FULL PAPER *Biochemistry*

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 $Pdgfr\alpha^+/CD34^+/Sca1^+$ 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
	5′-primer	3′-primer	accession number
aP2	5′-AAGGTGAAGAGCATCATAACCCT-3′	5′-TCACGCCTTTCATAACACATTCC-3′	NM_024406
C/ebpα	5′-CAAGAACAGCAACGAGTACCG-3′	5′-GTCACTGGTCAACTCCAGCAC-3′	NM_007678
C/ebpβ	5′-ACGACTTCCTCTCCGACCTCT-3′	5′-CGAGGCTCACGTAACCGTAGT-3′	NM_009883
Cidea	5′-AAACCATGACCGAAGTAGCC-3′	5′-AGGCCAGTTGTGATGACTAAGAC-3′	NM_007702
Cited1	5′-CGCTTCGTCCGTACCTCAGCT-3′	5′-CAGCTGGGCCTGTTGGTCTC-3′	NM_007709
Cox7a1	5′-AAAGTGCTGCACGTCCTTG-3′	5′-TTCTCTGCCACACGGTTTTC-3′	NM_009944
Ear2	5′-CAACCAGCCCTAAGTTCCAC-3′	5′-TGAGGCAAGCATTAGGACAA-3′	NM_007895
Pgc1α	5′-TGTGGAACTCTCTGGAACTGC-3′	5′-GCCTTGAAAGGGTTATCTTGG-3′	NM_008904
Pgc1β	5′-CTGACGGTGGAGCTTTGC-3′	5′-AGGCTGGGAGCTGTGTCTT-3′	NM_133249
Pparγ2	5′-TGCTGTTATGGGTGAAACTCTG-3′	5′-CTGTGTCAACCATGGTAATTTCTT-3′	NM_011146
Slc27a1	5′-GACAAGCTGGATCAGGCAAG-3′	5′-GAGGCCACAGAGGCTGTTC-3′	NM_011977
Tfam	5′-CAAAGGATGATTCGGCTCAG-3′	5′-AAGCTGAATATATGCCTGCTTTTC-3′	NM_009360
Tbp	5′-CCAATGACTCCTATGACCCCTA-3′	5′-CAGCCAAGATTCACGGTAGAT-3′	NM_013684
Ucp1	5′-ACTGCCACACCTCCAGTCATT-3′	5′-CTTTGCCTCACTCAGGATTGG-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 μM), IBMX (0.5 mM) and Ins (10 μg/m*l*)) for 2 days, followed by culture in growth medium supplemented with Ins (5 μg/m*l*). According to the protocol, 3T3-L1 cells are differentiated to white adipocytes on day 8 [41]. In addition to the control protocol, Rosi (1 μM), 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 μM) 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 μM) 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 ΔΔCt method, using *TATA-binding protein*(*Tbp*) as the normalization gene [8].

Statistical analyses: The data are expressed as the mean ± 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/ebpα* 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,

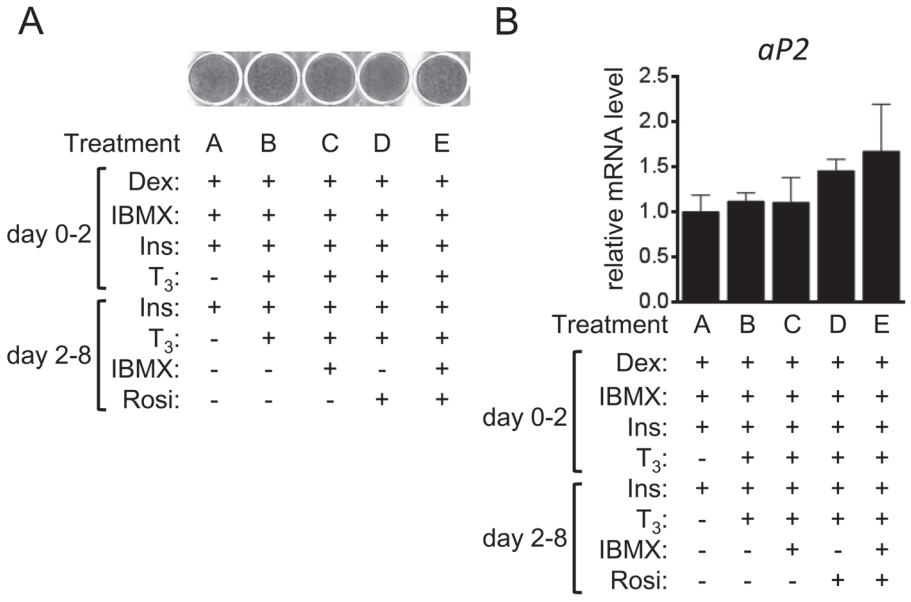

Fig. 1. Adipocyte differentiation in 3T3-L1 cells. 3T3-L1 cells were differentiated into adipocytes in the presence or absence of T_3, IBMX and Rosi. (A) The lipid accumulation in cells without Iso treatment on day 8 was examined using oil red O staining. (B) The gene transcript level of *aP2* in cells treated without Iso on day 8 was examined by RT-qPCR and expressed as ratios to *Tbp* levels with the level in the control 3T3-L1 cells (treatment A) set to 1. The data shown are the mean ± SE (n=6).

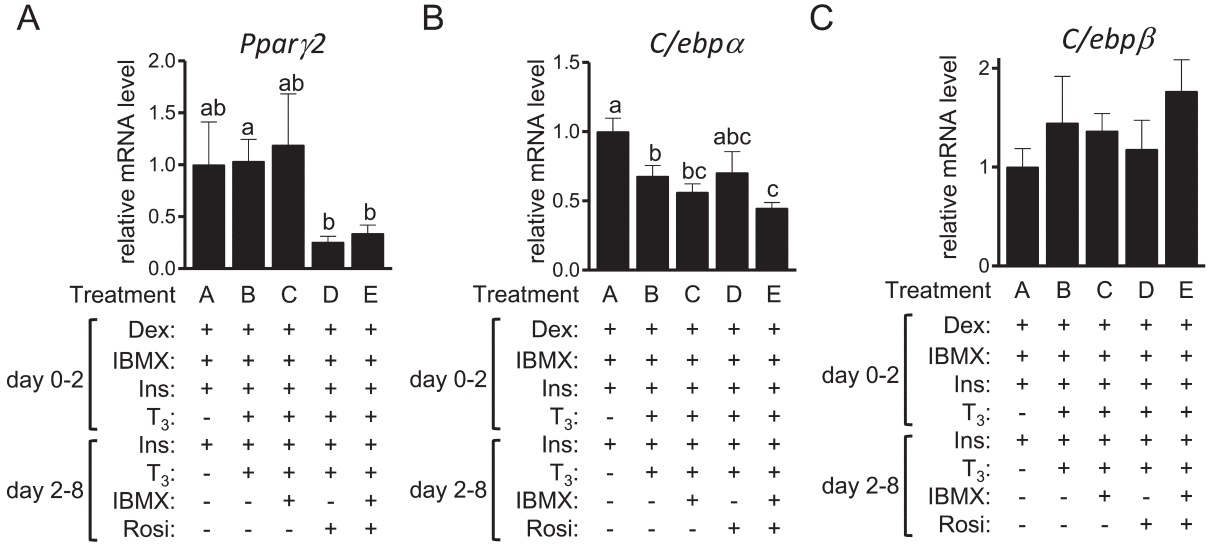

Fig. 2. The expression of adipogenic transcription factors in 3T3-L1 cells. 3T3-L1 cells were differentiated into adipocytes in the presence or absence of T_3, IBMX and Rosi. The gene transcript levels of *Pparγ2*(A), *C/ebpα* (B) and *C/ebpβ* (C) in cells treated without Iso on day 8 were examined by 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 ± 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), although 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]

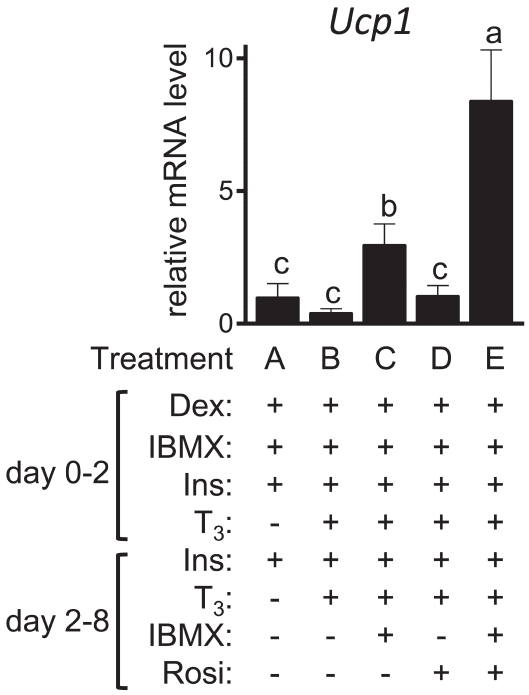

Fig. 3. The expression of *Ucp1* in 3T3-L1 cells. 3T3-L1 cells were differentiated into adipocytes in the presence or absence of the indicated factors. On day 8, the cells were further treated with Iso for 4 hr. *Ucp1* expression was examined by RT-qPCR and expressed as ratios to *Tbp* levels with the level in the control 3T3-L1 cells (treatment A) set to 1. The data shown are the mean ± 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 *Pgc1α*, *Pgc1β*, *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 T_3 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 T_3, 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 T_3, Rosi and IBMX are closer to mature beige adipocytes.

T_3, IBMX and Rosi are all needed for the efficient induction of *Ucp1* in response to β adrenergic receptor activation. However, whether T_3 is essential is not known, because the observed Iso-induced *Ucp1* expression was not examined in cells treated with IBMX and Rosi, but without T_3. In addition, the present results suggest that Rosi augments the effects of T_3 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 T_3, Rosi and IBMX in view of the following evidence: prolonged treatment with Rosi with or without T_3 in stromal vascular cells from white fat depots resulted in Iso-induced *Ucp1* induction [28, 30]. The overexpression of *C/ebpβ* enhanced cAMP-mediated *Ucp1* induction in 3T3-L1 cells [17]. Furthermore, IBMX is responsible for *C/ebpβ* 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/ebpβ* expression in treatments C and E that were treated with the prolonged IBMX. However, the expression level of *C/ebpβ* was comparable among treatments A, C and E (Fig. 2C), suggesting an activity of IBMX other than the regulation of *C/ebpβ* 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/ebpα* was lower in treatments B, C and E than in the control treatment A (Fig. 2B); the transcript level of *C/ebpα* 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

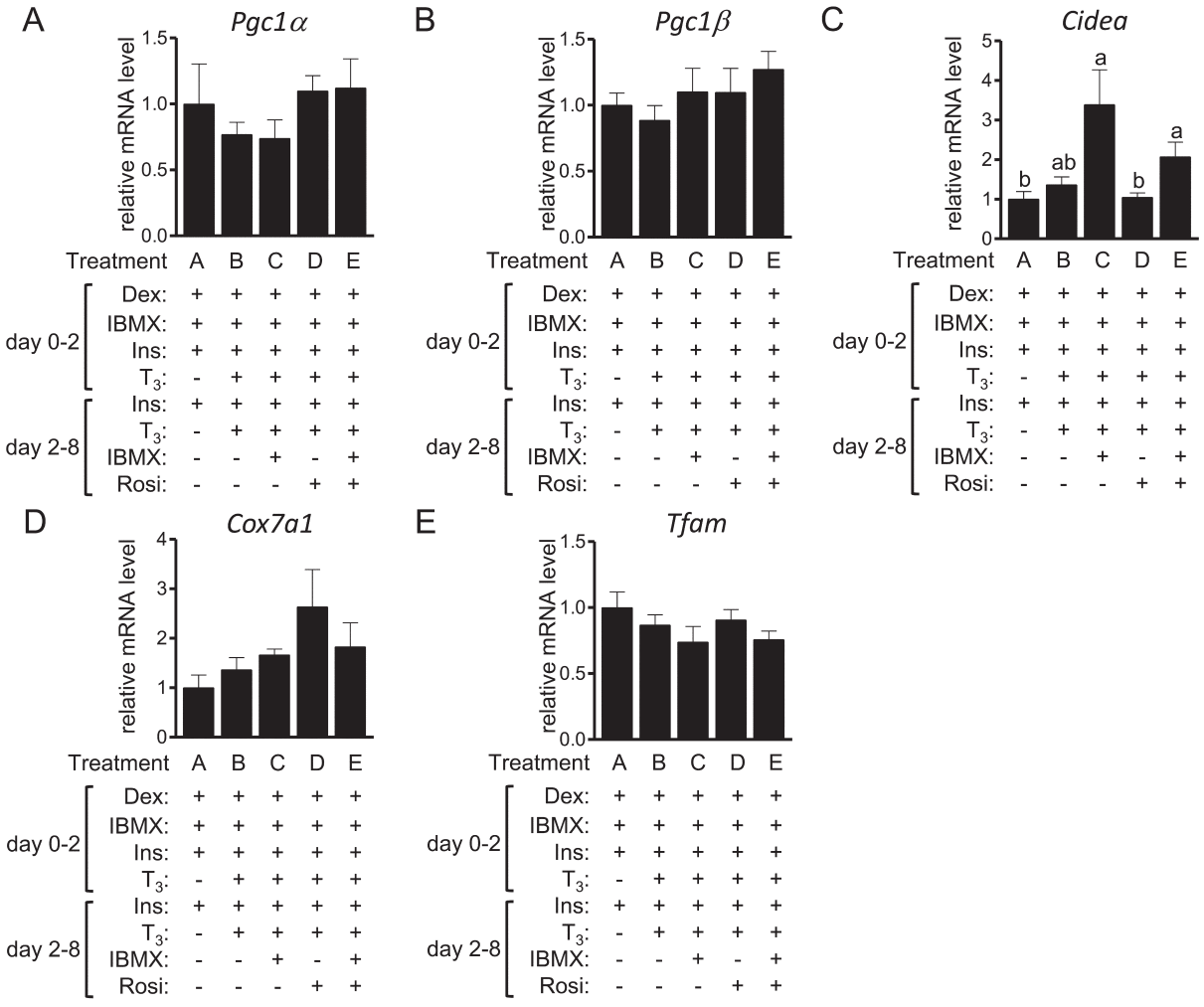

Fig. 4. The expression of brown fat-selective genes in 3T3-L1 cells. 3T3-L1 cells were differentiated into adipocytes in the presence or absence of T_3, IBMX and Rosi. On day 8, the expression of *Pgc1α* (A), *Pgc1β* (B), *Cidea*(C), *Cox7a1*(D) and *Tfam*(E) was examined by RT-qPCR and expressed as ratios to *Tbp* 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/ebpα* 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/ebpα* 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 T_3, 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 T_3-, IBMX- and Rosi-induced beige-like adipocytes from those developed by Sharp *et al.* [37]. *Cited1* expression was increased by prolonged treatment with T_3 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 Pparγ, 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 T_3- 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

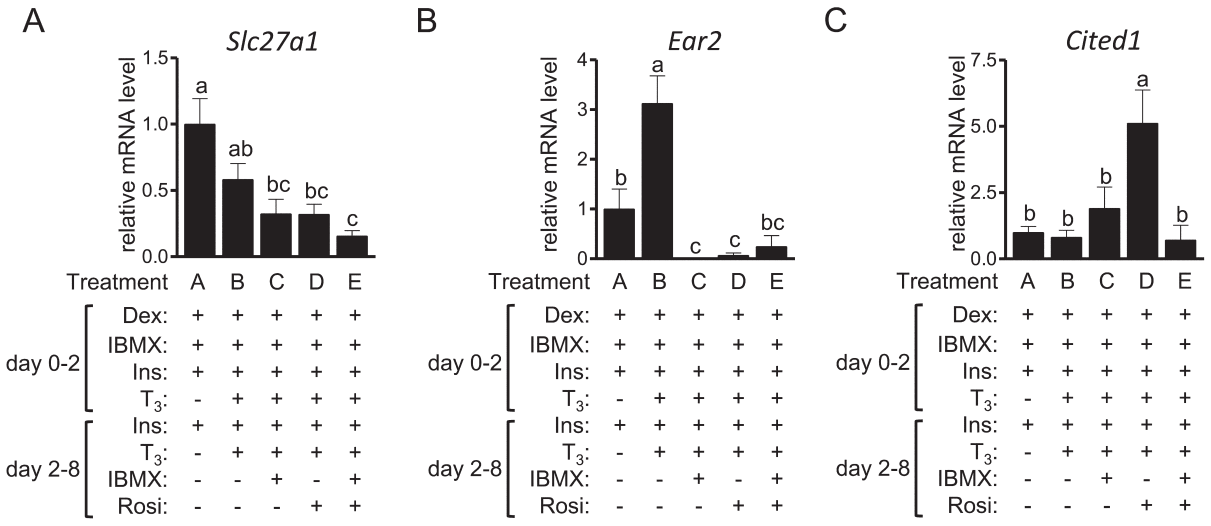

Fig. 5. The expression of beige adipocyte-selective genes in 3T3-L1 cells 3T3-L1 cells were differentiated into adipocytes in the presence or absence of T_3, IBMX and Rosi. On day 8, the expression of *Slc27a1*(A), *Ear2*(B) and *Cited1*(C) was examined by RT-qPCR and expressed as ratios to *Tbp*, with the level in the control 3T3-L1 cells (treatment A) set to 1. The data shown are the mean ± 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 Pdgfrα$^+$/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.

REFERENCES

1. Almind, K., Manieri, M., Sivitz, W. I., Cinti, S. and Kahn, C. R. 2007. Ectopic brown adipose tissue in muscle provides a mechanism for differences in risk of metabolic syndrome in mice. *Proc. Natl. Acad. Sci. U.S.A.* **104**: 2366–2371. [Medline] [CrossRef]
2. Asano, H., Yamada, T., Hashimoto, O., Umemoto, T., Sato, R., Ohwatari, S., Kanamori, Y., Terachi, T., Funaba, M. and Matsui, T. 2013. Diet-induced changes in Ucp1 expression in bovine adipose tissues. *Gen. Comp. Endocrinol.* **184**: 87–92. [Medline] [CrossRef]
3. Barbatelli, G., Murano, I., Madsen, L., Hao, Q., Jimenez, M., Kristiansen, K., Giacobino, J. P., De Matteis, R. and Cinti, S. 2010. The emergence of cold-induced brown adipocytes in mouse white fat depots is determined predominantly by white to brown adipocyte transdifferentiation. *Am. J. Physiol. Endocrinol. Metab.* **298**: E1244–E1253. [Medline] [CrossRef]
4. Cannon, B. and Nedergaard, J. 2004. Brown adipose tissue: function and physiological significance. *Physiol. Rev.* **84**: 277–359. [Medline] [CrossRef]
5. Cannon, B. and Nedergaard, J. 2012. Cell biology: Neither brown nor white. *Nature* **488**: 286–287. [Medline] [CrossRef]
6. Cypess, A. M., Lehman, S., Williams, G., Tal, I., Rodman, D., Goldfine, A. B., Kuo, F. C., Palmer, E. L., Tseng, Y. H., Doria, A., Kolodny, G. M. and Kahn, C. R. 2009. Identification and importance of brown adipose tissue in adult humans. *N. Engl. J. Med.* **360**: 1509–1517. [Medline] [CrossRef]
7. Distel, E., Penot, G., Cadoudal, T., Balguy, I., Durant, S. and Benelli, C. 2012. Early induction of a brown-like phenotype by rosiglitazone in the epicardial adipose tissue of fatty Zucker rats. *Biochimie* **94**: 1660–1667. [Medline] [CrossRef]
8. Duran, E. M., Shapshak, P., Worley, J., Minagar, A., Ziegler, F., Haliko, S., Moleon-Borodowsky, I. and Haslett, P. A. 2005. Presenilin-1 detection in brain neurons and FOXP3 in peripheral blood mononuclear cells: normalizer gene selection for real time reverse transcriptase PCR using the deltadeltaCt method. *Front. Biosci.* **10**: 2955–2965. [Medline] [CrossRef]
9. Elks, M. L. and Manganiello, V. C. 1984. Selective effects of phosphodiesterase inhibitors on different phosphodiesterases, adenosine 3′,5′-monophosphate metabolism, and lipolysis in 3T3-L1 adipocytes. *Endocrinology* **115**: 1262–1268. [Medline] [CrossRef]
10. Francis, S. H., Houslay, M. D. and Conti, M. 2011. Phosphodiesterase inhibitors: factors that influence potency, selectivity, and action. *Handb. Exp. Pharmacol.* **204**: 47–84. [Medline] [CrossRef]
11. Furutani, Y., Murakami, M. and Funaba, M. 2009. Differential responses to oxidative stress and calcium influx on expression of the transforming growth factor-β family in myoblasts and myotubes. *Cell Biochem. Funct.* **27**: 578–582. [Medline] [CrossRef]
12. García, B. and Obregón, M. J. 2002. Growth factor regulation of uncoupling protein-1 mRNA expression in brown adipocytes. *Am. J. Physiol. Cell Physiol.* **282**: C105–C112. [Medline]

[CrossRef]
13. Gesta, S., Tseng, Y. H. and Kahn, C. R. 2007. Developmental origin of fat: tracking obesity to its source. *Cell* **131**: 242–256. [Medline] [CrossRef]
14. Guerra, C., Koza, R. A., Yamashita, H., Walsh, K. and Kozak, L. P. 1998. Emergence of brown adipocytes in white fat in mice is under genetic control. Effects on body weight and adiposity. *J. Clin. Invest.* **102**: 412–420. [Medline] [CrossRef]
15. Guerra, C., Roncero, C., Porras, A., Fernández, M. and Benito, M. 1996. Triiodothyronine induces the transcription of the uncoupling protein gene and stabilizes its mRNA in fetal rat brown adipocyte primary cultures. *J. Biol. Chem.* **271**: 2076–2081. [Medline] [CrossRef]
16. Himms-Hagen, J., Melnyk, A., Zingaretti, M. C., Ceresi, E., Barbatelli, G. and Cinti, S. 2000. Multilocular fat cells in WAT of CL-316243-treated rats derive directly from white adipocytes. *Am. J. Physiol. Cell Physiol.* **279**: C670–C681. [Medline]
17. Karamanlidis, G., Karamitri, A., Docherty, K., Hazlerigg, D. G. and Lomax, M. A. 2007. C/EBPβ reprograms white 3T3-L1 preadipocytes to a brown adipocyte pattern of gene expression. *J. Biol. Chem.* **282**: 24660–24669. [Medline] [CrossRef]
18. Lee, Y. H., Petkova, A. P., Mottillo, E. P. and Granneman, J. G. 2012. *In vivo* identification of bipotential adipocyte progenitors recruited by β3-adrenoceptor activation and high-fat feeding. *Cell Metab.* **15**: 480–491. [Medline] [CrossRef]
19. Lee, J. Y., Takahashi, N., Yasubuchi, M., Kim, Y. I., Hashizaki, H., Kim, M. J., Sakamoto, T., Goto, T. and Kawada, T. 2012. Triiodothyronine induces UCP-1 expression and mitochondrial biogenesis in human adipocytes. *Am. J. Physiol. Cell Physiol.* **302**: C463–C472. [Medline] [CrossRef]
20. Lowell, B. B., S-Susulic, V., Hamann, A., Lawitts, J. A., Himms-Hagen, J., Boyer, B. B., Kozak, L. P. and Flier, J. S. 1993. Development of obesity in transgenic mice after genetic ablation of brown adipose tissue. *Nature* **366**: 740–742. [Medline] [CrossRef]
21. Lowell, B. B. and Spiegelman, B. M. 2000. Towards a molecular understanding of adaptive thermogenesis. *Nature* **404**: 652–660. [Medline]
22. Martinez de Mena, R., Scanlan, T. S. and Obregon, M. J. 2010. The T3 receptor beta1 isoform regulates UCP1 and D2 deiodinase in rat brown adipocytes. *Endocrinology* **151**: 5074–5083. [Medline] [CrossRef]
23. Mitschke, M. M., Hoffmann, L. S., Gnad, T., Scholz, D., Kruithoff, K., Mayer, P., Haas, B., Sassmann, A., Pfeifer, A. and Kilic, A. 2013. Increased cGMP promotes healthy expansion and browning of white adipose tissue. *FASEB J.* **27**: 1621–1630. [Medline] [CrossRef]
24. Mottillo, E. P. and Granneman, J. G. 2011. Intracellular fatty acids suppress β-adrenergic induction of PKA-targeted gene expression in white adipocytes. *Am. J. Physiol. Endocrinol. Metab.* **301**: E122–E131. [Medline] [CrossRef]
25. Murakami, M., Kawachi, H., Ogawa, K., Nishino, Y. and Funaba, M. 2009. Receptor expression modulates the specificity of transforming growth factor-β signaling pathways. *Genes Cells* **14**: 469–482. [Medline] [CrossRef]
26. Nedergaard, J., Bengtsson, T. and Cannon, B. 2007. Unexpected evidence for active brown adipose tissue in adult humans. *Am. J. Physiol. Endocrinol. Metab.* **293**: E444–E452. [Medline] [CrossRef]
27. Nishio, M., Yoneshiro, T., Nakahara, M., Suzuki, S., Saeki, K., Hasegawa, M., Kawai, Y., Akutsu, H., Umezawa, A., Yasuda, K., Tobe, K., You, A., Kubota, K., Saito, M. and Saeki, K. 2012. Production of functional classical brown adipocytes from human pluripotent stem cells using specific hemopoietin cocktail without gene transfer. *Cell Metab.* **16**: 394–406. [Medline] [CrossRef]
28. Ohno, H., Shinoda, K., Spiegelman, B. M. and Kajimura, S. 2012. PPARγ agonists induce a white-to-brown fat conversion through stabilization of PRDM16 protein. *Cell Metab.* **15**: 395–404. [Medline] [CrossRef]
29. Ouellet, V., Labbé, S. M., Blondin, D. P., Phoenix, S., Guérin, B., Haman, F., Turcotte, E. E., Richard, D. and Carpentier, A. C. 2012. Brown adipose tissue oxidative metabolism contributes to energy expenditure during acute cold exposure in humans. *J. Clin. Invest.* **122**: 545–552. [Medline] [CrossRef]
30. Petrovic, N., Walden, T. B., Shabalina, I. G., Timmons, J. A., Cannon, B. and Nedergaard, J. 2010. Chronic peroxisome proliferator-activated receptor γ (PPARγ) activation of epididymally derived white adipocyte cultures reveals a population of thermogenically competent, UCP1-containing adipocytes molecularly distinct from classic brown adipocytes. *J. Biol. Chem.* **285**: 7153–7164. [Medline] [CrossRef]
31. Rosen, E. D. and Spiegelman, B. M. 2006. Adipocytes as regulators of energy balance and glucose homeostasis. *Nature* **444**: 847–853. [Medline] [CrossRef]
32. Saito, M., Okamatsu-Ogura, Y., Matsushita, M., Watanabe, K., Yoneshiro, T., Nio-Kobayashi, J., Iwanaga, T., Miyagawa, M., Kameya, T., Nakada, K., Kawai, Y. and Tsujisaki, M. 2009. High incidence of metabolically active brown adipose tissue in healthy adult humans: effects of cold exposure and adiposity. *Diabetes* **58**: 1526–1531. [Medline] [CrossRef]
33. Schulz, T. J., Huang, T. L., Tran, T. T., Zhang, H., Townsend, K. L., Shadrach, J. L., Cerletti, M., McDougall, L. E., Giorgadze, N., Tchkonia, T., Schrier, D., Falb, D., Kirkland, J. L., Wagers, A. J. and Tseng, Y. H. 2011. Identification of inducible brown adipocyte progenitors residing in skeletal muscle and white fat. *Proc. Natl. Acad. Sci. U.S.A.* **108**: 143–148. [Medline] [CrossRef]
34. Seale, P., Conroe, H. M., Estall, J., Kajimura, S., Frontini, A., Ishibashi, J., Cohen, P., Cinti, S. and Spiegelman, B. M. 2011. Prdm16 determines the thermogenic program of subcutaneous white adipose tissue in mice. *J. Clin. Invest.* **121**: 96–105. [Medline] [CrossRef]
35. Seale, P., Kajimura, S., Yang, W., Chin, S., Rohas, L. M., Uldry, M., Tavernier, G., Langin, D. and Spiegelman, B. M. 2007. Transcriptional control of brown fat determination by PRDM16. *Cell Metab.* **6**: 38–54. [Medline] [CrossRef]
36. Seale, P., Bjork, B., Yang, W., Kajimura, S., Chin, S., Kuang, S., Scimè, A., Devarakonda, S., Conroe, H. M., Erdjument-Bromage, H., Tempst, P., Rudnicki, M. A., Beier, D. R. and Spiegelman, B. M. 2008. PRDM16 controls a brown fat/skeletal muscle switch. *Nature* **454**: 961–967. [Medline] [CrossRef]
37. Sharp, L. Z., Shinoda, K., Ohno, H., Scheel, D. W., Tomoda, E., Ruiz, L., Hu, H., Wang, L., Pavlova, Z., Gilsanz, V. and Kajimura, S. 2012. Human BAT possesses molecular signatures that resemble beige/brite cells. *PLoS One* **7**: e49452. [Medline] [CrossRef]
38. Spiegelman, B. M. and Flier, J. S. 2001. Obesity and the regulation of energy balance. *Cell* **104**: 531–543. [Medline] [CrossRef]
39. Student, A. K., Hsu, R. Y. and Lane, M. D. 1980. Induction of fatty acid synthetase synthesis in differentiating 3T3-L1 preadipocytes. *J. Biol. Chem.* **255**: 4745–4750. [Medline]
40. Suenaga, M., Matsui, T. and Funaba, M. 2010. BMP inhibition with dorsomorphin limits adipogenic potential of preadipocytes. *J. Vet. Med. Sci.* **72**: 373–377. [Medline] [CrossRef]

41. Tang, Q. Q. and Lane, M. D. 2012. Adipogenesis: from stem cell to adipocyte. *Annu. Rev. Biochem.* **81**: 715–736. [Medline] [CrossRef]
42. Tseng, Y. H., Kokkotou, E., Schulz, T. J., Huang, T. L., Winnay, J. N., Taniguchi, C. M., Tran, T. T., Suzuki, R., Espinoza, D. O., Yamamoto, Y., Ahrens, M. J., Dudley, A. T., Norris, A. W., Kulkarni, R. N. and Kahn, C. R. 2008. New role of bone morphogenetic protein 7 in brown adipogenesis and energy expenditure. *Nature* **454**: 1000–1004. [Medline] [CrossRef]
43. van Marken Lichtenbelt, W. D., Vanhommerig, J. W., Smulders, N. M., Drossaerts, J. M., Kemerink, G. J., Bouvy, N. D., Schrauwen, P. and Teule, G. J. 2009. Cold-activated brown adipose tissue in healthy men. *N. Engl. J. Med.* **360**: 1500–1508. [Medline] [CrossRef]
44. Vegiopoulos, A., Müller-Decker, K., Strzoda, D., Schmitt, I., Chichelnitskiy, E., Ostertag, A., Berriel Diaz, M., Rozman, J., Hrabe de Angelis, M., Nüsing, R. M., Meyer, C. W., Wahli, W., Klingenspor, M. and Herzig, S. 2010. Cyclooxygenase-2 controls energy homeostasis in mice by de novo recruitment of brown adipocytes. *Science* **328**: 1158–1161. [Medline] [CrossRef]
45. Virtanen, K. A., Lidell, M. E., Orava, J., Heglind, M., Westergren, R., Niemi, T., Taittonen, M., Laine, J., Savisto, N. J., Enerbäck, S. and Nuutila, P. 2009. Functional brown adipose tissue in healthy adults. *N. Engl. J. Med.* **360**: 1518–1525. [Medline] [CrossRef]
46. Wu, J., Boström, P., Sparks, L. M., Ye, L., Choi, J. H., Giang, A. H., Khandekar, M., Virtanen, K. A., Nuutila, P., Schaart, G., Huang, K., Tu, H., van Marken Lichtenbelt, W. D., Hoeks, J., Enerbäck, S., Schrauwen, P. and Spiegelman, B. M. 2012. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell* **150**: 366–376. [Medline] [CrossRef]
47. Yeh, W. C., Cao, Z., Classon, M. and McKnight, S. L. 1995. Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. *Genes Dev.* **9**: 168–181. [Medline] [CrossRef]