

Long-Term Direct Cold Stimulation of Mouse White Adipocytes Induces a Change to Beige Adipose Tissue-Like Cells

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Abstract

Background: Beige adipocytes and browning tissue appear in white adipocyte tissue through signal transduction via the brain when individuals are exposed to prolonged cold stimulation. In this study, we hypothesized that adipocyte browning may occur when mouse adipocytes are directly exposed to cold stimulation. **Methods:** White adipocytes were incubated under direct cold stimulation, and gene expression associated with transdifferentiation to beige adipocytes was examined. The effects of direct cold stimulation on Ca^{2+} uptake and gene expression were also examined to clarify the initial signal transduction. Moreover, long-term direct cold stimulation was conducted to examine its effects on transdifferentiation. **Results:** Under direct cold stimulation, gene expression associated with transdifferentiation to beige adipocytes was enhanced, and Ca^{2+} uptake occurred, suggesting that white adipocytes possess a pathway for changing to browning cells in response to direct cold stimulation and Ca^{2+} uptake relates to the initial signal transduction. Under long-term direct cold stimulation of white adipocytes, the color of the obtained cells was brownish, UCP1 and mitochondria content increased, and marker gene expression (*Cited1* and *Cd137*) and several batokines of beige adipocytes were enhanced. These results suggest that long-term cold stimulation induces a change from white adipocytes to beige adipose tissue-like cells.

Keywords

Beige Adipocytes, White Adipocytes, Cold Stimulation, Calcium Ions, Batokines

1. Introduction

Brown adipocytes are derived from Myf5+/Sca1+/Pax7+ cells in the dermal muscular layer of dermal precursors. In contrast, beige adipocytes were discovered as a novel a new type of thermogenic cell that differentiates from inguinal subcutaneous white adipose tissue [1]. Several studies have reported that beige adipocytes transdifferentiate from white adipocytes or preadipocytes [2] [3] and can be obtained from MyoD+ [4], smooth muscle-like [5], and PDGFR β + neural progenitors [6], suggesting that several beige adipocyte types may be present.

The characteristics of beige adipocytes are similar to those of brown adipocytes; they contain numerous mitochondria expressing uncoupling protein 1 (UCP1), which transports protons to the inner side of the inner mitochondrial membrane through signal transduction in response to cold stimulation, and the lost potential energy due proton transport is converted to non-shivering heat [7]. Cells with low mitochondrial potential energy restore the proton concentration gradient to its normal state, wherein fat is degraded to acetyl-CoA. Therefore, an increase in brown and beige adipocytes in mice and humans with obesity has an excellent effect on alleviating overweight [8] [9]. Moreover, batokines secreted by brown and beige adipocytes mediate crosstalk with various organs and act as paracrine agents to maintain sugar and lipid homeostasis, suggesting that increasing brown and beige adipocytes ameliorates metabolic syndrome by not only generating non-shivering heat but also through the roles of batokines [10] [11].

In humans, very few brown adipocyte precursors are present in older individuals, and increasing their levels is difficult [12]. Therefore, increasing beige adipocytes through continuous cold exposure is necessary to ameliorate obesity and type 2 diabetes. In cold stimulation pathway signaling, first, the vagus nerve receives the cold stimulation signal and transmits it to the brain, which then secretes adrenaline, which is in turn converted into noradrenaline and binds to the β 3-adrenergic receptor (β 3-AR). The signal received by β 3-AR activates adenylate cyclase, increases cyclic AMP, which activates protein kinase A (PKA), and finally, the activated PKA increases the expression of a series of genes, including Pparg, PGC1 α , and PRDM16 via p38MAPK. PR domain containing 16 (PRDM16) induces browning and increases UCP1, while peroxisome proliferative-activated receptor gamma coactivator 1 alpha (PGC1 α) increases the number of mitochondria [13] [14]. Browning in human adipose tissue occurs when cold stimulation at 17°C for 1 h is conducted continuously for approximately a month [8] [15].

Body temperature in humans is precisely controlled by the central nervous system. In contrast, because of insufficient control by the central nervous system in mice, their body temperature often changes in response to external temperature. Thus, mice are equipped with special supplementary thermogenic systems to protect the body from damage caused by cold stimulation. For example, UCP1 knock-out mice generate non-shivering heat through a different pathway [16] [17], namely the β 3-AR-independent pathway [4] [18] [19]. Given this background, we hypothesized that mouse cells may have an auxiliary mechanism to protect cold

stimuli and adapt themselves, and sought to verify this hypothesis. As a result, we found that long-term direct cold stimulation induces the change from white to beige adipose tissue-like cells.

2. Materials and Methods

2.1. Reagents and Antibodies

Ruthenium red (11103-72-3; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) [20], A967079 (1170613-55-4; TOCRIS Bioscience, Bristol, UK) [21] and ionomycin (56092-81-0; FUJIFILM Wako Pure Chemical Corporation) were used as ion channel inhibitors and inducers. Ruthenium red was dissolved in distilled water, and A967079 and ionomycin were dissolved in dimethyl sulfoxide. The final concentrations of ruthenium red, A967079, and ionomycin were 10, 100, and 200 mM, respectively. Rabbit anti-UCP1 polyclonal antibody (bs-1925R; Thermo Fisher Scientific, Waltham, MA, USA), rabbit anti-TRPA1 polyclonal antibody (NB110-40763; Novus Biologicals LLC, Centennial, CO, USA), and rabbit anti-TRPM8 polyclonal antibody (NBP1-97311; Novus Biologicals LLC) were used as primary antibodies for immunostaining. Cy3-conjugated anti-rabbit IgG goat antibody (AP132C; Merck, Darmstadt, Germany) was used as the secondary antibody.

2.2. Cell Lines and Media

3T3-L24 mouse white adipocytes is a cell line obtained by inducing 3T3-L1 mouse progenitor cells (ECACC strain cells, EC91031101-F0) into white adipocytes followed by isolation [22]. HPA primary human preadipocytes derived from subcutaneous adipocytes were obtained from ScienCell Research Laboratories (7220; Carlsbad, CA, USA) and induced to HA human white adipocytes using preadipocyte differentiation medium (ScienCell Research Laboratories; 7221). 3T3-L24 and HA adipocytes underwent 2-weeks passaging culture at 37°C before use.

Dulbecco's Minimum Essential Medium and Minimum Essential Medium Eagle (modified) with Earle's salts (MP Biomedicals Inc., Illkirch, France) adjusted to pH 7.0 were respectively mixed with fetal bovine serum (A5256701; Thermo Fisher Scientific, Waltham, MA, USA) at a ratio of 9:1, and penicillium-streptomycin solution (168-23191; FUJIFILM Wako Pure Chemical Corporation) was added at a final concentration of 25 µg/mL. These media are hereafter referred to as DMEM and MEM, respectively.

2.3. Measurement of Gene Expression by qPCR

An RNeasy Lipid Mini Kit (74804; Qiagen, Hilden, Germany) was used for mRNA purification, and cDNA was synthesized using a QuantiTect Reverse Transcription Kit (205311; Qiagen). qPCR was performed with the obtained cDNA using a Rotor-Gene device (Qiagen). Commercially available QuantiTect Primer Assays (Qiagen) were used as DNA primers for qPCR. The reaction solution comprised a Rotor-Gene SYBR Green PCR Kit (204443; Qiagen). Reactions were performed

for at least 50 cycles of 95°C for 5 s and 65°C for 30 s according to the manufacturer's instructions. β -actin was used as a standard for Ct values, and $\Delta\Delta$ Ct values were calculated using the Δ Ct values of control cells. The DNA primers used in this study are shown in **Table 1**.

Table 1. Primers used in this study.

(1) Mouse primers			
Symbol	Official name	Entrez Gene ID	Gene Globe ID
Actb	Actin, beta	1146	QT00095242
Prdm16	PR domain containing 16	70673	QT00148127
Ucp1	Uncoupling protein 1	22227	QT00097300
Pgc1a	Peroxisome activated receptor, gamma, coactivator 1 alpha	19017	QT00156303
Cidea	Cell death-inducing DNA fragment factor, alpha subunit-like effect	12683	QT00095249
Cox8b	Cytochrome C oxidase subunit VIIIb	12869	QT00100366
Cdk5	Cyclin-dependent kinase 5	12568	QT00164031
Cebpa	CCAAT/enhancer binding protein (C/EBP), alfa	12606	QT00311731
Pparg	Peroxisome proliferator activated receptor gamma	19016	QT00100296
Fabp4	Fatty acid binding protein 4, adipocyte	117760	QT00091532
Zfp516	Zinc finger protein 516	329003	QT01059324
Cited 1	Cbp/p300-interacting trans-activator with Glu/Asp-rich carboxy-terminal domain	12706	QT01059261
Tnfrsf9	Tumor necrosis factor receptor superfamily, member 9	21942	QT00147266
Fst	Follistatin	14313	QT00105483
Igf1	Insulin-like growth factor 1	16000	QT00154469
Fgf21	Fibroblast growth factor 21	56636	QT00132202
Il6	Interleukin 6	16193	QT00098875
Trpa1	Transient receptor potential cation channel, subfamily A, member 1	277328	QT00133791
Trpm8	Transient receptor potential cation channel, subfamily M, member 8	171382	QT00137256
(2) Human primers			
Symbol	Official name	Entrez Gene ID	Gene Globe ID
ACTB	Actin, beta	60	QT01680476
PRDM16	PR domain containing 16	63976	QT00016975
UCP1	Uncoupling protein 1	7350	QT01670641
CIDEA	Cell death-inducing DNA fragment factor, alpha subunit-like effect	1149	QT00038192
PGC1A	Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	10891	QT00095578
PPARG	Peroxisome proliferator activated receptor gamma	5468	QT00029841
CEBPA	CCAAT/enhancer binding protein (C/EBP), alfa	1050	QT00203357

2.4. Short-Term Direct Cold Stimulation

3T3-L24 adipocytes (1×10^5 cells/flask) and HA human adipocytes (4×10^4 cells/well) were seeded into 25 cm²-culture flasks containing 5 mL DMEM and 6-well plates containing 4 mL MEM, respectively, and cultured at 37°C for 2 days. The respective medium was quickly changed to medium at 37°C and 15°C, and incubated for 1 h in an incubator set at 37°C and 15°C, respectively. After incubation, the cells were washed once with phosphate-buffered saline (PBS), detached using 0.25% trypsin, and collected by centrifugation ($800 \times g$, 3 min). Thereafter, mRNA was immediately extracted to examine gene expressions by qPCR. All solutions used for cell collection were preheated at 37°C, and the operation was performed on aluminum plates (WakenBtech, Kyoto, Japan) at 37°C and room temperature (30°C) within 15 min.

The amount of NADH per cell, which corresponds to mitochondrial activity, was measured using a Cell Counting Kit 8 (341-07761; FUJIFILM Wako Pure Chemical Corporation), according to the manufacturer's instructions. Briefly, 10,000 cells were inoculated into a 96-well plate containing 0.1 mL of DMEM and attached for 16 h through incubation. Subsequently, the medium was replaced with 0.1 mL DMEM containing 10 μ L CCK-8 solution at 15°C, and the plate was incubated at 15°C. The plates were sequentially removed from the incubator at 0, 45, 90, and 120 min, and absorbance was measured at 450 nm. The total cell number was determined using a cell counter (WC2-100; WakenBtech) after the cells were detached with 0.25% trypsin and collected using centrifugation. The increase in the percentage absorbance per cell at 45, 90, and 120 min relative to that of the control cells (0 min) was calculated.

2.5. Effect of Direct Cold Stimulation on Calcium Ion Uptake

Calcium ion (Ca²⁺) uptake in response to direct cold stimulation was examined using a Calcium Kit-Fluo4 (348-91281; FUJIFILM Wako Pure Chemical Corporation). Briefly, 3T3-L24 cells (2×10^4 cells) were attached by 16 h of incubation at 37°C in a glass bottom dish (D141400; Matsunami Glass, Osaka, Japan) containing 2 mL DMEM and precultured in loading buffer (20 mM HEPES, 115 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl₂, 1.8 mM CaCl₂, 13.8 mM glucose, pH 7.6, 1.25 mM probenecid, 0.04% of 5% pluronic F-127, and 50 μ L Flo4-AM solution) for 1 h at 37°C. After the sample was placed in the insulation device set on an EVOS[®] FL Auto Cell Imaging System (Thermo Fisher Scientific, Massachusetts, USA), the cells were washed with 2 mL of recording buffer (loading buffer without Flo4-AM) at 37°C. Thereafter, the buffer was replaced with recording buffer at 37°C, recording buffer containing 5 μ M ionomycin at 37°C, recording buffer at 15°C, and recording buffer without Ca²⁺ at 15°C, and the fluorescence intensity was measured every 10 s. For the inhibition experiments, the cells were precultured with loading buffers containing 10 μ M ruthenium red or A97079 at 37°C for 1 h, and recording medium containing 10 μ M ruthenium red or A967079 at 15°C was used for fluorescence intensity analysis.

Next, immunostaining of transport receptor potential ankyrin 1 (TRPA1) and transport receptor potential melastatin 8 (TRPM8) was conducted. Briefly, the cells cultured in glass bottom dishes were washed twice with 2 mL PBS solution, fixed in phosphate buffer containing 4% formaldehyde for 10 min, washed thrice with PBS solution, incubated with PBS solution containing 0.2% TritonX-100 for 10 min, and washed thrice with PBS solution. Subsequently, the sample was blocked with PBS containing 1% BSA for 1 h and washed thrice with PBS solution. Next, rabbit anti-TRPA1 and anti-TRPM8 polyclonal antibody solutions diluted to 1/200 volume in PBS containing 0.1% albumin were added to the pretreated cells, which were then incubated for 1 h at 30°C and washed four times with PBS. Thereafter, secondary antibody (Cy3-conjugated anti-rabbit IgG goat polyclonal antibody) solution diluted to 1/500 volume in PBS solution containing 0.1% albumin was incubated at 30°C for 1 h and washed four times with PBS solution. The cells were then observed for fluorescence intensity using an EVOS® FL Auto Cell Imaging System.

Next, the effect of Ca^{2+} uptake on gene expression was assessed. Briefly, 3T3-L24 cells (1×10^5 cells) were cultured in 24-well plates at 37°C for 16 h, after which the cells were replaced with recording medium and Ca^{2+} -free recording medium (recording medium without Ca^{2+}) and incubated for 30 s at 15°C; these were replaced with 5 mL DMEM at 37°C and incubated at 37°C for 1 h. Subsequently, the resulting cells were rapidly collected, mRNA was extracted, and gene expression was analyzed using qPCR.

2.6. Long-Term Direct Cold Stimulation

Preliminary experiments showed that gene expression after short-term cold stimulation at 15°C for 1 hour was maintained for more than a day, so this was repeated for long-term cold stimulation. 3T3-L24 adipocytes (5×10^4 cells/flask) cultured in 25-cm² culture flasks were replaced with 5 mL DMEM at 15°C, maintained for 1 h in an incubator, and then replaced with 5 mL DMEM at 37°C for 23 h. This cold stimulation procedure was repeated for 5 days, and on the fifth day, the cold-stimulated cells were collected, and mRNA was extracted from a portion of the cells in the same manner as that for short-term direct cold stimulation. The rest of the cells (5×10^4 cells) were inoculated into 25-cm flasks containing 6 mL DMEM and cultured at 37°C for 2 days. Long-term direct cold stimulation with one cycle of 7 days was continued for 4 weeks.

2.7. Characteristics of Long-Term Cold-Stimulated Cells

Long-term cold-stimulated cells were collected weekly, mRNA was extracted, and gene expression was analyzed by qPCR, according to the previously described method. Cells subjected over 2 weeks of long-term direct cold stimulation and 3T3-L24 white adipocytes were cultured for 16 h at 37°C in 35-mm glass bottom dishes containing 4 mL DMEM, and cell color, mitochondrial content, and UCP1 expression were evaluated. Cell color was observed under an optical microscope

(EVOS® FL Auto Cell Imaging System). For UCP1 immunostaining, rabbit anti-UCP1 polyclonal antibody was used, and immunostaining was conducted similarly to that of TRPA1. For fluorescent mitochondrial staining, cells in 35-mm glass bottom dishes were fixed in 3.7% formamide in PBS for 30 min, washed with PBS, incubated in Molt View Green solution (100 nM Molt View Green in PBS) for 30 min, washed with PBS, and Molt View Green-stained cells were observed for fluorescence intensity using the EVOS® FL Auto Cell Imaging System.

NADH amounts in beige and white adipocytes were measured using a Cell Counting Kit 8 as a measure of mitochondrial activity. Briefly, 5,000 cells were seeded in 96-well plates, and 3 h later, the medium was replaced with 100 μ L DMEM containing 10 μ L CCK-8, and after 2 h of incubation at 37°C, absorbance at 450 nm was measured. Glucose uptake rate was measured using a Glucose Uptake Cell-Based Kit (600470; Cayman Chemical, Ann Arbor, MI, USA). Briefly, cells (3×10^4) were inoculated in 96-well plates and cultured in 100 μ L DMEM for 16 h, after which the medium was replaced with 100 μ L DMEM containing 2 μ g/mL insulin and 1/100 volume μ L of Cell Base Assay NDB Glucose (Cayman Chemical), and incubated for 1 h at 37°C. After washing twice with 100 μ L Cell Base Assay Buffer (Cayman Chemical), fluorescence (485/538 nm) was measured.

2.8. Statistical Analysis

More than three independent experiments were performed to confirm the reproducibility of the results (the total independent experiments number is indicated as n). For statistical evaluation, p-value based on a two-tailed Student's t-test were calculated using KaleidaGraph software (HULINKS Inc., Tokyo, Japan). Results with a p-value greater than 0.05 are not shown in the figures. To determine relative fluorescence intensity, several fluorescence micrographs were obtained under the same imaging conditions as those of the controls, and the fluorescence intensities of approximately 60 - 100 cells were calculated and compared using ImageJ software (NIH, Bethesda, MD, USA).

3. Results and Discussion

3.1. Effect of Short-Term Direct Cold Stimulation on Gene Expression Related to Beige Adipocyte

The effects of direct short-term cold stimulation on the gene expression were examined. **Figure 1(a)** and **Figure 1(b)** show the ratio of gene expression in 3T3-L24 white adipocytes exposed to 1 h of direct cold stimulation at 15°C relative to that in 3T3-L24 cells cultured at 37°C (control cells). Specific beige adipocyte genes (*Ucp1* and *Prdm16*), genes associated with the amounts of mitochondria (*PGC1a*, *Cider*, *Cdk5*, and *Cox8b*), and browning induction genes (*Cebpa*, *Pparg*, *Fabp4*, and *Zfb516*) were used as marker genes [23] [24]. The average expression values of specific beige adipocyte genes and genes associated with the amount of mitochondria in cells exposed to direct cold stimulation were approximately 5–10-fold higher than those in control cells (**Figure 1(a)**). Moreover, in the cells ex-

posed to direct cold stimulation, *Pparg* and *Zfp516* genes [25] [26], which are the most important genes for initial induction to beige adipocyte, were upregulated relative to those in control cells (**Figure 1(b)**). **Figure 1(c)** shows the increased NADH per cell when cells were exposed to direct cold stimulation at 15°C, and the value corresponds to mitochondrial activity. A 15% increase occurred during 2 h of direct cold stimulation. These results suggest that direct cold stimulation of white adipocytes may activate a signaling pathway that induces beige adipocytes.

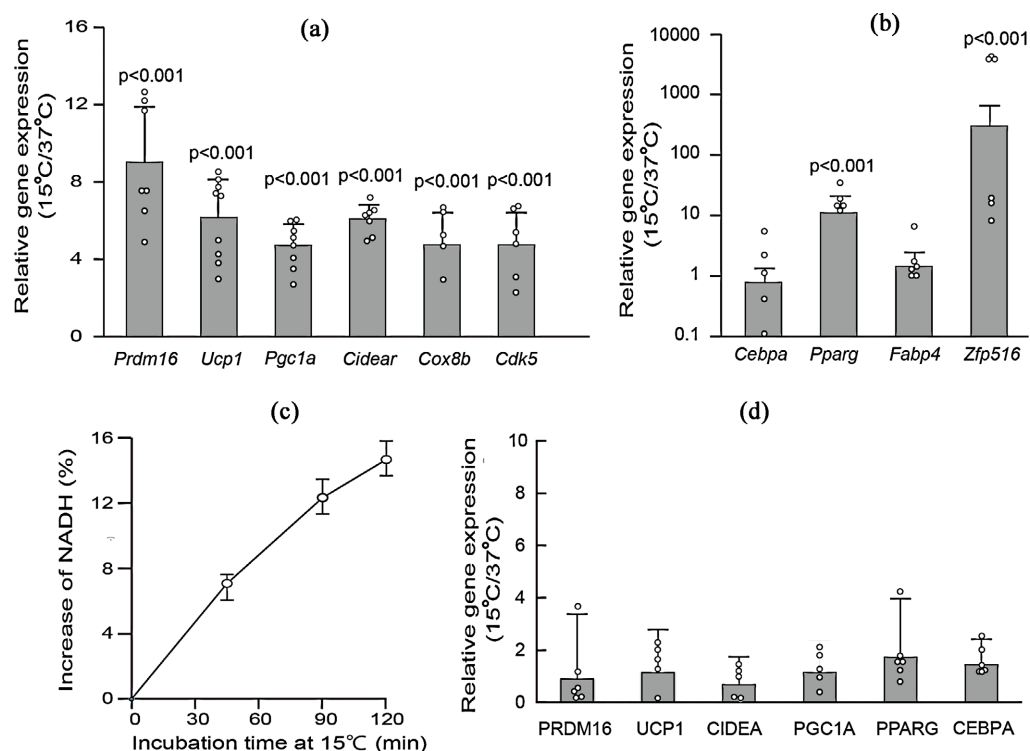


Figure 1. Effects of direct cold stimulation for 1 h at 15°C on gene expression related to differentiation into beige adipocytes. Ratio of gene expression in 3T3-L24 adipocytes incubated at 15°C for 1 h to that of those incubated at 37°C for 1 h (mean $\pm$ SD, n = 6 - 9) of beige adipocyte marker genes (a) and initial induction and maintenance factors of beige adipocytes (b). (c) Increased ratio of the amount of NADH in 3T3-L24 adipocytes incubated at 15°C to those incubated at 37°C (mean $\pm$ SD, n = 5). (d) Ratio of gene expression of HA adipocytes incubated at 15°C for 1 h to those incubated at 37°C for 1 h (mean $\pm$ SD, n = 5 - 6).

Whether this pathway is present in human cells was examined. **Figure 1(d)** shows the ratio of gene expression in human HA white adipocytes exposed to 1 h of direct cold stimulation at 15°C to that in HA cells cultured at 37°C (control cells). In the case of human HA white adipocytes, the expression of genes related to direct cold stimulation at 15°C varied but did not significantly increase ($p > 0.05$). Thus, the response mechanism to short-term direct cold stimulation differs between human and mouse adipocytes.

3.2. Involvement of Ca^{2+} in the Signaling Response to Short-Term Direct Cold Stimulation

Next, the mechanism of the signaling response to short-term direct cold stimula-

tion in mouse adipocytes was examined. Studies have reported an increase in Ca^{2+} when adipocytes or progenitor adipocytes change to brown adipocytes [27], and TRPA1 and TRPM8, which are activated at temperatures below around 20 and 10°C, respectively, are present in vagus nerve cells and take up Ca^{2+} for activation [28]. Based on these findings, we surmised that a small number of TRP channels may be present in mouse adipocytes, and these channels take up Ca^{2+} in response to direct cold stimulation.

First, the relationship between direct cold stimulation and Ca^{2+} uptake by TRP channels was examined. **Figure 2(a)** shows the amount of Ca^{2+} influx when 3T3-L24 adipocytes were incubated with recording buffer solutions containing ionomycin, ruthenium red, and A967079 at 15°C for 10 s; an increase in Ca^{2+} results in green fluorescence. **Figure 2(b)** shows the average fluorescence intensity calculated from the experiment shown in **Figure 2(a)** using ImageJ software. At 37°C, a small amount of Ca^{2+} influx was observed even after 4 min (sample A), but at 15°C, much Ca^{2+} influx occurred within 10 s (sample D), and the amount

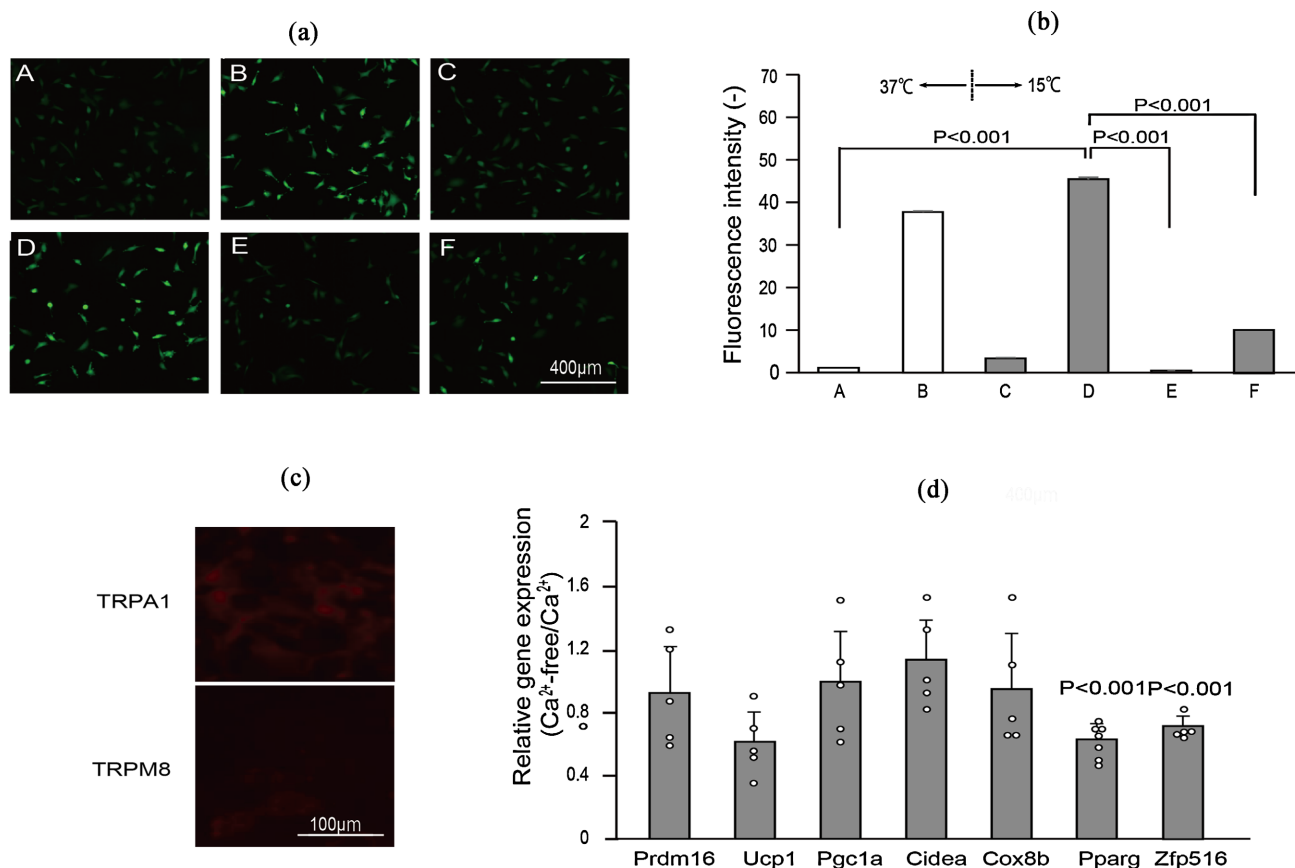


Figure 2. Involvement of Ca^{2+} in signaling in short-term direct cold stimulation. (a) Fluorescence micrographs of cells uptaking Ca^{2+} at 10 s. (b) Relative fluorescence intensity calculated from several images (mean $\pm$ SD, $n = 80$ –100). (A) Recording medium at 37°C (negative control); (B) Recording medium + ionomycin at 37°C (positive control); (C) Recording medium without Ca^{2+} at 15°C; (D) Recording medium with Ca^{2+} at 15°C; (E) Recording medium with Ca^{2+} + ruthenium red at 15°C; (F) Recording medium with Ca^{2+} + A967079 at 15°C. (c) Immunostaining of 3T3-L24 adipocytes using anti-TRPA1 and anti-TRPM8 antibodies. (d) Ratio of the gene expression of 3T3-L24 adipocytes incubated in a solution without Ca^{2+} at 15°C for 30 s to those incubated in a solution with Ca^{2+} at 15°C for 30 s (mean $\pm$ SD, $n = 5$ –7).

reached levels approximately similar to those of the positive control (sample B). In contrast, a small amount of influx was observed even in the case of direct cold stimulation at 15°C with recording solution without Ca²⁺ (sample C), suggesting that the increase in Ca²⁺ at 15°C was not only due to uptake from outside the cells but also due to efflux from the intracellular smooth endoplasmic reticulum. Moreover, Ca²⁺ influx was strongly suppressed when ruthenium red, a TRP channel inhibitor, was added and incubated at 15°C for 10 s (sample E), and when A967079, a specific TRPA1 channel inhibitor, was added, Ca²⁺ influx was suppressed to approximately 20% of that without A967079 addition (sample F). These results suggest that Ca²⁺ is most likely primarily taken up into the cell by several TRP channels, including TRPA1 channels. Moreover, whether TRPA1 and TRPM8 were present on the cell surface was examined. **Figure 2(c)** shows the results of immunostaining of mouse white adipocytes using anti-TRPA1 and anti-TRPM8 antibodies. While TRPA1 and TRPM8 expression was low, TRPA1 was shown to be slightly expressed.

Next, we examined whether Ca²⁺ influx into the cells caused increased gene expression. 3T3-L24 adipocytes were subjected to direct cold stimulation at 15°C for 30 s with Ca²⁺-free buffer and Ca²⁺-containing buffer, which were replaced with DMEM for 1 h at 37°C, and gene expression levels were compared. **Figure 2(d)** shows the ratio of gene expression in cold-stimulated cells with the buffer without Ca²⁺ at 15°C to that in cold-stimulated cells with the buffer containing Ca²⁺ at 15°C. *Pparg* and *Zfp516* gene expression in cells treated with Ca²⁺-free buffer was significantly downregulated, whereas a reduction in the expression of the other genes was observed but was not significant. Direct cold stimulation at 15°C may not have resulted in a predominant difference in gene expression ratios because a small amount of Ca²⁺ is secreted from intracellular organelles. In summary, although the detailed mechanism is still unclear, short-term direct cold stimulation likely causes Ca²⁺ uptake via TRP channels, which in turn induces genes such as *Pparg* and *Zfp516*.

3.3. Change from White Adipocytes to Beige Adipose Tissue-Like Cells by Long-Term Direct Cold Stimulation

Finally, we investigated whether prolonged direct cold stimulation induces a change to beige adipose tissue-like cells. Since cell browning and UCP1 protein expression could not be observed following only one cycle of direct cold stimulation, repeated long-term direct cold stimulation (1 h of direct cold stimulation at 15°C once a day for 5 days, followed by an interval of 2 days of incubation at 37°C after the cells were transplanted) was conducted. **Figure 3(a)** shows the ratio of *Cited1* and *Tnfrsf9* (*Cd137*) gene expression in 3T3-L24 adipocytes exposed to long-term direct cold stimulation to that of 3T3-L24 cells cultured at 37°C (control). *Cited1* and *Tnfrsf9* are marker genes specifically expressed in beige adipocytes [23] [24]. **Figure 3(b)** shows the average expression levels of these genes in long-term cold-stimulated cells from week 2 to week 4. The expression values of *Cited1* and

Tnfrsf9 genes gradually increased with long-term direct cold stimulation, almost reaching their maximum after 2 weeks of culture. The average expression values (2 - 4 weeks) of *Cited1* and *Tnfrsf9* in cells exposed to long-term direct stimulation were 1.2×10^5 - and 7.4-fold higher than those in the control. The strain of cells obtained following long-term cold stimulation for more than 2 weeks was designated 3T3-Beige (**Figure 3(c)**). The color of 3T3-Beige cells was more brownish than that of 3T3-L24 adipocytes (photos A and B) and dark brown spots were observed after more than 3 weeks of culture (photo C).

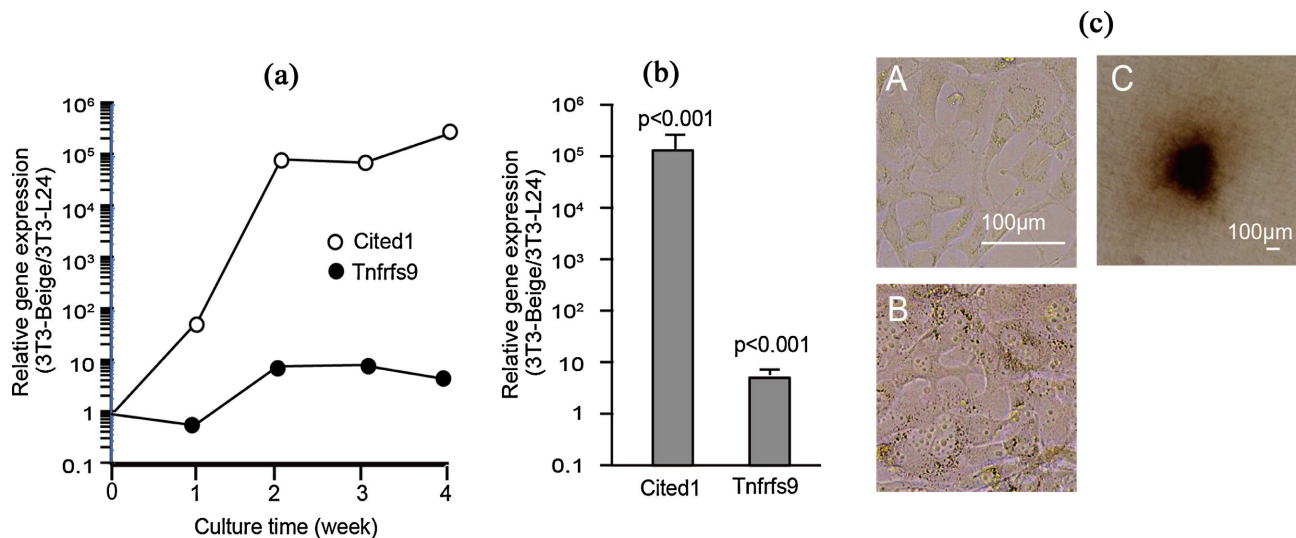


Figure 3. Effects of long-term direct cold stimulation on a change of 3T3-L24 adipocytes. (a) Ratio of gene expression of 3T3-L24 adipocytes incubated at 15°C for 1 h to those incubated at 37°C during long-term direct cold stimulation ($n = 3$). (b) Gene expression ratio averaged from week 2 to week 4 in **Figure 3(a)** (mean $\pm$ SD, $n = 3$). (c) Micrographs of 3T3-L24 adipocytes (A), 3T3-Beige cells (B), and brown spots observed in cells cultured by long-term direct cold stimulation (C).

Moreover, the characteristics of 3T3-Beige cells and 3T3-L24 white adipocytes were compared. **Figure 4(a)** shows the results of UCP1 protein immunostaining and fluorescent staining of mitochondria with anti-UCP1 antibody and Molt View Green; UCP1 and mitochondria emitted red and green fluorescence, respectively. **Figure 4(b)** shows their fluorescence levels calculated from several images of the experiments shown in **Figure 4(a)** using ImageJ software. The amounts of UCP1 and mitochondria in 3T3-Beige cells were approximately 1.4- and 1.3-fold higher than those in 3T3-L24 cells, respectively.

The mitochondrial activity and glucose uptake rates were also compared (**Figure 4(c)** and **Figure 4(d)**). The amount of NADH, corresponding to mitochondrial activity, in 3T3-Beige cells increased by approximately 1.8-fold, and glucose uptake also increased by approximately 1.7-fold. In addition, we investigated whether 3T3-Beige cells produced the four typical batokines, *i.e.*, fibroblast growth factor 21 (fgf21), insulin-like growth factor 1 (Igf1), follistatin (Fst), and interleukin-6 (IL-6) [10] [11]. **Figure 4(e)** shows the ratio of the gene expression of batokines in 3T3-Beige cells to that in 3T3-L24 adipocytes. Significant *Fgf21* and *Igf1* upregulation was observed, approximately 6- and 42-fold on average,

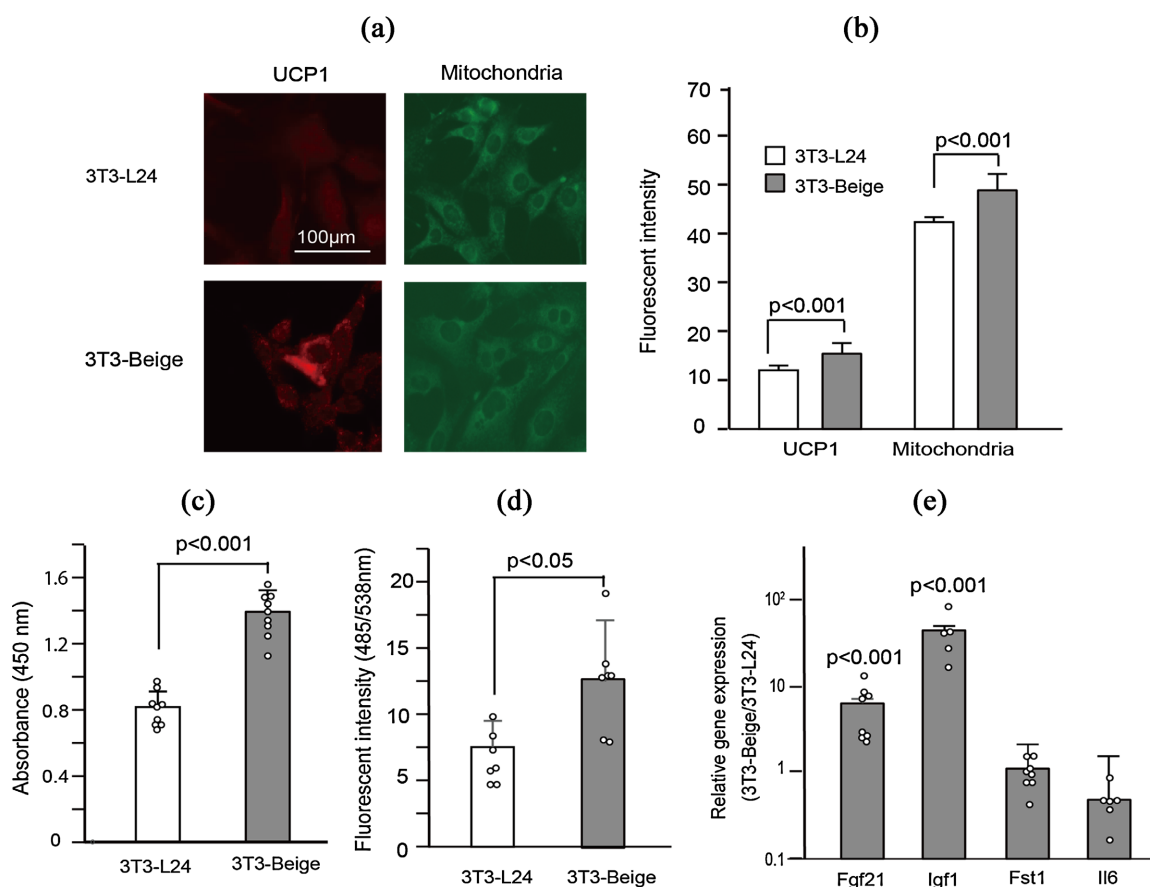


Figure 4. Characteristics of 3T3-Beige cells. (a) Comparison of UCP1 and mitochondria amounts in 3T3-L24 adipocytes and 3T3-Beige cells. Fluorescence staining of UCP1 and mitochondria with anti-UCP1 antibody and MoltVeiw Green. (b) Relative fluorescence values calculated from images of the experiments shown in **Figure 4(a)** using ImageJ software. (c) Amount of NADH in 3T3-L24 adipocytes and 3T3-Beige cells measured by using a Cell Counting Kit 8 (mean $\pm$ SD, $n = 9$). (d) Glucose uptake in 3T3-L24 adipocytes and 3T3-Beige cells measured using a Glucose Uptake Cell-Based Kit (mean $\pm$ SD, $n = 7$). (e) Ratio of batokine gene expression in 3T3-Beige cells to those in 3T3-L24 adipocytes (mean $\pm$ SD, $n = 5 - 7$).

whereas no increase in gene expression was observed in *Fst1* and *Il-6*, which are induced by adrenergic receptors. These results indicate that 3T3-Beige cells possess properties very similar to beige adipocytes, and that prolonged direct cold stimulation can induce a change from white adipocytes to beige adipose tissue-like cells.

4. Discussion

In this study, based on our hypothesis that mouse adipocytes may possess a previously unknown pathway to adapt to direct cold stimulation, a study to verify the hypothesis was conducted. We first examined gene expression when mouse white adipocytes were subjected to direct cold stimulation at 15°C for 1 h and found that gene expression for transdifferentiation into beige adipocytes was induced. We further examined the effects on long-term direct cold stimulation and revealed that white adipocytes changed to the beige adipose tissue-like cell after 2 weeks of

direct cold stimulation. Recent studies using knockout mice clarified that mice have several auxiliary thermogenic systems in response to cold stimulation. Incidentally, a pathway to generate non-shivering thermogenesis without using UCP1 has been identified [16] [17], and several β -AR-independent pathways, such as the pathway for beige adipocyte differentiation from MyoD+ progenitor adipocytes [4], the pathway via the adenosine A2A receptor [18], and the pathway via mineralocorticoid receptor [19], have also been identified. However, few studies on direct cold stimulation have been conducted, and our finding that direct cold stimulation induces the change to beige adipose tissue-like cells is novel and remarkable.

Next, the mechanism of this cold stimulation response was examined, and we found that a few TRP channels are present on the surface of mouse white adipocytes, and Ca^{2+} uptake in response to direct cold stimulation occurred, enhancing the gene expression of the initial factors inducing beige adipocytes, such as *Pparg* and *Zfp516*. Several studies have suggested a relationship between Ca^{2+} and browning. For example, Ca^{2+} transport in brown adipose tissue [27] and muscle [29] have been shown to activate sarcoplasmic reticulum ATPase to promote non-shivering thermogenesis. In addition, the UCP1-independent thermogenic mechanism in beige adipocytes is reportedly dependent on SDRCA2b and Ca^{2+} cycling [30]. Furthermore, FGF21 signaling markedly increases intracellular Ca^{2+} levels and induces white adipose tissue browning [31] [32]. Collectively, these studies corroborate our results that Ca^{2+} influx in response to direct cold stimulation induces browning.

Finally, we clarified the characteristics of the beige adipose tissue-like cells (3T3-Beige) obtained by long-term direct cold stimulation. The amounts of UCP1 and mitochondria in 3T3-Beige cells were approximately 40% and 30% higher than those in 3T3-L24 white adipocytes, with a concomitant increase in mitochondrial activity and glucose metabolism rate. Although these increased amounts were much less than those of brown adipocytes [33], they were similar to those in 3T3-BA1 beige adipose tissue-like cells induced using Kikyo extract in a previous study [34]. The change to beige adipose tissue-like cells is solely caused by an increase in cellular components, such as PRDM16, UCP1, and PGC1 α , due to sustained expression; therefore, we believe it is a reversible change, not a differentiation. Moreover, batokine gene expression in the 3T3-Beige cells was examined, and an increased *Fgf21* and *Igf1* expression was observed, although a small increase in *Fst1* and *IL6* expression was observed. Batokines are secreted from brown adipose tissue and are known to play a role in paracrine crosstalk with other organs [10] [11]. Since FGF21 plays a role in releasing excess energy as heat, this suggests that 3T3-Beige beige adipose tissue-like cells promote heat generation through FGF21.

The limitations of this study are as follows: 1) Identifying beige adipocytes is difficult because various types have been proposed. 3T3-Beige cells express the most important beige cell marker genes and several batokine genes, but further research is needed to determine whether they are beige adipocytes. 2) In this study,

it was thought that Ca^{2+} uptake is involved in the browning of 3T3-L24 adipocytes. Several reports have pointed to a relationship between browning and calcium ions, but the mechanism is not yet clear, so it is necessary to clarify the mechanism in the future.

5. Conclusion

This study revealed that direct cold stimulation in mouse cells increases gene expression that induces differentiation into beige adipocytes, and that prolonged direct cold stimulation causes white adipocytes to brown and become beige adipocyte-like cells. These results suggest the existence of a novel pathway in mice that responds to direct cold stimulation.

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Availability of Data and Materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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