

Induction of Beige-Like Adipocytes in 3T3-L1 Cells

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(Received 21 July 2013/Accepted 5 September 2013/Published online in J-STAGE 20 September 2013)

ABSTRACT. There are two types of brown adipocytes: classical brown adipocytes that form the brown fat depots and beige adipocytes that emerge in the white fat depots. Beige adipocytes have a low level of uncoupling protein 1 (Ucp1) expression in the basal state, but Ucp1 expression is increased in response to β adrenergic receptor activation. The present study explored the factors responsible for the differentiation of 3T3-L1 white preadipocytes to beige adipocytes. Significant expression of *Ucp1* was not detected under any tested conditions in the absence of isoproterenol (Iso), an agonist of β adrenergic receptor. Iso-induced *Ucp1* expression was significantly higher in the cells treated with a mixture of triiodothyronine (T_3) and 3-isobutyl-1-methylxanthine (IBMX) for days 0–8 than in the control cells. Chronic IBMX treatment was indispensable for the enhanced Iso-induced *Ucp1* expression, and treatment with additional rosiglitazone (Rosi) for days 0–8 further increased the *Ucp1* expression. Recently, genes were identified that are predominantly expressed in beige adipocytes, which were induced from stromal vascular cells in white fat depots. However, the expression levels of the beige adipocyte-selective genes in the adipocytes induced by the mixture of T_3 , IBMX and Rosi did not differ from those in the control adipocytes. The present study indicates that 3T3-L1 cells can differentiate to beige-like adipocytes by prolonged treatment with the mixture of T_3 , IBMX and Rosi and that the gene expression profile of the adipocytes is distinct from those previously induced from white fat depots.

KEY WORDS: adipocyte, beige adipocyte, cell culture, cellular differentiation, Ucp1.

doi: 10.1292/jvms.13-0359; *J. Vet. Med. Sci.* 76(1): 57–64, 2014

There are two major types of adipocytes in mammals: white and brown. White adipocytes are specialized for the storage of excess energy [31]. In contrast, brown adipocytes dissipate chemical energy in the form of heat as a reaction against cold exposure or excess feeding [1, 4, 14, 20, 21, 38]. This thermogenic function of brown adipocytes results from the expression of a series of genes related to a high mitochondrial content and elevated cellular respiration that is largely uncoupled from ATP synthesis [35]. This uncoupling occurs through mitochondrial uncoupling protein 1 (Ucp1), a mammalian brown adipocyte-specific protein that promotes proton leak across the inner mitochondrial membrane [4, 13].

There are at least 2 origins of Ucp1-positive adipocytes in mice: brown adipocytes consisting of the classical brown fat depots, which are located in the interscapular region, and beige adipocytes residing in white fat depots. Both cell types up-regulate Ucp1 expression in response to β adrenergic receptor activation [3, 28, 37, 46]. However, beige adipocytes resemble white adipocytes in having extremely low basal expression of *Ucp1*, whereas classical brown adipocytes

constitutively express *Ucp1* [46]. In accordance with the differential regulation of *Ucp1* expression, a distinct commitment/differentiation process is suggested between classical brown adipocytes and beige adipocytes in mice; classical brown adipocytes are derived from Myf-5-positive myoblast precursors, whereas beige adipocytes arise from non-Myf-5 lineage cells [36]. Certain studies have explored the cell origin of beige adipocytes and showed a direct conversion from white adipocytes [16], differentiation from beige preadipocytes located in white fat depots [46], commitment/differentiation of Sca1⁺/CD45[−]/Mac1[−] stem cells [33] and commitment/differentiation from Pdgfra⁺/CD34⁺/Sca1⁺ stellate-like cells, which can be bipotentially differentiated into white adipocytes and beige adipocytes [18]. Thus, beige adipocytes may be induced from multiple types of cells.

Recent findings that adult humans have functional brown adipocytes [6, 26, 32, 43, 45] have triggered a focus on brown adipocyte activation as a novel therapeutic treatment for obesity [5]. In fact, the activation of human brown adipocytes is responsible for energy expenditure during acute cold exposure [29]. Comprehensive profiles of gene expression indicate a similar pattern between human brown adipocytes and mouse beige adipocytes but not mouse classical brown adipocytes, suggesting that human brown adipocytes have compatible characteristics to mouse beige adipocytes [37, 46]. In mice, increases in the number of beige adipocytes in the white fat depots are associated with protection against diet-induced obesity and metabolic dysfunction, including insulin resistance in mice [34, 44]. Therefore, clarification of the factors affecting the development of beige adipocytes is a prerequisite to basic information on beige adipocyte-me-

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Table 1. Oligonucleotide PCR primers for RT-qPCR

	Oligonucleotide		GenBank accession number
	5'-primer	3'-primer	
<i>aP2</i>	5'-AAGGTGAAGAGCATCATAACCCT-3'	5'-TCACGCCTTTCATAACACATTCC-3'	NM_024406
<i>C/ebpa</i>	5'-CAAGAACAGCAACGAGTACCG-3'	5'-GTCAGTGGTCAACTCCAGCAC-3'	NM_007678
<i>C/ebpβ</i>	5'-ACGACTTCCTCTCCGACCTCT-3'	5'-CGAGGCTCACGTAACCGTAGT-3'	NM_009883
<i>Cidea</i>	5'-AAACCATGACCGAAGTAGCC-3'	5'-AGGCCAGTTGTGATGACTAAGAC-3'	NM_007702
<i>Cited1</i>	5'-CGCTTCGTCCGTACCTCAGCT-3'	5'-CAGCTGGGCCTGTTGGTCTC-3'	NM_007709
<i>Cox7a1</i>	5'-AAAGTGCTGCACGTCCTTG-3'	5'-TTCTCTGCCACACGGTTTTC-3'	NM_009944
<i>Ear2</i>	5'-CAACGAGCCCTAAGTTCCAC-3'	5'-TGAGGCAAGCATTAGGACAA-3'	NM_007895
<i>Pgc1α</i>	5'-TGTGGAACCTCTGGAAGTGC-3'	5'-GCCTTGAAAGGGTTATCTTGG-3'	NM_008904
<i>Pgc1β</i>	5'-CTGACGGTGGAGCTTTGC-3'	5'-AGGCTGGGAGCTGTGTCTT-3'	NM_133249
<i>Pparγ2</i>	5'-TGCTGTTATGGGTGAACTCTG-3'	5'-CTGTGTCAACCATGGTAATTTCTT-3'	NM_011146
<i>Slc27a1</i>	5'-GACAAGCTGGATCAGGCAAG-3'	5'-GAGGCCACAGAGGCTGTTC-3'	NM_011977
<i>Tfam</i>	5'-CAAAGGATGATTCGGCTCAG-3'	5'-AAGCTGAATATATGCCTGCTTTTC-3'	NM_009360
<i>Tbp</i>	5'-CCAATGACTCCTATGACCCCTA-3'	5'-CAGCCAAGATTCACGGTAGAT-3'	NM_013684
<i>Ucp1</i>	5'-ACTGCCACACCTCCAGTCATT-3'	5'-CTTTGCCCTCACTCAGGATTGG-3'	NM_009463

diated regulation of energy metabolism. The present study explores the conditions to induce beige adipocytes without exogenous gene transfer in 3T3-L1 white preadipocytes.

MATERIALS AND METHODS

Materials: The following reagents were purchased from Sigma (St. Louis, MO, U.S.A.): dexamethasone (Dex), 3-isobutyl-1-methylxanthine (IBMX), insulin (Ins), triiodothyronine (T_3), rosiglitazone (Rosi) and isoproterenol (Iso).

Cell culture: The 3T3-L1 preadipocytes were cultured as described previously [40]. The standard protocol of differentiation in 3T3-L1 cells [39] was treated as the control: two days after reaching confluence (day 0), the cells were cultured in DMEM with 10% FBS and antibiotics (growth medium) in the presence of differentiation inducers (Dex (0.25 μ M), IBMX (0.5 mM) and Ins (10 μ g/ml)) for 2 days, followed by culture in growth medium supplemented with Ins (5 μ g/ml). According to the protocol, 3T3-L1 cells are differentiated to white adipocytes on day 8 [41]. In addition to the control protocol, Rosi (1 μ M), T_3 (50 nM) and IBMX (0.5 mM) treatments were used for the indicated period. On day 8, lipid accumulation was examined using oil red O staining as described previously [40]. On day 8, the cells were further treated with or without Iso (10 μ M) for 4 hr.

The concentrations of the additional reagents used to induce the beige adipocytes were determined on the basis of the results from previous studies. Treatment with Rosi (1 μ M) for 5 to 7 days induced *Ucp1* expression in stromal vascular cells isolated from white fat depots [28, 30]. Treatment with T_3 (50 nM) enhanced norepinephrine-induced *Ucp1* expression in primary brown adipocytes [22]. In addition, T_3 is frequently used during brown adipocyte differentiation at concentrations of 1–250 nM [12, 15, 19, 28, 42].

Real-time RT-quantitative PCR: RNA isolation and real-time RT-quantitative PCR (qPCR) were performed as described previously [2, 11, 25]. The oligonucleotide primers for RT-qPCR are presented in Table 1. The Ct value

was determined, and the abundance of gene transcripts was analyzed using the $\Delta\Delta$ Ct method, using *TATA-binding protein (Tbp)* as the normalization gene [8].

Statistical analyses: The data are expressed as the mean $\pm$ SEM. The data on gene expression were log-transformed to provide an approximation of a normal distribution before analysis. The differences between the groups were examined by ANOVA. $P < 0.05$ was considered to be significant.

RESULTS

The 3T3-L1 preadipocytes were differentiated by treatments with Rosi and T_3 in addition to the reagents used in the control protocol for differentiation to white adipocytes [39]. Oil red O staining on day 8 showed that the 3T3-L1 cells were efficiently differentiated to adipocytes, irrespective of the treatment (Fig. 1A). Expression of *aP2*, a fatty-acid binding protein expressed in adipocytes, was comparable among groups, which was verified by RT-qPCR analyses (Fig. 1B) [47].

We also examined expression level of transcription factors related to adipogenesis [41]. The expression level of *Pparγ2* in treatments D and E was significantly lower than that in treatment B (Fig. 2A). Compared to the control treatment A, the gene transcript levels of *C/ebpa* were lower in treatments B, C and E (Fig. 2B). The expression level of *C/ebpβ* was comparable among treatments (Fig. 2C).

Ucp1 expression is restricted in brown/beige adipocytes in mammals [4, 13]. The expression of *Ucp1* was not reproducibly detected in any cells without β adrenergic activation (data not shown). In contrast, significant *Ucp1* expression was detected in all the cells treated with Iso (Fig. 3). Treatment with the mixture of T_3 , IBMX and Rosi (treatment E) enhanced Iso-induced *Ucp1* expression; the expression level of *Ucp1* in treatment E was ~8-fold higher than that in the control treatment A ($P = 0.003$). The prolonged IBMX treatment was essential for the increased expression of *Ucp1* in response to Iso treatment; the expression in treatment D,

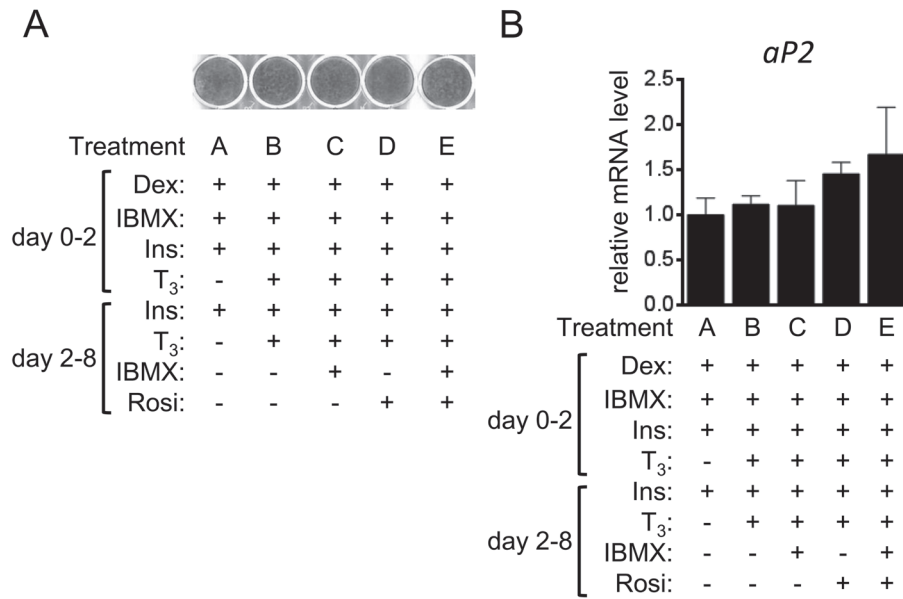


Fig. 1. Adipocyte differentiation in 3T3-L1 cells. 3T3-L1 cells were differentiated into adipocytes in the presence or absence of T₃, IBMX and Rosi. (A) The lipid accumulation in cells without Iso treatment on day 8 was examined using oil red O staining. (B) The gene transcript level of *aP2* in cells treated without Iso on day 8 was examined by RT-qPCR and expressed as ratios to *Thp* levels with the level in the control 3T3-L1 cells (treatment A) set to 1. The data shown are the mean $\pm$ SE (n=6).

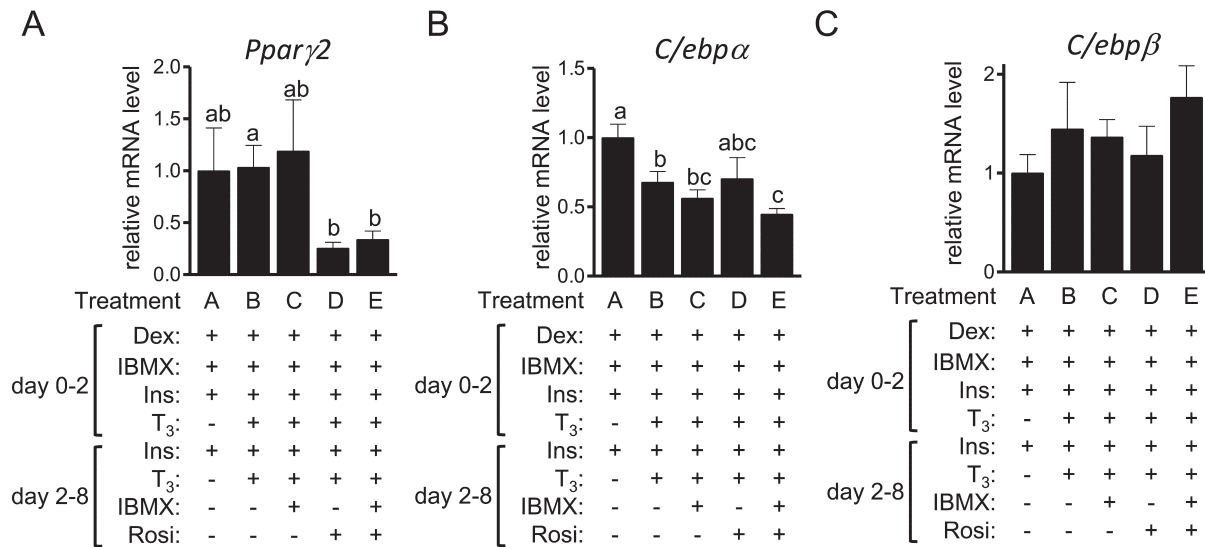


Fig. 2. The expression of adipogenic transcription factors in 3T3-L1 cells. 3T3-L1 cells were differentiated into adipocytes in the presence or absence of T₃, IBMX and Rosi. The gene transcript levels of *Ppar γ 2* (A), *C/ebp α* (B) and *C/ebp β* (C) in cells treated without Iso on day 8 were examined by RT-qPCR and expressed as ratios to *Thp* levels with the level in the control 3T3-L1 cells (treatment A) set to 1. The data shown are the mean $\pm$ SE (n=6). ^{a,b,c}Means that do not have a common letter above the bars differ significantly ($P < 0.05$).

which lacked the IBMX used in treatment E, was not different from that in the control treatment A. *Ucp1* expression in treatment C, which is devoid of Rosi unlike treatment E, was still higher than that in the control treatment A ($P=0.04$), al-

though the extent of the induction in treatment C was smaller than that in treatment E ($P=0.03$).

The expression of genes that are predominantly expressed in brown fat depots compared with white fat depots [35]

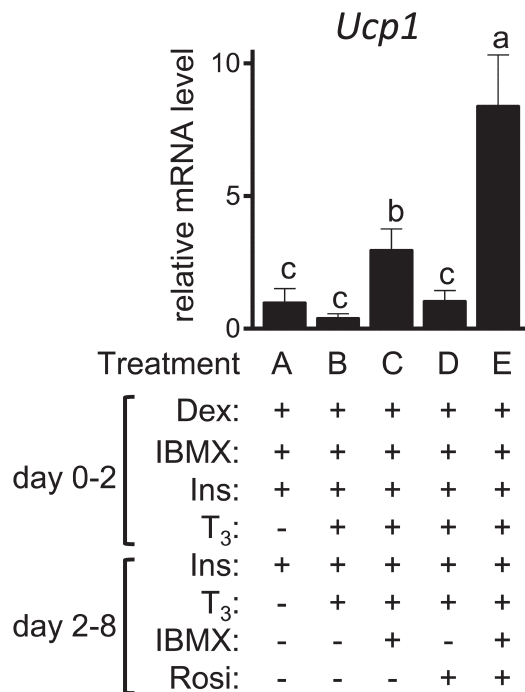


Fig. 3. The expression of *Ucp1* in 3T3-L1 cells. 3T3-L1 cells were differentiated into adipocytes in the presence or absence of the indicated factors. On day 8, the cells were further treated with Iso for 4 hr. *Ucp1* expression was examined by RT-qPCR and expressed as ratios to *Tbp* levels with the level in the control 3T3-L1 cells (treatment A) set to 1. The data shown are the mean $\pm$ SE ($n=6$). ^{a,b,c}Means that do not have a common letter above the bars differ significantly ($P<0.05$).

was next examined (Fig. 4). The expression levels of *Pgc1 α* , *Pgc1 β* , *Cox7a1* and *Tfam* were comparable among treatments, whereas the expression of *Cidea* was higher in the cells of treatments C ($P=0.02$) and E ($P=0.04$) than in treatment A.

Wu *et al.* [46] identified genes expressed selectively in beige adipocytes, but not brown adipocytes and white adipocytes, including *CD137*, *Slc27a1*, *Ear2*, *Tbx1* and *Tmem26*. Among these genes, significant expression of *CD137*, *Tbx1* and *Tmem26* was not detected in the 3T3-L1 cells, irrespective of the treatment (data not shown). The expression level of *Slc27a1* was not higher in treatments B-E than in treatment A (Fig. 5A); rather, it was significantly lower in treatments C ($P=0.004$), D ($P=0.005$) and E ($P=0.001$) than in the control treatment A. The expression level of *Ear2* was higher in treatment B ($P=0.04$) than in the control treatment A (Fig. 5B). However, the expression was lower in treatments C ($P=0.001$) and D ($P=0.03$). Sharp *et al.* [37] independently identified the beige adipocyte-selective gene in cells following prolonged treatment with T₃ and Rosi; they revealed *Cited1* as a novel beige adipocyte marker. The gene transcript level of *Cited1* in treatment D was significantly higher than that in the other treatments (Fig. 5C).

DISCUSSION

The present results indicate that 3T3-L1 adipocytes treated with T₃, Rosi and IBMX express higher *Ucp1* in response to β adrenergic activation. Basal expression of *Ucp1* in beige adipocytes is as low as that in white adipocytes, whereas *Ucp1* expression is enhanced in response to β adrenergic activation [46]. Significant expression of *Ucp1* was also detected in the control 3T3-L1 adipocytes (treatment A) when the cells were treated with Iso; the result is consistent with that by Mottillo and Grannerman [24]. Thus, the control 3T3-L1 adipocytes meet the definition of beige adipocytes by Wu *et al.* [46]. It is possible that the differences between white adipocytes and beige adipocytes are not discrete, but continuous. Our results suggest that 3T3-L1 cells chronically treated with the mixture of T₃, Rosi and IBMX are closer to mature beige adipocytes.

T₃, IBMX and Rosi are all needed for the efficient induction of *Ucp1* in response to β adrenergic receptor activation. However, whether T₃ is essential is not known, because the observed Iso-induced *Ucp1* expression was not examined in cells treated with IBMX and Rosi, but without T₃. In addition, the present results suggest that Rosi augments the effects of T₃ and IBMX on the *Ucp1* induction in response to Iso treatment. The increase in *Ucp1* expression in white fat depots has been shown in mice chronically treated with Rosi [7, 28], implying a role of Rosi as an enhancer of beige adipocyte induction.

We focused on T₃, Rosi and IBMX in view of the following evidence: prolonged treatment with Rosi with or without T₃ in stromal vascular cells from white fat depots resulted in Iso-induced *Ucp1* induction [28, 30]. The overexpression of *C/ebp β* enhanced cAMP-mediated *Ucp1* induction in 3T3-L1 cells [17]. Furthermore, IBMX is responsible for *C/ebp β* induction during mitotic clonal expansion, i.e., days 0–2, in 3T3-L1 cells, which allows for the cells to differentiate to white adipocytes [47]; therefore, we expected up-regulation of the *C/ebp β* expression in treatments C and E that were treated with the prolonged IBMX. However, the expression level of *C/ebp β* was comparable among treatments A, C and E (Fig. 2C), suggesting an activity of IBMX other than the regulation of *C/ebp β* expression. There are nearly 100 cyclic nucleotide phosphodiesterases that catalyze cAMP or cGMP or both [10]. The non-selective phosphodiesterase inhibitor IBMX actually increased cytosolic concentration of cAMP in 3T3-L1 cells [9] and possibly increases cGMP concentration. It was recently revealed that cGMP-dependent protein kinase I in white adipocytes acts induces beige adipocytes [23] and may be involved in the IBMX-induced development of beige adipocytes.

Expression level of *C/ebp α* was lower in treatments B, C and E than in the control treatment A (Fig. 2B); the transcript level of *C/ebp α* reflects adipocyte differentiation [41]. The precise reason for the decreased expression is unknown, although there are at least 2 possibilities: 1) the adipocyte differentiation was partially inhibited by treatments B, C and E, although lipid accumulation was unaffected; or 2) the decreased expression is partially related to the induction of

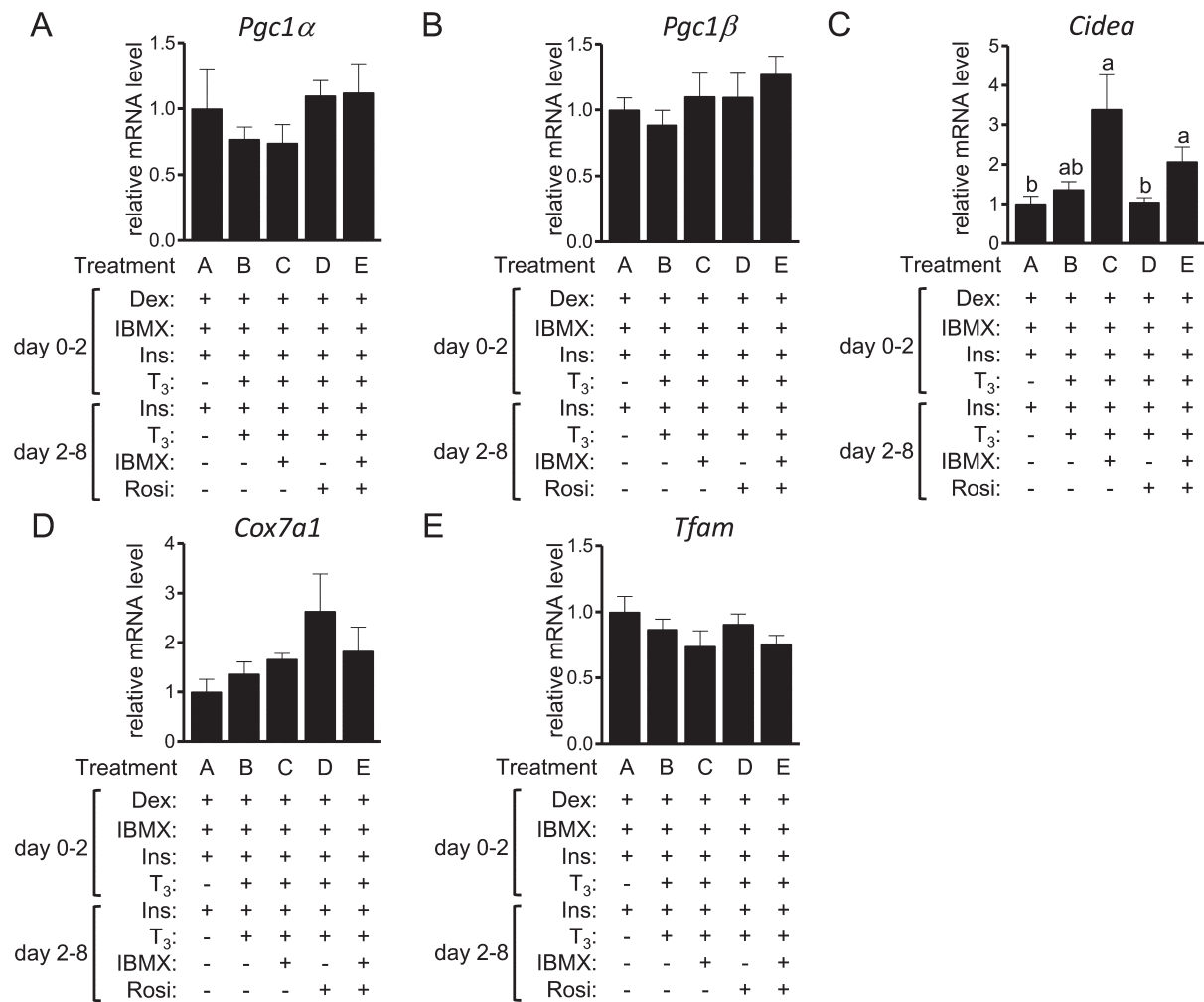


Fig. 4. The expression of brown fat-selective genes in 3T3-L1 cells. 3T3-L1 cells were differentiated into adipocytes in the presence or absence of T₃, IBMX and Rosi. On day 8, the expression of *Pgc1α* (A), *Pgc1β* (B), *Cidea* (C), *Cox7a1* (D) and *Tfam* (E) was examined by RT-qPCR and expressed as ratios to *Thp* levels with the level in the control 3T3-L1 cells (treatment A) set to 1. The data shown are the mean ± SE (n=6). ^{a,b}Means that do not have a common letter above the bars differ significantly ($P < 0.05$).

beige (brown) adipocytes. The expression level of *C/ebpa* in beige (brown) adipocytes may be lower than that in white adipocytes. The results of the transcriptomic analyses (NCBI gene expression omnibus accession number: GSE8044), which were performed in the study by Seale *et al.* [35], indicated that the expression level of *C/ebpa* was lower in brown fat depots than in white fat depots.

Wu *et al.* [46] showed that the expression level of genes highly expressing in brown fat depots [35] was comparable between beige adipocytes and white adipocytes, although others observed higher expression of these genes in beige adipocytes [28, 30, 33]. Thus, the expression pattern of the brown fat-selective genes in 3T3-L1 cells treated with the mixture of T₃, IBMX and Rosi essentially resembles that of the beige adipocytes identified by Wu *et al.* [46]. However, expression pattern of beige adipocyte-selective genes was different from the results by Wu *et al.* [46], suggesting that

the characteristics of the beige-like adipocytes induced in this study are distinct from those developed by Wu *et al.* [46].

Our results also suggest the distinct cell context of the T₃-, IBMX- and Rosi-induced beige-like adipocytes from those developed by Sharp *et al.* [37]. *Cited1* expression was increased by prolonged treatment with T₃ and Rosi (treatment D: $P=0.001$) in the 3T3-L1 cells, which was similar to the results by Sharp *et al.* [37]. Thus, *Cited1* is likely to be induced by the activation of Ppar γ , and the expression level of *Cited1* does not reflect the development of beige-like adipocytes, at least in the 3T3-L1 cell model. In addition, the T₃- and Rosi-induced *Cited* expression is blocked by co-treatment with IBMX.

The present study clarifies the differentiation of 3T3-L1 white preadipocytes into beige-like adipocytes. As described above, beige adipocytes could be differentiated from white

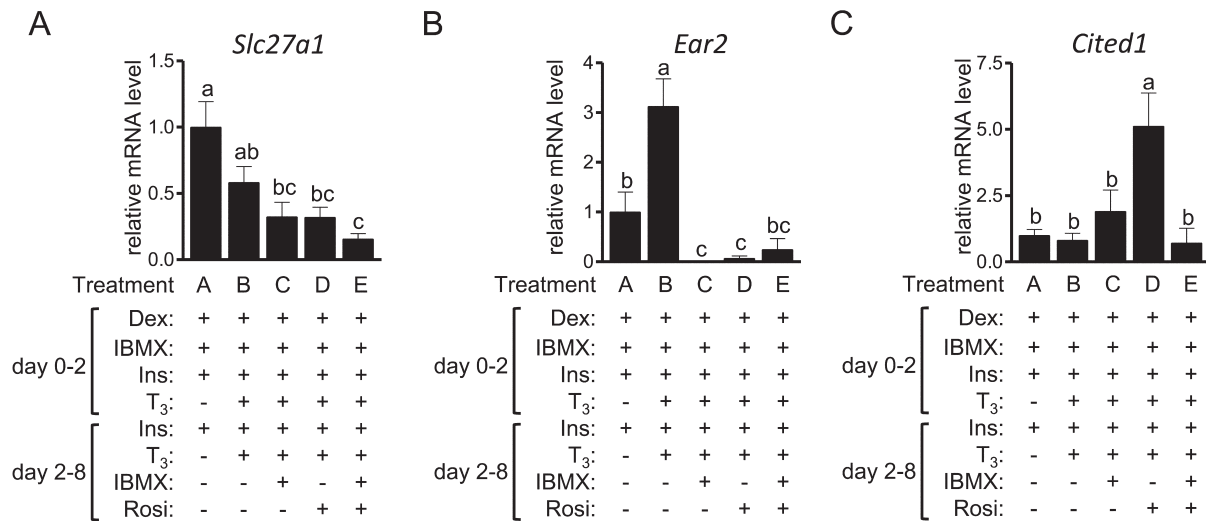


Fig. 5. The expression of beige adipocyte-selective genes in 3T3-L1 cells. 3T3-L1 cells were differentiated into adipocytes in the presence or absence of T₃, IBMX and Rosi. On day 8, the expression of *Slc27a1*(A), *Ear2*(B) and *Cited1*(C) was examined by RT-qPCR and expressed as ratios to *Tbp*, with the level in the control 3T3-L1 cells (treatment A) set to 1. The data shown are the mean $\pm$ SE (n=6).
a,b,c Means that do not have a common letter above the bars differ significantly ($P < 0.05$).

adipocytes [16], beige preadipocytes [46], Sca1⁺/CD45⁻/Mac1⁻ stem cells [33], pluripotent stem cells [27] and Pdgfra⁺/SD34⁺/Sca1⁺ stellate-like cells [18]. Considering all the previous results with the present results, beige adipocytes could be developed from a variety of cells through their specific regulation. It was recently reported that a hematopoietin cocktail composed of stem cell factor, interleukin-6, fms-related tyrosine kinase 3 ligand and vascular endothelial growth factor efficiently differentiates human pluripotent stem cells to brown adipocytes [27]. Further studies are needed to pursue efficient beige adipocyte development, which would provide basic information on the differentiation of white preadipocytes to beige adipocytes.

ACKNOWLEDGMENT. This work was supported by a Grant-in-Aid for Scientific Research (23580368) from The Japan Society for the Promotion of Science.

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