, we investigate whether TH could regulate UCP-1 expression in WAT using multipotent cells isolated from human adipose tissue. T_3 treatment increased the mRNA expression level of mitochondrial biogenesis genes, including PPAR-γ coactivator-1α (*PGC-1α*), nuclear respiratory factor 1 (*NRF1*), and cytochrome *c* (*Cyt c*), in adipocyte differentiation of hMADS cells. T_3 treatment also increased *UCP-1* mRNA

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expression level via a TR-mediated pathway, resulting in the increase in OCR.

MATERIALS AND METHODS

Materials and cell culture. 3,3′,5-Triiodo-L-thyronine sodium salt was purchased from Sigma and dissolved in DMSO as a stock solution. All of the other chemicals used were from Sigma or Nacalai Tesque (Kyoto, Japan) and guaranteed to be of reagent or tissue-culture grade.

hMADS cells were established by Rodriguez et al. (30) and supplied by Stem Cell Sciences KK (Kobe, Japan). We modified the culture protocol from the published protocol (9, 30). hMADS cells were maintained in a maintenance medium (MM; 10% FBS and 10 mg/ml penicillin/streptomycin in DMEM) at 37°C in 5% CO_2/95% air under a humidified condition. Under this condition, the concentrations of free T_3 and thyroxine in FBS were 2.1 ± 0.006 and 165.6 ± 0.285 nM, respectively. The determinations of total and free T_3 concentrations were performed by Mitsubishi Chemical Mediense (Tokyo, Japan). For white adipocyte differentiation, hMADS cells were seeded at a high density (25,000 cells/cm^2). Two days after the seeding, the cells were incubated in differentiation medium (DM), which was MM supplemented with 1 μM dexamethasone, 0.5 mM 1-methyl-3-isobutylxanthine, 0.85 μM insulin, and 1 μM pioglitazone. Three days after the incubation, the cell culture medium was changed to a post-DM, which was MM supplemented with 0.85 μM insulin and 1 μM pioglitazone, and then the medium was changed with a fresh one every 2 days. Cells cultured on 6- or 12-well tissue culture plates were prepared for biochemical assays.

RNA preparation and quantification of gene expression. RNA samples of the differentiated hMADS cells were prepared using cells cultured on 12-well tissue culture plates 10 days after the differentiation induction by Sepasol-RNA-I Super (Nacalai Tesque), in accordance with the manufacturer's protocol. Aliquots of total RNA were reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen), in accordance with the manufacturer's instructions using a thermal cycler (Takara PCR Thermal Cycler SP, Takara Shuzo, Kyoto, Japan). To determine the mRNA expression levels of genes, quantitative PCR was performed using a fluorescence temperature cycler (LightCycler System, Roche Diagnostics, Mannheim, Germany), as previously described (36, 40). The primer sets used in this study are listed in Table 1. To compare mRNA expression levels among samples, the copy number of each transcript was divided by that of ribosomal protein large P0 (36B4), showing a constant mRNA expression level. The mRNA expression levels are presented as a ratio compared with a control in each experiment.

Western blotting analysis. Total cellular proteins were solubilized in lysis buffer (50 mM Tris·HCl, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS; pH 7.4), and a protease inhibitor cocktail. Proteins were diluted with Laemmli SDS-PAGE sample buffer. Equal amounts of proteins were separated by SDS-PAGE, and separated proteins were transferred to Immobilon-P membrane (Millipore). The membranes were blocked with 5% skim milk in PBS overnight at 4°C and incubated with indicated primary antibodies diluted with 5% skim milk in PBS for 2 h at room temperature. The bound primary antibodies were detected with a peroxidase-conjugated secondary antibody and enhanced chemiluminescence (Amersham Biosciences).

Mitochondrial content and microscopic analysis. Mitochondrial staining for analyzing the mass of mitochondria was performed using an oxidized MitoTracker Red probe (CM-XRos, Invitrogen), in accordance with the manufacturer's protocol. Ten days after the differentiation induction, hMADS cells were incubated at 37°C for 30 min with 200 nM MitoTracker Red and washed twice with PBS. Fluorescence signals were imaged using an IX71 fluorescence microscope (Olympus, Tokyo, Japan).

To examine the effects of T_3 on mitochondrial content, mitochondrial DNA (mtDNA) and genomic DNA samples of hMADS cells were prepared using cells cultured 10 days after differentiation induction. Nuclear and mitochondrial extracts were prepared in accordance with a protocol described by Busch et al. (4). The differentiated hMADS cells (2×10^8) were homogenized using a motor-driven homogenizer in 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 1% protease inhibitor cocktail. The homogenates were centrifuged at 228 *g* for 5 min at 4°C. Pellets (nuclear fraction) were stored for genomic DNA isolation. Supernatants were centrifuged at 20,000 *g* for 20 min at 4°C. Pellets (mitochondrial and

Table 1. *Primers for real-time PCR*

Gene	Forward Primer	Reverse Primer	Association No.
aP2	GCATGGCCAAACCTAACATGA	CCTGGCCCAGTATGAAGGAAA	NM_001442
Adiponectin	CCTAAGCCAGACATCGGTGA	GTAAAGCGAATGGGCATGTT	NM_001177800
PPAR-γ	TGAATGTGAAGCCCATTGAA	CTGCAGTAGCTGCACGTGTT	NM_005037
C/EBP-α	TGTATACCCCTGGTGGGAGA	TCATAACTCCGGTCCCTCTG	NM_004364
FATP1	CCACTTGGATGTCACACTG	GTGGGACCCTCCAGTAGACA	NM_198580
LPL	GTGGCCAAATAGCACATCCT	CCGAAAGATCCAGAATTCCA	NM_000237
UCP-2	CTTTCCCCACCTCTTCCTTC	AGGACGAAGATTCTGGCTGA	NM_003355
UCP-1	TGCCCAACTGTGCAATGAA	TCGCAAGAAGGAAGGTACCAA	NM_021833
PGC-1α	CTGTGTCACCACCCAAATCCTTAT	TGTGTCGAGAAAAGGACCTTGA	NM_013261
NRF1	CGTTGCCCAAGTGAATTATTCTG	ACTGTAGCTCCCTGCTGCAT	NM_005011
TFAM	CCCAGATGCAAAAACTACAGAACTAA	TCCGCCCTATAAGCATCTTGA	NM_003201
PRDM16	TCCTGAAGACATTCCGATCC	CCGAAGTCTGTCTCCTTTGC	NM_022114
Cidea	CTTAACGTGAAGGCCACCAT	CCCTATCCACACGTGAACCT	NM_001279
Elovl3	AAGGACATGAGGCCCTTTTT	AAGATTGCAAGGCAGAAGGA	NM_152310
Cyt c	AGGCCCCTGGATACTCTTACACAG	TCAGTGTATCCTCTCCCCAGATG	NM_018947
COX4	CCAGAAGGCATTGAAGGAGA	GGGCCGTACACATAGTGCTT	NM_001861
COX8a	TGTACTCCGTGCCATCATGT	AGAACGGACCCCTTCACTCT	NM_004074
CPT-1b	CTCCTTTCCTTGCTGAGGTG	TCTCGCCTGCAATCATGTAG	NM_001145134
AOX	CTGAAGGCTTTCACCTCCTG	CATGCCACACACCAACTTTC	NM_004035
36B4	AAACTGCTGCCTCATATCCGG	TTGTAGATGCTGCCATTGTCGA	NM_001002

aP2, adipocyte lipid-binding protein; *PPAR-γ*, peroxisome proliferator-activated receptor-γ; *C/EBP-α*, CCAAT/enhancer binding protein-α; *FATP1*, fatty acid transporter-1; *LPL*, lipoprotein lipase; *UCP-2*, uncoupling protein-2; *UCP-1*, uncoupling protein-1; *NRF1*, nuclear respiratory factor 1; *TFAM*, mitochondrial transcription factor-A; *PGC-1α*, PPAR-γ coactivator-1α; *PRDM16*, PR domain containing-16; *Cidea*, cell death-inducing DFFA-like effector-a; *Elovl3*, ELOVL fatty acid elongase-3; *Cyt c*, cytochrome *c*; *COX4*, Cyt c oxidase subunit IV; *COX8a*, Cyt c oxidase subunit VIIIa; *CPT-1b*, human carnitine palmitoyltransferase-1b; *AOX*; acyl-CoA oxidase; *36B4*, ribosomal protein large P0.

cytosol fraction) were stored for mtDNA isolation. Each fraction was resuspended in cell lysis buffer (containing 50 mM NaCl, 5 nM EDTA, 0.1% SDS, 10 mM HEPES; pH 7.9). The fractions were incubated with Protease K (60 μg/ml) at 55°C for 30 min. Phenol-chloroform-isoamyl alcohol (25:24:1; Nacalai Tesque) was added using the same volume for each fraction. After centrifugation (at 12,000 *g* for 10 min), isopropanol (Nacalai Tesque) was added using the same volume for each fraction. After centrifugation, the pellets containing mtDNA and genomic DNA were solublized in DNase-free water containing RNase A. The concentrations of mtDNA and genomic DNA were measured using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). The concentration of mtDNA was normalized by those of genomic DNA. The mtDNA levels are presented as a ratio compared with a control in each experiment.

TH antagonist and small interfering RNA-mediated TR knockdown. hMADS cells were cultured in MM supplemented with 5 μM 1–850, a TR antagonist (Merck KGaA, Darmstadt, Germany) (33), 12 h before the start of T_3 treatment. hMADS cells were then incubated in DM containing 50 nM T_3 and 5 μM 1–850 for 3 days. Small interfering RNA (siRNA) was transfected using Lipofectamine 2000 (Invitrogen) in accordance with manufacturer's protocol. Experiments using fluorescein isothiocyanate-conjugated siRNA (BLOCK-iT Alexa Fluor Red Fluorescent Oligo; Invitrogen) demonstrated that the transcription efficacy of each nontargeting siRNA was >90% (data not shown). hMADS cells were seeded at a high density (25,000 cells/cm^2) in antibiotic-free DMEM containing siRNA (Qiagen) and then incubated at 37°C for 3 days. Three days after the transfection, the cells were incubated with DM for differentiation induction. The sequences of the siRNA against *TR-α* and *TR-β* were 5′-TCCCAC-CTATTCCTGCAAATA-3′ and 5′-AAGTGAGACTTTAACCTT-GAA-3′, respectively.

OCR. OCR indicative of mitochondrial respiration was determined using an XF24 Extracellular Flux Analyzer (Seahorse Bioscience). The XF24 device created transient 7-μl chambers above target cells in which OCR was monitored in real time, as previously reported (46). hMADS cells were seeded at a density of 12,500 cells/well on the customized Seahorse 24-well plates (Seahorse Bioscience). Ten days after the differentiation induction on the customized plates, the cells were incubated in prewarmed XF24 assay media for 1 h. The assay media consisted of DMEM containing 10% FBS, 1 mM L-glutamine, 1 mM sodium pyruvate, 3.7 g/l NaCl, and 25 mM glucose. OCR was calculated by plotting the O_2 tension of the media in the chamber as a function of time (pmol/min). Values were divided by the protein amount in each well.

To test the effect of β-agonist, 1 μM of isoproterenol was added in the transient chambers, and similar experiments as described above were then carried out.

Fatty acid oxidation measurement. Ten days after differentiation induction, hMADS cells were incubated in DMEM containing 0.2 mM palmitic acid, 2.5% fatty acid (FA)-free BSA, 200 μM l-carnitine, and [^{14}C]palmitic acid (1 μCi) (American Radiolabeled Chemicals) for 16 h. FA oxidation products were assessed as previously described (40) with modification. Briefly, the labeling medium was collected and centrifuged, and the supernatant was transferred to a 50 ml polypropylene tube. An uncapped Eppendorf tube containing a piece of filter paper soaked in benzethonium hydroxide was placed inside a 50-ml tube. After the tube was sealed, 200 μl of 70% perchloric acid were added to the medium sample to release [^{14}C]CO_2. The tube was then shaken at 37°C for 1 h. The saturated filter paper containing trapped [^{14}C]CO_2 was assessed for radioactivity in a liquid scintillation counter (LS6500, Beckman Coulter). The acidified medium was centrifuged twice to remove particulate matter, and 200 μl of supernatant were assessed for the amount of [^{14}C]-labeled acid soluble metabolites (ASMs), which include labeled ketone bodies.

Statistical analyses. The data are presented as means ± SE. Data of two experimental groups were compared using unpaired Student's *t*-test. Multiple comparisons were carried out using ANOVA, followed by the Bonferroni test. Differences were considered significant when P was < 0.05.

RESULTS

T_3 treatment did not induce adipocyte differentiation but induced UCP-1 mRNA expression in hMADS cells. To determine the effects of T_3 on the mRNA expression of *UCP-1*, confluent hMADS cells were incubated in DM with 250 nM T_3 for 3 days. Although the concentrations of T_3 were very high, the concentrations of free T_3, an active form of T_3, were 1.20 and 2.30 nM in the medium containing 100 and 250 nM T_3, respectively. Ten days after the T_3 treatment to induce adipocyte differentiation, the mRNA expression level of CCAAT/enhancer binding protein (*C/EBP-α*) significantly increased, whereas the white adipocyte marker genes, adipocyte lipid-binding protein (*aP2*, also known as fatty acid binding protein 4, *FABP4*), adiponectin, FA transporter-1 (fatty acid transport protein 1, *FATP1*), lipoprotein lipase (*LPL*), and *UCP-2* were not affected by T_3 treatment (Fig. 1*A*). On the other hand, treatment with T_3 increased the mRNA expression levels of *UCP-1* (4.6-fold increase), cell death-inducing DFFA-like effector-a (*Cidea*), and *ELOVL* FA elongase-3 (*Elovl3*), as shown in Fig. 1*B*. The T_3 treatment increased the mRNA expression levels of *PGC-1α* (3.2-fold increase) and *NRF1*, a specific marker gene involved in mitochondrial biogenesis (1.9-fold increase). The mRNA expression level of PR domain containing-16 (*PRDM16*), a marker of brown adipocytes was not altered by T_3 treatment (Fig. 1*B*). The mRNA expression of *D2*, a gene encoding an enzyme that converts T_4 to T_3, was not detected on *days 0*, *3*, and *10* of adipocyte differentiation of hMADS cells (data not shown). Next, to examine the phase dependence of the effects of T_3 treatment, we treated hMADS cells with 50 nM T_3 in different phases (Fig. 2*A*). No significant induction of *UCP-1* mRNA expression was observed in the treatment in either the −5/−2, −2/0, proliferation, or confluent phase (Fig. 2*B*). Treatment with T_3 from *day 0* to *day 3* (0/3) showed a maximal induction of *UCP-1* mRNA expression. Interestingly, T_3 treatment during differentiation (from *day 7* to *day 10*) or the entire experimental period (from *day 0* to *day 10*) slightly but significantly increased *UCP-1* mRNA expression level. These findings indicate that T_3 induces the mRNA expression of *UCP-1* and other genes related to mitochondria biogenesis in hMADS cells in a differentiation stage-dependent manner. Importantly, the effects of T_3 treatment between *day 0* and *day 3* on *UCP-1* mRNA expression examined on *day 10* suggest that adipocyte transcriptional factors may be involved in the T_3-induced *UCP-1* expression.

UCP-1 mRNA expression was induced in hMADS cells in a T_3 dose-dependent manner. To examine the details of the T_3 effects on mRNA induction, we exposed hMADS cells to T_3 at various concentrations for 3 days (from *day 0* to *day 3*). The T_3 treatment increased the mRNA expression level of *UCP-1* in a dose-dependent manner (Fig. 3*A*). The mRNA expression levels of *PGC-1α* and *NRF1* were increased only at the highest concentration of T_3 (250 nM) (Fig. 3, *B* and *C*). The mRNA expression of mitochondrial transcription factor A *(TFAM)*, a key regulator of mitochondrial biogenesis (5), tended to be higher in the presence of 250 nM T_3, although the difference was not statistically significant (Fig. 3*D*). Next, we examined *UCP-1* mRNA expression at various time points (Fig. 4*A*). T_3

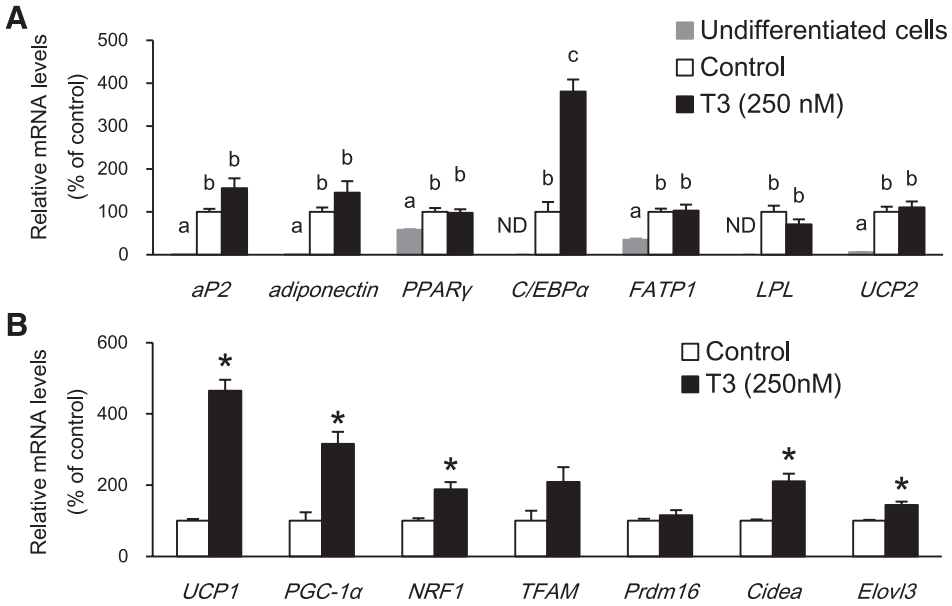

Fig. 1. Effects of triiodothyronine (T_3) treatment on mRNA expression levels of adipogenic marker genes, *UCP-1*, and mitochondrial biogenesis genes in human multipotent adipose-derived stem (hMADS) cells. hMADS cells were incubated in differentiation medium (DM) supplemented with vehicle or T_3 (250 nM) for 3 days. Ten days after adipocyte differentiation, the mRNA expression levels of adipogenic marker genes (*PPAR-γ*, *aP2*, *adiponectin*, *C/EBP-α*, *FATP1*, *LPL*, and *UCP-2*; *A*) and thermogenic genes and mitochondrial biogenesis genes (*UCP-1*, *PGC-1α*, *NRF1*, *TFAM*, *PRDM16*, *Cidea*, and *Elovl3*; *B*) were determined. Values are means ± SE ($n = 4$). [a,b,c] Bars without a common letter (*A*), or bars with asterisk (*B*): $P < 0.05$ compared with control, as analyzed by the unpaired *t*-test or one-way ANOVA and Bonferroni test. ND, not detected. See text for definition of gene acronyms.

(50 nM) treatment for 3 days (from *day 0* to *day 3*) significantly increased the mRNA expression levels of *UCP-1* and *C/EBP-α* on *day 8* (Fig. 4, *A* and *E*). These expression levels were maintained up to *day 10*. The mRNA expression of TFAM was decreased from *day 8* in hMADS cells. The decrease was inhibited by the T_3 treatment. On the other hand, those of the other genes did not show significant differences at this concentration of T_3.

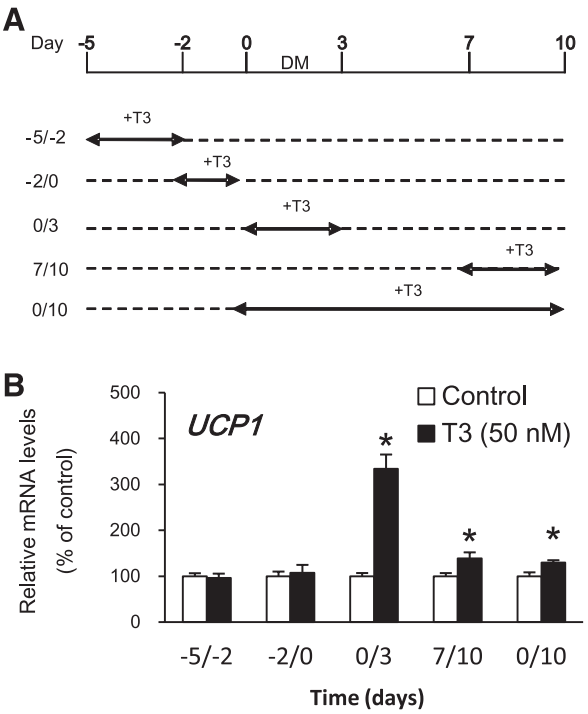

Fig. 2. Phase-dependent effects of T_3 treatment on mRNA expression levels of *UCP-1* and mitochondrial biogenesis genes in adipocyte differentiation. *A*: schematic diagram of protocols to examine phase dependence of effects of T_3 treatment (50 nM). hMADS cells were exposed to DM in the absence or presence of T_3, as indicated. *B*: T_3 was added between indicated days. Ten days after adipocyte differentiation, the mRNA expression level of *UCP-1* was determined. Values are means ± SE ($n = 4$). *$P < 0.05$ compared with control, as analyzed by the unpaired *t*-test.

T_3 treatment increased mitochondrion-related gene expression and mitochondrial biogenesis. To further clarify the effects of T_3 on mitochondrial biogenesis, nucleus-encoded mitochondrial genes, *Cyt c*, *Cyt c* oxidase subunit IV (*COX4*), and *Cyt c* oxidase subunit VIIIa (*COX8a*), were measured by real-time PCR. The *Cyt c* mRNA level at the 10th day was decreased in the control cells, and the decrease was significantly inhibited in the T_3-treated cells. At protein levels, Cyt c expression was also higher in cultured cells treated with T_3 than in control at the 10th day (Fig. 5*C*). The protein levels of Cyt c and UCP-1 showed 1.65- and 3.34-fold amounts, respectively, in the T_3-treated cells. The mRNA levels of *COX4* and *COX8a* were higher in the T_3-treated cells than in control (Fig. 5*D*). Moreover, the treatment with T_3 also increased amounts of mtDNA, indicating higher levels of mitochondrial amounts (Fig. 5*E*). These findings suggest that the T_3 treatment augments mitochondrial biogenesis in hMADS cells. Indeed, staining of differentiated hMADS cells with MitoTracker Red revealed stronger staining of T_3-treated cells than that of control cells (Fig. 5*F*), indicating higher levels of membrane potential by T_3 treatment. Increased membrane potential is created by the respiratory chain reaction to drive ATP synthesis, suggesting that T_3 treatment may increase glucose and FA oxidation in hMADS cells.

T_3-induced UCP-1 mRNA expression was mediated by TR. To investigate the involvement of TR in *UCP-1* mRNA expression, the TR antagonist 1–850 (5 μM) was added to the medium throughout the T_3 treatment period (50 nM). As expected, T_3 in the absence of 1–850 significantly increased the *UCP-1* mRNA expression level in cultured cells. The addition of 1–850 significantly decreased the T_3-mediated *UCP-1* mRNA expression level (Fig. 6*A*). To further verify the TR dependence of the effects of T_3, we decreased *TR* expression level by siRNA-mediated knockdown. Quantitative RT-PCR revealed the decrease of the *TR-α* mRNA level by siRNA treatment, although that of *TR-β* mRNA level was not signif-

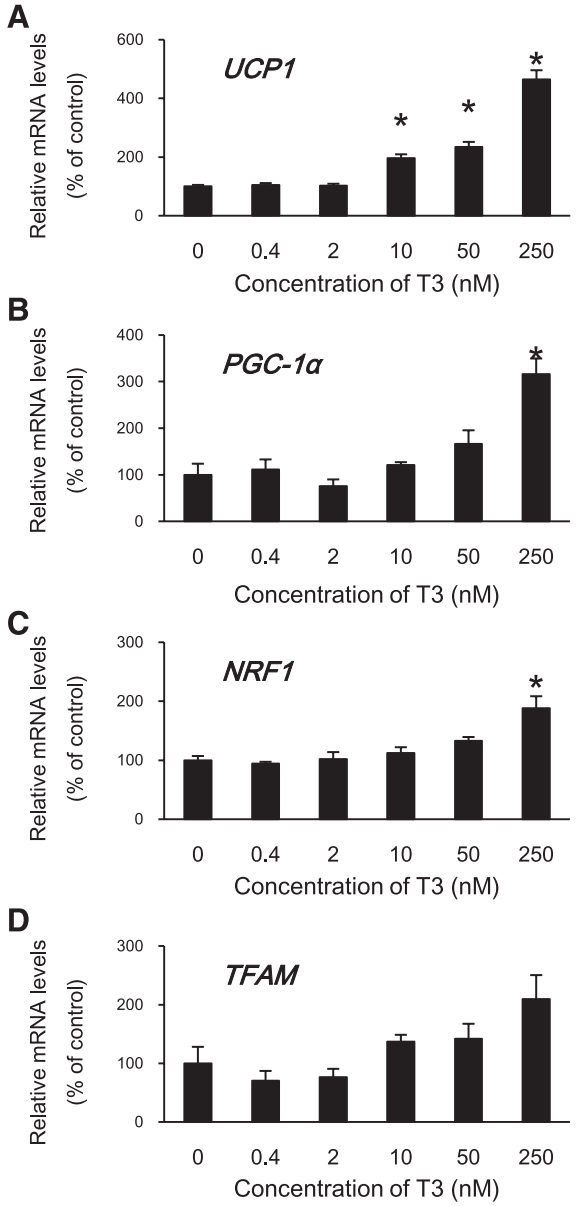

Fig. 3. Dose-dependent effects of T_3 treatment on mRNA expression levels of *UCP-1* and mitochondrial biogenesis genes. hMADS cells were differentiated into adipocytes and exposed to the indicated concentrations of T_3 from *day 0* to *day 3*. Ten days after adipocyte differentiation, the mRNA expression levels of *UCP-1* (*A*), *PGC-1α* (*B*), *NRF1* (*C*), and *TFAM* (*D*) were determined. Values are means ± SE ($n = 4$). *$P < 0.05$ compared with control, as analyzed by one-way ANOVA and Bonferroni test.

icant (Fig. 6*B*). However, the TR-β protein level was significantly decreased (43.1%), as shown in Fig. 6*C*. The knockdown of TR attenuated the TR-dependent induction of *UCP-1* mRNA expression (Fig. 6*D*). Specificity of TR isoforms was not observed under our experimental conditions. These findings indicate that T_3 induces *UCP-1* mRNA expression by TR activation, and TR mediates the T_3-induced *UCP-1* mRNA expression.

T_3 treatment increased OCR and FA oxidation. To determine whether mitochondrial biogenesis is accompanied by functional changes in cellular metabolism, we compared OCR of the T_3-treated hMADS cells with that of nontreated cells. OCR was significantly increased by the T_3 treatment (Fig. 7*A*). The T_3 treatment showed a 1.2-fold increase in OCR. To determine the UCP-1-mediated respiration, hMADS cells were treated with isoproterenol. OCR was increased in T_3-treated cells (Fig. 7*B*).

Next, we measured the mRNA expression levels of FA oxidation-related genes. T_3 treatment increased the mRNA

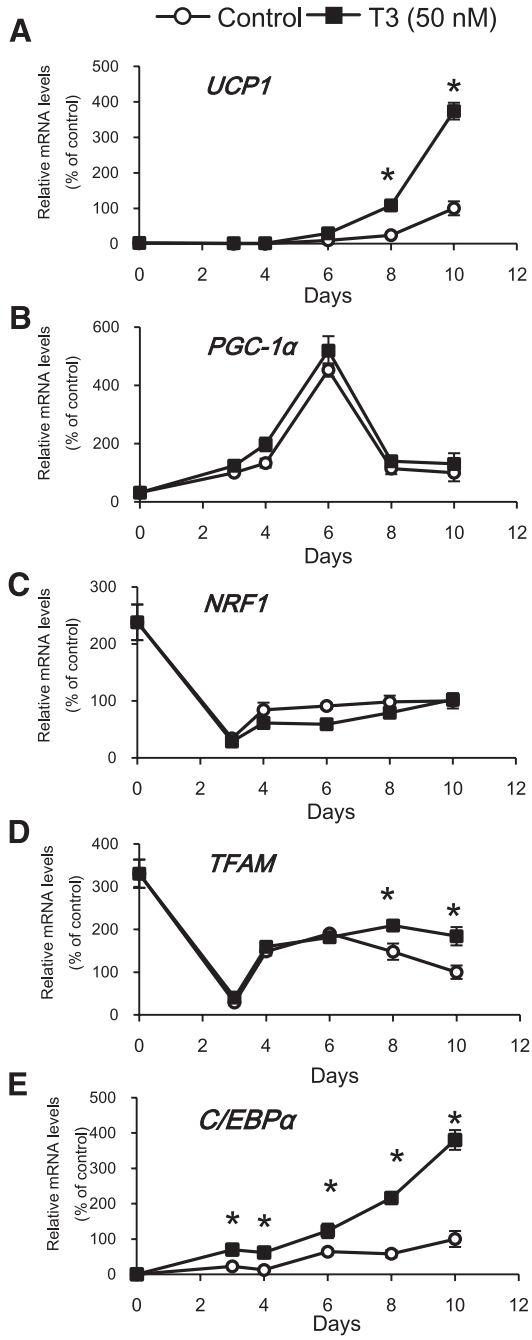

Fig. 4. Time course of mRNA expression levels of *UCP-1* and mitochondrial biogenesis genes during adipocyte differentiation. hMADS cells were differentiated into adipocytes and exposed to T_3 (50 nM) from *day 0* to *day 3*. The mRNA expression levels of *UCP-1* (*A*), *PGC-1α* (*B*), *NRF1* (*C*), *TFAM* (*D*), and *C/EBP-α* (*E*) were quantified by a real-time PCR at indicated time points. Values are means ± SE ($n = 4$). *$P < 0.05$ compared with control, as analyzed by the unpaired *t*-test. The control at *day 10* was set in each experiment to 100%.

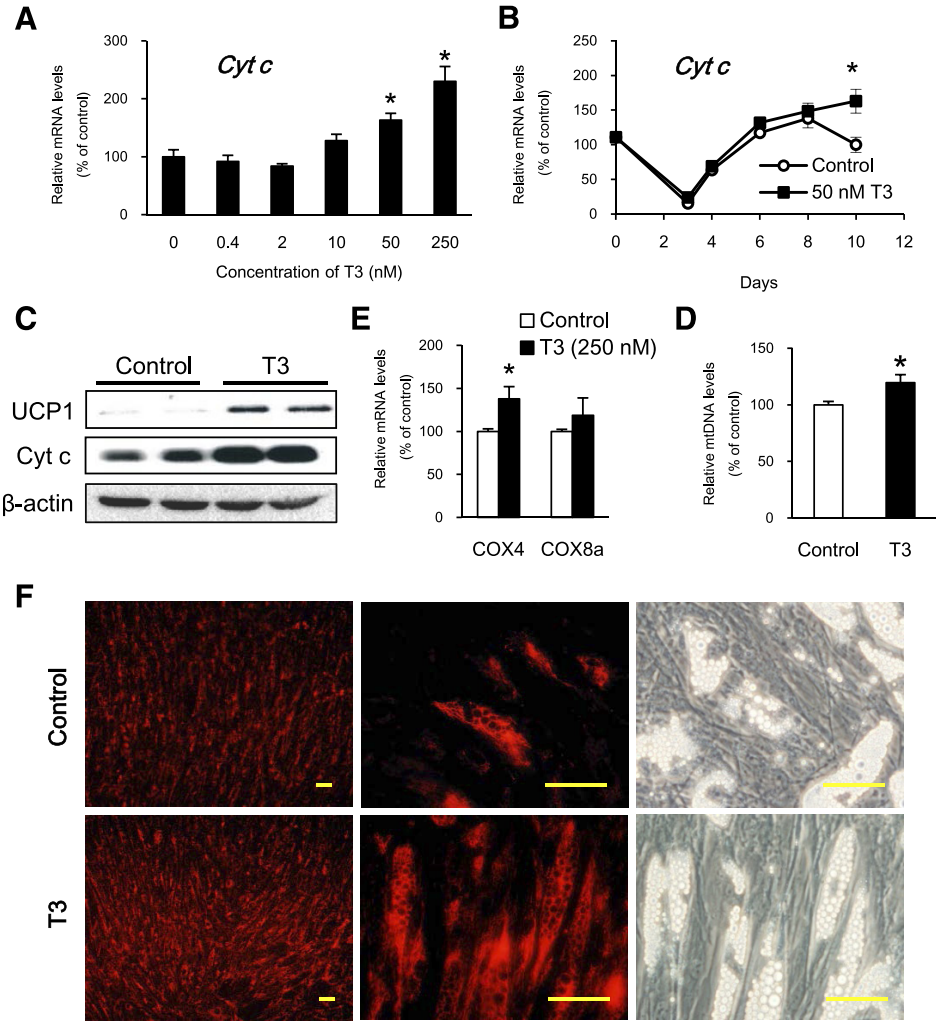

Fig. 5. Mitochondrion-related gene expression and amounts of mitochondria in the T_3-treated hMADS cells. *A*: hMADS cells were differentiated into adipocytes and exposed to various concentrations of T_3 from *day 0* to *day 3*. The mRNA expression level of *Cyt c* was determined by real-time PCR on *day 10* of culture. Values are means ± SE ($n = 4$). *$P < 0.05$ compared with control, as analyzed by one-way ANOVA and Bonferroni test. *B*: hMADS cells were incubated in DM in the absence or presence of T_3 (50 nM) from *day 0* to *day 3*. The mRNA expression level of *Cyt c* was determined at the indicated time points. The control at *day 10* was set in each experiment to 100%. *C*: total cell lysates (30 μg protein/lane) were examined by Western blot assay using indicated antibodies. *D*: the mRNA expression levels of *COX4* and *COX8a* were determined by real-time PCR on *day 10* of culture. Values are means ± SE ($n = 4$). *$P < 0.05$ compared with control, as analyzed by the unpaired *t*-test. *E*: the concentration of mitochondrial DNA (mtDNA) was determined by spectrophotometry on *day 10* of culture. The concentration of mtDNA was normalized by those of genomic DNA. The value of a control was set at 100%, and the relative value was presented as fold induction with respect to that of the control. *F*: differentiated hMADS cells were analyzed for mitochondrial abundance by MitoTracker Red staining. The *center* and *right* panels show images of the same areas. The yellow scale bars in panels represent 100 μm. See text for definition of gene acronyms.

expression levels of human carnitine palmitoyltransferase-1b (*CPT-1b*) and acyl-CoA oxidase (*AOX*), which are rate-limiting enzymes in mitochondria and peroxisomes, respectively (Fig. 7*C*). Moreover, the evaluation of CO_2 and ASM releases using [^{14}C]palmitic acid revealed that T_3 treatment increased the amounts of released CO_2 and ASMs, which are products of FA oxidation, in hMADS cells (Fig. 7, *D* and *E*). These findings suggest that T_3 treatment augmented oxidative capacity in addition to mitochondrial biogenesis in hMADS cells.

DISCUSSION

In this study, T_3 induced the UCP-1 expression in hMADS cells under the conditions for white adipocyte differentiation. UCP-1, a brown adipocyte-specific protein, consumes lipids without ATP production in brown adipocytes, suggesting that the increase in UCP-1 expression level suppresses the accumulation of lipids, thus regulating body weight (1, 13). Therefore, understanding of the mechanism of the increase in UCP-1 expression level contributes to the management of obesity and obesity-related diseases. The expression of UCP-1 is classically considered to be a physiological difference between brown and white adipocytes, which have distinct developmental origins (38). However, recent studies have shown that UCP-1 expression is observed in WAT under chronic β-adrenergic receptor activation or PPAR-γ activation in animals (17, 26). White preadipocytes isolated from WAT can differentiate into UCP-1-expressing cells without mRNA expression of brown adipocyte markers (27). Elabd et al. (9) have also shown that hMADS cells differentiate into functional brown adipocytes, depending on the duration of PPAR-γ activation. The observations suggest that the UCP-1-expressing cells in WAT are descendants of white adipocyte precursors, but not of brown adipocyte precursors. In this study, T_3-treated hMADS cells after differentiation showed the phenotype of white adipocytes, indicating the involvement of TH in the regulation of UCP-1 expression in white adipocytes. The concentrations of T_3 used in this study (50–250 nM) were higher than the physiological concentrations. However, the concentrations of free T_3, an active form of TH, were ~10% of total T_3 in serum. Indeed, the free T_3 concentrations were 1.20 and 2.30 nM in our used medium containing 100 and 250 nM T_3, respectively. The data suggest a possibility that T_3 affects the UCP-1 expression at lower concentrations. The actual concentrations of the free T_3 were much lower than expected. This is because changes of culture medium increase D3 activity to a great extent and lead to a rapid decrease in T_3 concentrations in a few hours under in vitro conditions (15). In brown adipocytes, the amount of T_3 is increased by D2. The increase in the local

concentration of T_3 in brown adipocytes is essential for BAT activation (8). In addition, it has been reported that D2 is expressed in WAT, and its expression level is increased by cold exposure (48), although hMADS cells showed no mRNA expression of *D2* (data not shown). Moreover, there are many reports showing that important genomic actions of T_3 usually require higher concentrations (10–100 nM) in in vitro experiments than in intact organisms (22, 35). Although further investigations are required to elucidate the in vivo effects of T_3

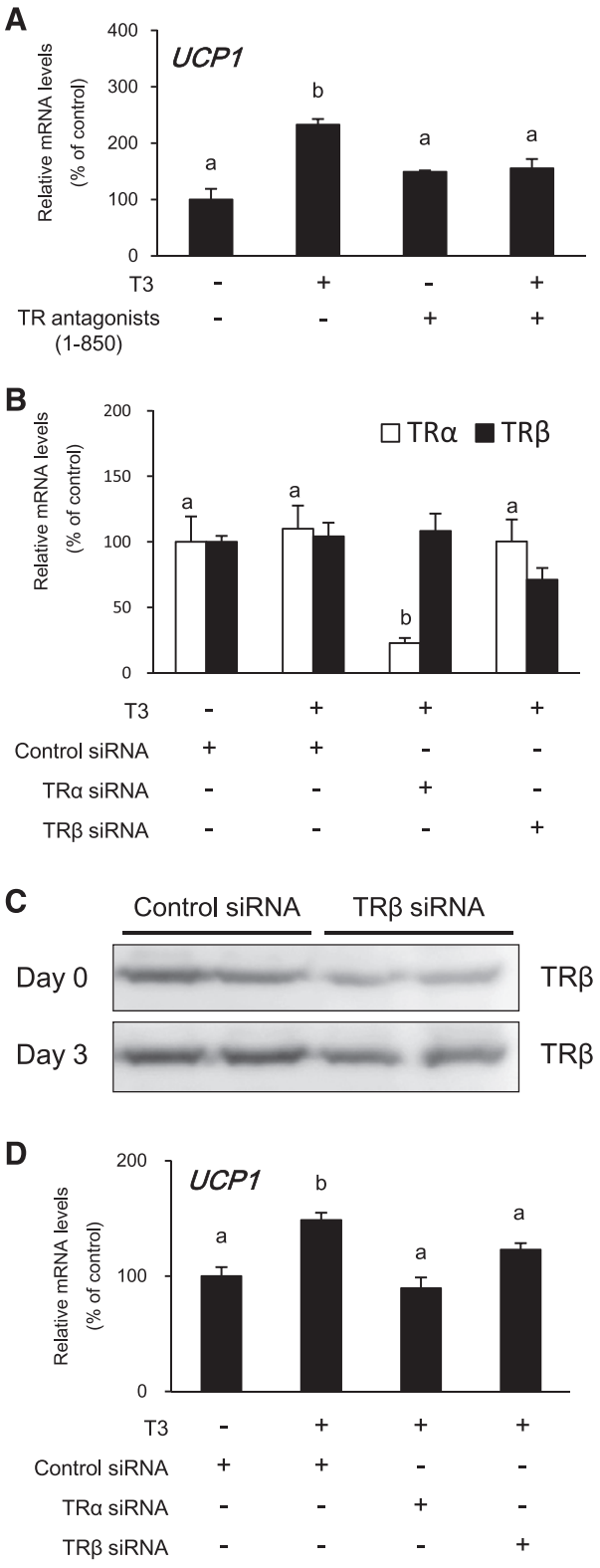

Fig. 6. Involvement of thyroid hormone receptors (TR) in T_3-induced *UCP-1* mRNA expression. *A*: hMADS cells were differentiated into adipocytes and treated with T_3 (50 nM) from *day 0* to *day 3* in the presence or absence of a TR antagonist, 1–850 (5 μM). Ten days after differentiation induction, the mRNA expression level of *UCP-1* was quantified by real-time PCR. Values are means ± SE ($n = 4$). *B* and *C*: TRs were knocked down with a small interfering RNA (siRNA), as described in MATERIALS AND METHODS. The protein levels of TR-β are representative data. Quantification of the TR-β protein expression at *day 3* is described in the text. *D*: 10 days after differentiation induction, the mRNA expression level of *UCP-1* was quantified. Values are means ± SE ($n = 4$). $^{a,b|}$ Bars without a common letter, $P < 0.05$ compared with control, as analyzed by the one-way ANOVA and Bonferroni test.

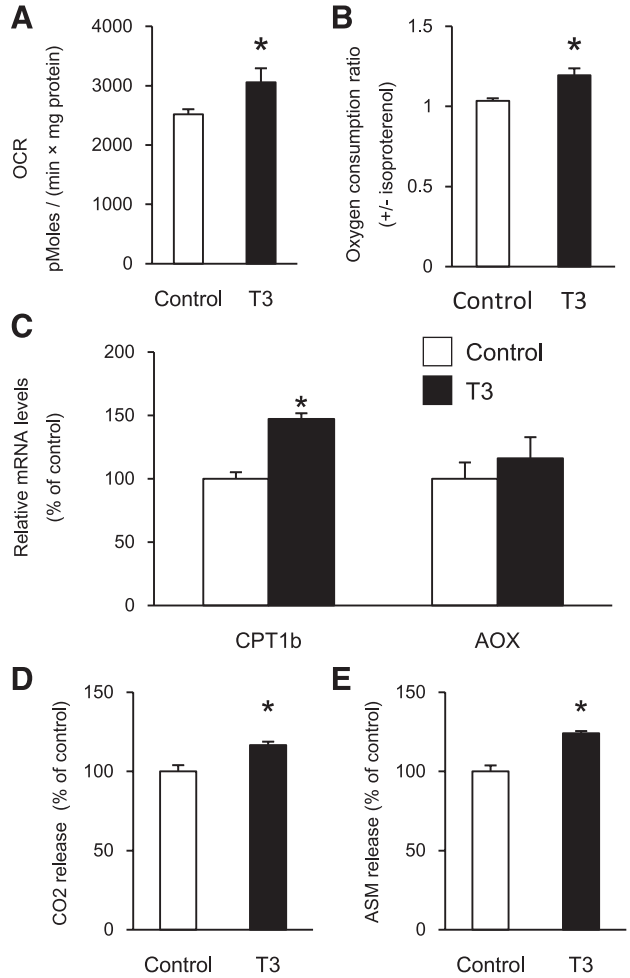

Fig. 7. Effects of T_3 treatment on oxygen consumption rate (OCR) and fatty acid oxidation in hMADS cells. hMADS cells were differentiated into adipocytes and treated with T_3 (250 nM) from *day 0* to *day 3*. Ten days after differentiation induction, OCR (*A*) and β-agonist-stimulated respiration (the ratio of OCR before and after addition of 1 μM isoproterenol; *B*) were determined by the method described in MATERIALS AND METHODS. The mRNA expression levels of adipocyte differentiation marker genes (*CPT-1B* and *AOX*; *C*), CO_2 release (*D*), and acid-soluble metabolite (ASM; *E*) release in T_3-treated hMADS cells were determined. The value of a control was set at 100%, and the relative value was presented as fold induction with respect to that of the control. Values are means ± SE ($n = 10$). *$P < 0.05$ compared with control, as analyzed by the unpaired *t*-test. See text for definition of gene acronyms.

and the requirement of high concentrations in in vitro experiments, our findings may provide a clue to understanding the effects of T_3 on UCP-1 induction in white adipocytes.

TH has been shown to regulate a wide range of genes involved in metabolisms in adipose tissues (14, 19, 43). Chronic TH treatment has increased heat production in rodent animals, indicating that TH is involved in thermogenesis (16, 24). The effects of TH on thermogenesis are both direct and indirect. Several transcriptional factors have been suggested as intermediary factors for the indirect effects (18). For example, C/EBP-α is a transcriptional activator of the promoter sequences of *UCP-1* (49) and ablating C/EBP-α causes defects in *UCP-1* expression (44) in BAT of mice. The overexpression of *C/EBP-α* induces *UCP-1* expression in fetal brown adipocytes (42). These findings suggest a possibility that C/EBP-α can play an important role in the induction of *UCP-1* expression in white adipocytes. In the present study, T_3 treatment enhanced the induction of *C/EBP-α* mRNA expression, and *C/EBP-α* mRNA expression level was continuously increased during adipocyte differentiation of hMADS cells. The findings suggest that T_3 exposure in the early phase of adipocyte differentiation induces UCP-1 expression examined on *day 10* via the induction of *C/EBP-α* mRNA expression. It has been reported that T_3 can induce *C/EBP-α* expression by a direct transcriptional regulation in a brown adipocyte cell line (25). Moreover, C/EBP-α permits autoregulation by direct binding of the *C/EBP-α* promoter region (6), as well as by an indirect mechanism (37). Thus our findings may provide evidence that transcriptional factors regulated by T_3 may play an intermediary role in T_3-induced gene expression. On the other hand, PRDM16 has been reported as an important factor indispensable for differentiation into brown adipocytes (20). Recently, it has been demonstrated that PRDM16 determines the thermogenic program of WAT, as well as BAT (20). However, the *PRDM16* mRNA expression was not changed by the T_3 treatment in our study (Fig. 1). The data might suggest a possibility that there is a PRDM16-independent pathway for inducing *UCP-1* expression in white adipocytes or that the basal expression of *PRDM16* in hMADS cells is enough to stimulate the T_3-dependent *UCP-1* expression. Petrovic and colleagues (27) have demonstrated that chronic PPAR-γ activation of mouse primary culture of white adipocytes induces *UCP-1* expression, but only modest increase of *PRDM16*, and described that it is apparently not *PRDM16* gene expression that confers white adipocytes with the ability to express *UCP-1*. Further investigations are needed for examining a role of PRDM16 in the *UCP-1* expression in white adipocytes.

In this study, we demonstrated that *PGC-1α* mRNA expression was also induced by T_3 treatment. Many studies have shown that PGC-1α, which is predominantly expressed in BAT and skeletal muscle, is a regulator of mitochondrial biogenesis (41, 47). The overexpression of *PGC-1α* in a mouse muscle cell line induces *NRF1* mRNA expression and stimulates mitochondrial biogenesis via the induction of *NRF1* (47). Although the *PGC-1α* mRNA expression level is lower in white adipocytes than that in brown adipocytes and muscle cells, a similar transcriptional regulation of *PGC-1α* expression has also been observed in human white adipocytes (39). Thus the regulation of *PGC-1α* and *NRF1* expression may also play an important role in mitochondrial biogenesis in white adipocytes. In this study, T_3 treatment increased the mRNA expression levels of *PGC-1α*, *NRF1*, and *UCP-1* in hMADS cells. These findings suggest that T_3 treatment enhances mitochondrial biogenesis in human white adipocytes. The results of MitoTracker probe staining and immunoblottings of UCP-1 and Cyt c also supported the idea that T_3-mediated gene regulation induces mitochondrial biogenesis in human white adipocytes. The ectopic expression of *PGC-1α* in human adipocytes leads to an increase in *UCP-1* mRNA expression level (39). Importantly, when comparing the mRNA expression levels of *UCP-1*, the minor T_3 effects on the mRNA expression levels of *Cyt c* are contrasted with the general concern that *UCP-1* expression and mitochondrial biogenesis should overlap to a certain extent. However, Petrovic et al. (27) recently showed that a proportion of mitochondrion-rich cells are UCP-1 positive, whereas numerous mitochondrion-rich cells are negative for UCP-1 during the induction of brown adipocytes in WAT by PPAR-γ activation. A recent study also showed that, in a primary culture of β-adrenoceptor knockout brown adipocytes, *UCP-1* mRNA expression level is markedly decreased, whereas *PGC-1α* and *TFAM* mRNA expression levels are not (23). Moreover, in the same experiment, UCP-1, PGC-1α, and COX IV protein expression levels are decreased by 97, 62, and 22%, respectively. The previous observations suggest that the amount of mitochondria is not a limiting factor for the regulation of *UCP-1* expression. These findings suggest that the T_3-mediated mitochondrial biogenesis in human white adipocytes is partly due to the induction of *PGC-1α* and *NRF1* expression, in addition to the direct effect of T_3 through TR activation. However, the results shown in the present study could not demonstrate the increase in mitochondrial uncoupling induced by the T-dependent induction of UCP-1 expression. It is very important for understanding the thermogenic effect of T_3 on energy consumption in white adipocytes to elucidate changes in the uncoupling of mitochondria. Therefore, further investigations must be performed for clarifying the changes in white adipocytes.

In conclusion, the present study demonstrated that T_3 treatment induced UCP-1 expression and mitochondrial biogenesis accompanied by the induction of *PGC-1* and *NRF1* in differentiated hMADS white adipocytes. The effects of T_3 on UCP-1 induction were dependent on TR-β. Moreover, T_3 treatment resulted in the increase in cellular OCR. The findings suggest a possibility that TH is a physiological modulator that induces energy utilization in white adipocytes through the induction of UCP-1 expression. However, the molecular mechanism underlying the effects T_3 on the regulation of gene expression remains to be clarified.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: J.-Y.L., N.T., and T.K. conception and design of research; J.-Y.L., N.T., M.Y., H.H., and T.S. performed experiments; J.-Y.L., N.T., M.Y., H.H., and T.S. analyzed data; J.-Y.L., N.T., M.Y., Y.-I.K., H.H., M.-J.K., T.S., T.G., and T.K. interpreted results of experiments; J.-Y.L., N.T., and Y.-I.K. prepared figures; J.-Y.L. and N.T. drafted manuscript; J.-Y.L., N.T., and T.K. edited and revised manuscript; J.-Y.L., N.T., M.Y., Y.-I.K., H.H., M.-J.K., T.S., T.G., and T.K. approved final version of manuscript.

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