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Tumor suppressor candidate 5 (TUSC5) is expressed in brown adipocytes

Hisashi Koide [a], Takahisa Shibata [a], Nobuko Yamada [a], Toshiyuki Asaki [b], Toshitaka Nagao [c], Tomohiko Yoshida [a], Yoshihiko Noguchi [a], Tomoaki Tanaka [a], Yasushi Saito [a], Ichiro Tatsuno [a,*]

[a] *Department of Clinical Cell Biology, Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan*
[b] *Laboratory of Neurobiophysics, School of Pharmaceutical Sciences, The University of Tokyo, Tokyo 113-0033, Japan*
[c] *Department of Clinical Pathology, Tokyo Medical University Hospital, Tokyo 160-0023, Japan*

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Abstract

Rat brain endothelial cell derived gene-1 (BEC-1) had considerable homology with tumor suppressor candidate 5 (TUSC5). TUSC5 was expressed abundantly, and its mRNA was inhibited by cold exposure in rat brown adipose tissue (BAT). In the present study, we investigated its regulatory mechanism using primary cultured rat brown preadipocytes (RBPA) and Zucker lean rats (ZL). We found that: (1) TUSC5 mRNA began to increase in a manner similar to C/EBP-α, PPAR-γ, and adiponectin during differentiation in RBPA; (2) neither β3-adrenoceptor agonist BRL 37344 nor dexamethasone affected TUSC5 mRNA in RBPA; (3) propranolol did not block the decrease of TUSC5 mRNA by cold exposure in ZL; (4) BRL 37344 did not influence TUSC5 mRNA in ZL; and (5) dexamethasone inhibited TUSC5 mRNA in a dose-dependent manner similar to UCP-1 in ZL. These data suggested that TUSC5 is involved in the differentiation, and its expression is regulated independently of the β-adrenergic pathway in BAT.

Keywords: BEC-1; TUSC5; Adipocyte; Brown adipose tissue; Obese

Brain endothelial cell derived gene-1 (BEC-1) was identified as a gene dominantly expressed in rat brain endothelial cells using suppression subtractive hybridization technique [1,2]. The complementary deoxyribonucleic acid (cDNA) sequence of BEC-1 messenger ribonucleic acid (mRNA) was completely determined with a full length of 3410 base pairs. The open reading frame within the sequence consisted of 522 base pairs. BEC-1 protein consisted of 173 amino acids and its predicted molecular weight was 18.7 kDa. BEC-1 gene was thought to be a rat tumor suppressor candidate 5 (TUSC5), since this gene had considerable homology with both mouse TUSC5 [3] and human located at seventeen-p-thirteen point three 1 (LOST1) [4] categorized as human TUSC5 (identities of 97% and 75%, respectively), which were recently identified as a novel tumor suppressor gene candidate [1].

Expressional analyses for both BEC-1 mRNA with real-time RT-PCR and its protein by Western blotting demonstrated that both were dominantly expressed in adipose tissues such as subcutaneous, mesenteric, and epididymal white adipose tissue (WAT), and interscapular brown adipose tissue (BAT) in Zucker rats [1]. In addition, cold exposure significantly suppressed BEC-1 mRNA expression in BAT. These data indicated that BEC-1, a rat TUSC5, might be involved in the regulation of adipose tissues [1].

In rodents, BAT is a major site of adaptive thermogenesis [5]. It has nonshivering thermogenic activity that transfers energy from food into heat. Its activity is controlled by the sympathetic nervous system, and brown adipocytes are dependent on adequate delivery of oxygen and lipids. Both food intake and cold exposure induce BAT thermogenesis

* Corresponding author. Address: Department of Clinical Cell Biology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan. Fax: +81 43 226 209.
E-mail address: ichiro-tatsuno@faculty.chiba-u.jp (I. Tatsuno).

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via the sympathetic nervous system [6]. In brown adipocytes, norepinephrine interacts with all three types of adrenergic receptors, β, α1, and α2, among which β-adrenergic stimulation through β3-adrenoceptor is the most significant pathway for thermogenesis in mature brown adipocytes of rodents [7–9]. β3-adrenergic receptors are also expressed on WAT, but its thermogenic capacity is supposedly so low that they can be ignored in this context [5,7]. Blockade of the sympathetic nervous system or a lack of noradrenline and adrenaline reportedly results in the inability to maintain body temperature during cold exposure [10,11].

UCP-1, a mitochondrial inner-membrane protein that uncouples proton entry from ATP synthesis, with the energy stored in proton-motive force then being released as heat, also plays a critical role for the thermogenesis in BAT. Cold exposure increased UCP-1 expression for thermogenesis, and UCP1-deficient mice were sensitive to cold exposure [12]. The increment of UCP-1 mRNA expression induced by cold exposure was dependent on the β-adrenergic pathway, since it was abolished by the presence of β-adrenergic antagonist [12], and the stimulation of β-adrenergic receptor increased UCP-1 mRNA in BAT [13–15].

In the present study, we investigated the regulatory mechanism for TUSC5 (BEC-1) in brown adipocytes in comparison with UCP-1 using both primary cultured rat brown preadipocytes (RBPA) and wild-type (+/+) Zucker lean rats (ZL).

Materials and methods

Cell culture. Primary cultured rat brown preadipocytes (RBPA) isolated from BAT of SD rats were purchased from TaKaRa Bio (Tokyo, Japan) [16]. The differentiation of RBPA was performed as previously reported [16]. Briefly, brown adipocytes were cultured in Dullbecco's modified Eagle's medium supplemented with 10% fetal calf serum, plated on Type I collagen-coated dishes, and were grown at 37 °C in a humidified atmosphere with 5% CO2. Differentiation into adipocytes was achieved by incubating subconfluent undifferentiated brown adipocytes with 10 μg/ml insulin, 2.5 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) for 2 days. The medium was then replaced with medium containing 10 μg/ml of insulin. All the experiments were performed with brown adipocytes within two passages. On the seventh day of the brown adipocyte differentiation, cells were added with BRL37344 (1 μM) or dexamethasone (10, 100, 1000 nM) or medium as control, and they were collected 3 h later.

Animals. Eight-week-old male Sprague–Dawley (SD) rats and 10-week-old male wild-type (+/+) Zucker lean rats (ZL) were purchased from Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan). They were bred in our laboratory and maintained at a constant temperature (23 °C) with a fixed 12-h artificial light cycle (lights on at 7 a.m.) and had free access to water and standard laboratory chow. They were housed in cages from 1 week before each experiment.

All experiments were performed according to the guidelines of the Animal Care and Experimentation Committee of Chiba University, the University of Tokyo, and the National Institute for Basic Biology.

Total RNA extraction and real-time PCR. Interscapular BAT was surgically removed and immediately frozen and stored at −80 °C until use. Total RNA was prepared from BAT using the QIAzol Lysis Reagent (RNeasy Lipid Tissue) isolation method according to the manufacturer's protocol (Qiagen, Valencia, CA, USA). Total RNA was isolated from undifferentiated or differentiated brown adipocytes using RLT buffer (RNeasy) isolation method according to the manufacturer's instructions (Qiagen). The ABI PRISM 7500 instrument (Applied Biosystems, Foster City, CA, USA) was used for quantitative real-time RT-PCR amplification and detection. Quantitative PCR was performed using duplicates or triplicates of 50-μl reaction mixture in MicroAmp optical 96-well reaction plates and sealed with optical caps (Applied Biosystems). Each reaction well contained 1 μl of template RNA, 25 μl of 10× SYBR Green PCR Master Mix, 0.5 μl of RT Mix (Qiagen), and 25 pmol each of forward and reverse primers.

Real-time RT-PCR was conducted with the following cycling conditions: 50 °C for 30 min, 95 °C for 15 min, followed by 40 cycles at 94 °C for 15 s, 60 °C for 35 s, and 72 °C for 30 s each. The oligonucleotide primers for rat BEC-1, rat UCP-1, and β-actin were as follows: BEC-1 forward, GGA GAA CAA GGA TGA CCA AGC; BEC-1 reverse, CGG AGA TGA CCT TGA AGG GTA; UCP-1 forward, TCC CTC AGG ATT GGC CTC TAC; UCP-1 reverse, GTC ATC AAG CCA GCC GAG AT; β-actin forward, GTA GCC ATC CAG GCT GTG TT; β-actin reverse, CCC TCA TAG ATG GGC ACA GT; C/EBPβ forward, CCG GGC CCT GAG TAA TCA CT; C/EBPβ reverse, AAC ATC AAC AGC AAC AAC CCC; C/EBPα forward, ACG TGG AGA CGC AGC AGA A; C/EBPα reverse, AGG CGG TCA TTG TCA CTG G; PPARγforward, CAT TCT GGC CCA CCA ATC TC; PPARγreverse, TCA AAG GAA TGC CAG TGG TCT T; adiponectin forward, AAT CCT GCC CAG TCA TGA AG; adiponectin reverse, TCT CCA GGA GTG CCA TCT CT ; 18s forward, CTT AGA GGG ACA AGT GGC G; 18s reverse, ACG CTG AGC CAG TCA GTG TA.

Expressional analysis of TUSC5 (BEC-1) mRNA in ZL. To examine the effect of β3-adrenoceptor agonist, Zucker lean rats were injected intraperitoneally twice a day with a β3-adrenoceptor agonist, BRL 37344 (Tocris, Ellisville, US) (0.5 mg/kg body weight), or saline vehicle for 3 days. The animals were killed 12 h after the last dose. To examine the effect of β-adrenergic antagonist in the decreasing expression of BEC-1 mRNA by cold exposure, Zucker lean rats were injected intraperitoneally with propranolol (Tocris, Ellisville, US) (20 mg/kg body weight/day) for 3 days and then suddenly exposed to 4 °C for 30 min in individual cages. The animals were then killed to collect samples. For the study of dexamethasone (Nacalai Tesque, Kyoto, Japan), it was injected intraperitoneally at doses of 0.5 mg, 0.05 mg and 0.005 mg/kg body weight. BAT was sampled 3 h after injection.

Statistical analysis. Statistical differences within each experiment were determined by analysis of variance, and differences between groups were calculated by Fisher's PLSD. A rejection level of $p < 0.05$ was considered significant. This analysis was carried out on a personal computer using the "StatView J4.02" software statistical package (Abacus Concepts, Inc., Berkeley, CA, USA).

Results

TSUC5 (BEC-1) increases during differentiation in primary cultured rat brown preadipocytes

RBPA isolated from BAT of SD rats were differentiated for 7 days (Fig. 1A) as previously reported [16]. Differentiation into adipocytes was achieved by incubating subconfluent undifferentiated brown adipocytes with 10 μg/ml insulin, 2.5 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) for 2 days. The medium was then replaced with medium containing 10 μg/ml of insulin. After this treatment, more than 95% of cells were differentiated, showing drastic morphological changes consisting of multiple small intracytoplasmic lipid droplets (Fig. 1A).

The mRNA expressions of various adipocyte markers were analyzed during the differentiation in RBPA by real-time PCR (Fig. 1B). The expression of C/EBPβ mRNA increased transiently in the early period of differentiation.

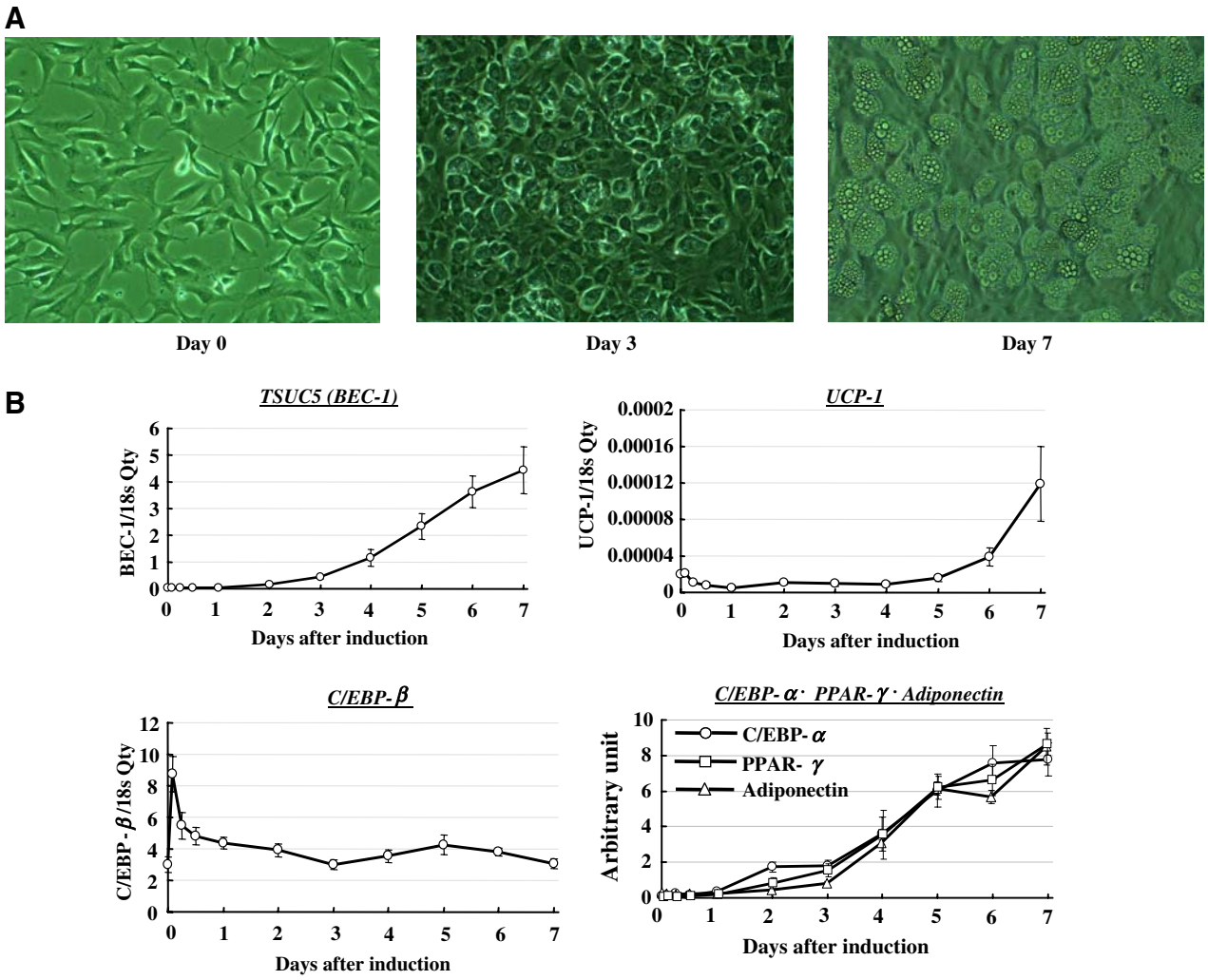

Fig. 1. Adipocyte differentiation in primary cultured rat brown preadipocytes. (A) Rat brown preadipocytes (RBPA) were cultured to differentiation as described in Materials and methods. The morphological changes of RBPA at days 0, 3, and 7 of culture are shown, revealing multiple small intracytoplasmic lipid droplets. (B) Real-time RT-PCR was performed to analyze the mRNA levels of TUSC5 (BEC-1), UCP-1, C/EBPβ, C/EBPα, PPARγ, and adiponectin at the indicated time points. Data represent means ± SE of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Other adipocyte markers including C/EBPα, PPARγ, and adiponectin increased as lipid droplets accumulated in the cells. TSUC5 (BEC-1) mRNA also increased during the differentiation in a manner similar to C/EBPα, PPARγ, and adiponectin, with its increment being earlier than that of UCP-1 (Fig. 1B).

Neither β3-adrenoceptor agonist nor dexamethasone affects TSUC5 (BEC-1) mRNA in primary cultured rat brown preadipocytes

We examined the regulatory mechanism of TSUC5 (BEC-1) in RBPA at day 7 of culture. BRL37344, a β3-adrenoceptor agonist, increased UCP-1 mRNA for 3 h in differentiated RBPA, as expected, but not TSUC5 (BEC-1) mRNA (Fig. 2A). In addition, dexamethasone decreased the mRNA expression of UCP-1, but not TUSC5 (BEC-1) in RBPA (Fig. 2B).

Cold exposure decreased TSUC5 (BEC-1) mRNA independently of β-adrenergic pathway in brown adipose tissue in Zucker lean rats (ZL)

Since TSUC5 (BEC-1) mRNA was demonstrated to decrease during cold exposure in BAT, we also examined the role of the β-adrenergic pathway in the expression of BEC-1 mRNA in BAT using an in vivo model of cold exposure in ZL.

TSUC5 (BEC-1) mRNA was reconfirmed to decrease with cold exposure in BAT (Fig. 3). The treatment of propranolol, a β-adrenergic antagonist, did not have any effect on the decreasing mRNA expression of TSUC5 (BEC-1) induced by cold exposure in BAT, although its treatment significantly inhibited the increasing mRNA expression of UCP-1 as expected (Fig. 3). Its treatment had no effect on the mRNA expression of either UCP-1 or TSUC5 (BEC-1) at room temperature.

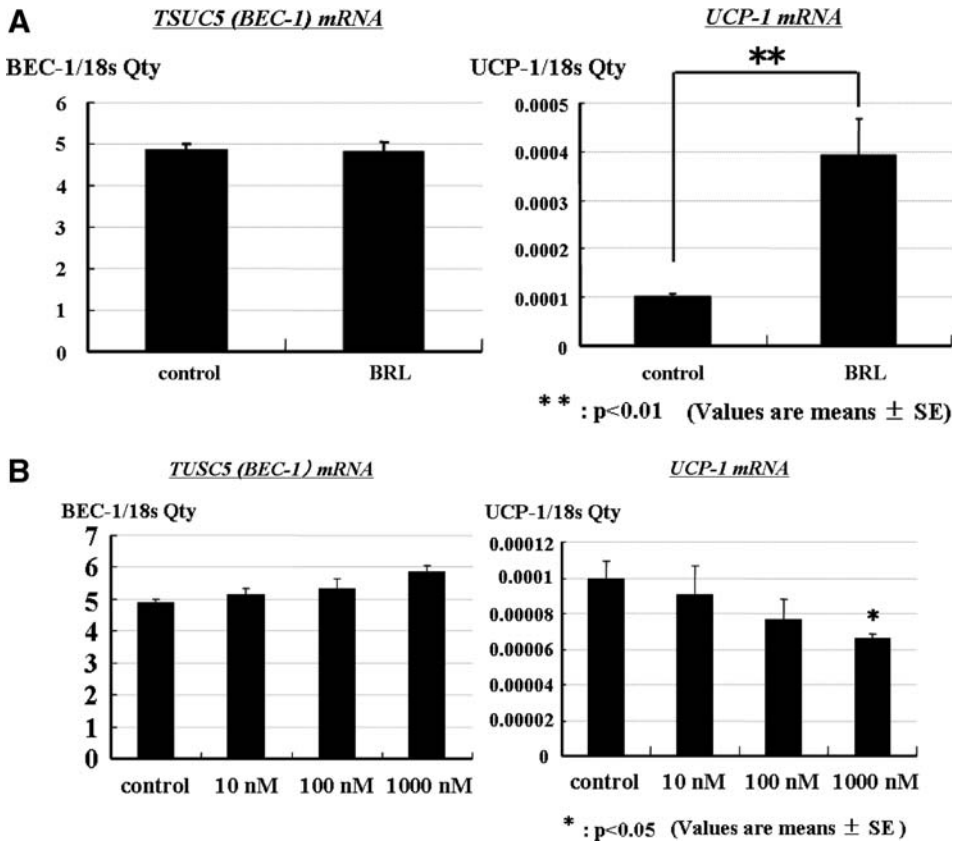

Fig. 2. Effects of β 3-adrenoceptor agonist and dexamethasone on mRNA expression of TUSC5 (BEC-1) and UCP-1 in primary cultured rat brown preadipocytes. RBPA at day 7 of culture were stimulated by BRL37344 (1 μM) and dexamethasone (10, 100, 1000 nM) for 3 h. (A) BRL37344, and (B) dexamethasone. The mRNA expressions of TUSC5 (BEC-1) and UCP-1 were analyzed by real-time RT-PCR in comparison with the expression of 18s rRNA as described in Materials and methods. Data represent means ± SE of three independent experiments. $^{**}p < 0.01$ vs. control, $^{*}p < 0.05$ vs. control.

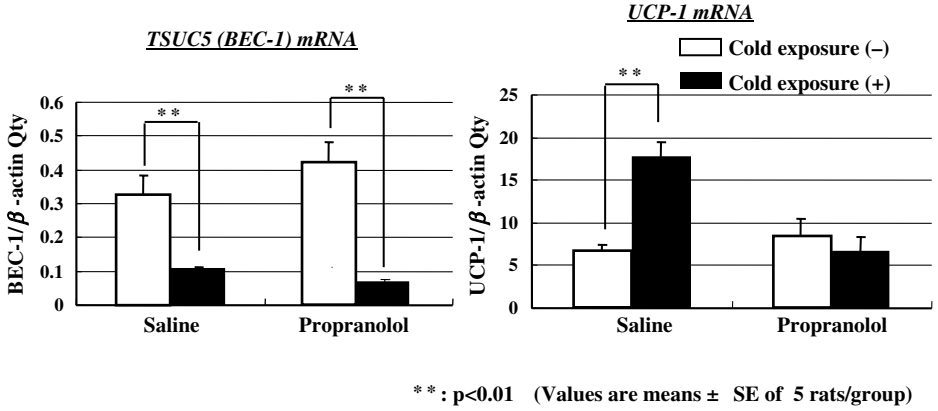

Fig. 3. Effects of propranolol on mRNA expressions of TUSC5 (BEC-1) and UCP-1 stimulated by cold exposure in BAT of ZL. Wild-type (+/+) Zucker lean rats (ZL) were injected intraperitoneally with propranolol (20 mg/kg body weight/day) or vehicle for 3 days, and then were exposed to 4 °C for 30 min after the final injection for 3 h. TUSC5 (BEC-1) mRNA and UCP-1 mRNA were analyzed by real-time PCR in comparison with β-actin expression as described in Materials and methods. Values are means ± SE for five Zucker lean rats. $^{**}p < 0.01$ vs. cold exposure.

We also examined the effect of BRL 37344, a specific β3-adrenoceptor agonist, on the expression of TSUC5 (BEC-1) mRNA in BAT of ZL. BRL 37344 significantly increased the mRNA expression of UCP-1, but it did not have any effect on the mRNA of TSUC5 (BEC-1) (Fig. 4A). However, dexamethasone decreased UCP-1 mRNA and TSUC5 (BEC-1) mRNA in BAT in a dose-dependent manner in ZL (Fig. 4B).

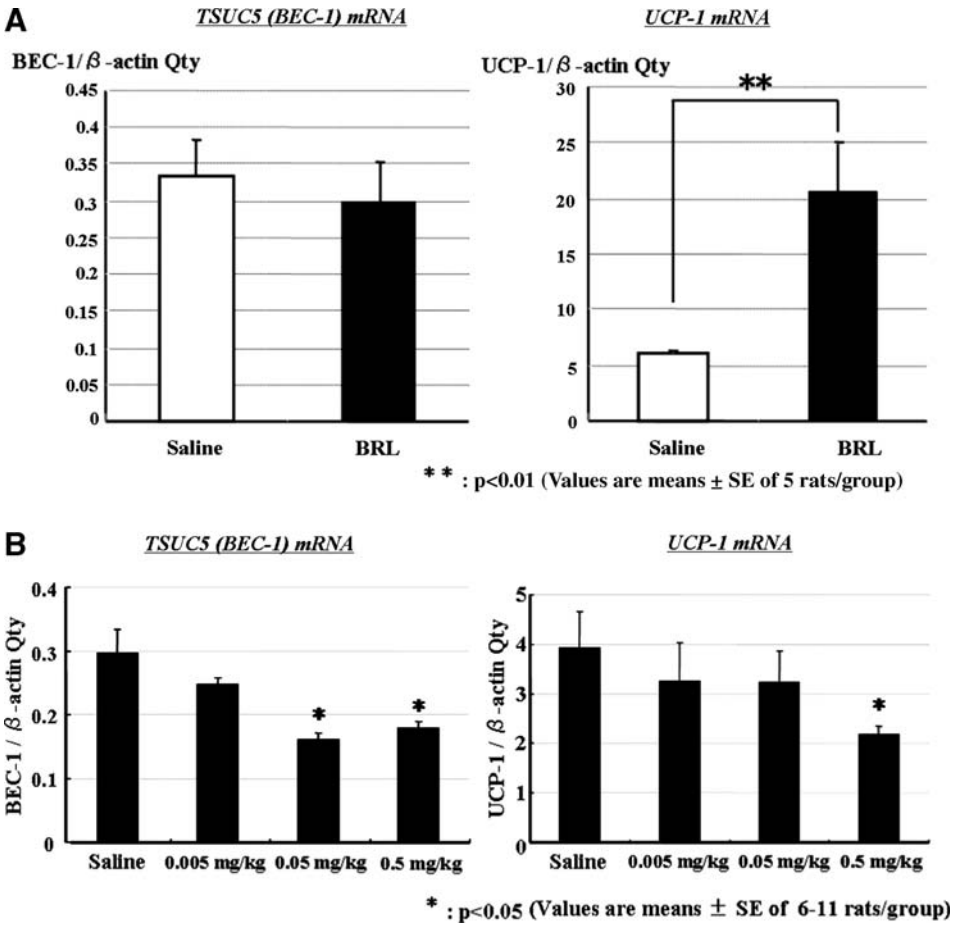

Fig. 4. Effects of β3-adrenoceptor agonist and dexamethasone on mRNA expressions of TUSC5 (BEC-1) and UCP-1 in BAT of ZL. (A) Wild-type (+/+) Zucker lean rats were injected intraperitoneally twice a day with BRL 37344 (0.5 mg/kg body weight) or saline vehicle for 3 days. (B) ZL were injected intraperitoneally with dexamethasone at doses of 0.5 mg, 0.05 mg and 0.005 mg/kg body weight. TUSC5 (BEC-1) mRNA and UCP-1 mRNA were analyzed by real-time PCR in comparison with β-actin expression as described in Materials and methods. Values are means ± SE for five Zucker lean rats. $^{**}p < 0.01$ vs. saline, $^{*}p < 0.05$ vs. saline.

Discussion

We reported that both TSUC5 (BEC-1) mRNA and protein were expressed abundantly in rat brown adipose tissue (BAT) and that the mRNA expression of TSUC5 (BEC-1) in BAT was significantly inhibited by cold exposure in ZL [1], indicating the significant roles of TUSC5 (BEC-1) in brown adipose tissues.

First, we examined TSUC5 (BEC-1) mRNA expression during the differentiation of brown preadipocytes. The hormonal induction for differentiation triggered the mRNA expression of C/EBPβ, followed by C/EBPα and PPARγ in the brown preadipocytes. It is known that the transcriptional factors of C/EBPα, β, and PPARγ are important players in adipocyte differentiation [17–19]. C/EBPα is a transcriptional activator of adipocyte genes and C/EBPβ is a transcriptional activator of the C/EBPα gene. In the present study, increasing expression of TSUC5 (BEC-1) mRNA was observed during the differentiation of RBPA. The expression of TSUC5 (BEC-1) was observed in a manner similar to that of adiponectin, and earlier than UCP-1, as lipid droplets accumulated in the cells. It was suggested that BEC-1 might play a role in lipid droplet accumulation at the later period of differentiation in brown adipocytes.

Significant roles of the sympathetic nervous system have been demonstrated in BAT. Mice lacking the gene for dopamine β-hydroxylase (*dbh*−/− mice) are cold-intolerant because they are unable to induce thermogenesis in BAT through UCP-1 [11]. The induction of UCP-1 by cold exposure was dependent on the β-adrenergic pathway, since it was abolished by the presence of β-adrenergic antagonist [12], and the stimulation of β-adrenergic receptor increased UCP-1 mRNA in BAT [13–15]. In order to gain further insights into the regulatory mechanism of TSUC5 (BEC-1), we examined the role of the β-adrenergic pathway in regulating the expression of TSUC5 (BEC-1) mRNA in cultured rat brown adipocytes. As expected, BRL37344, a β3-adrenoceptor agonist, stimulated the expression of UCP1 mRNA in the primary cultured rat brown adipocytes as reported previously [20]. However, the expression of TUSC5 (BEC-1) mRNA was not affected by BRL37344.

Then, to further understand the regulatory mechanism of TSUC5 (BEC-1), we examined the role of the β-adrenergic pathway using an in vivo model of ZL with cold expo-

sure. Although the cold-induced increment in UCP-1 mRNA of BAT was abolished by propranolol, it did not influence the decrement of BEC-1 mRNA caused by cold exposure in ZL. In addition, the specific β3-adrenoceptor agonist BRL 37344 increased the expression of UCP-1 mRNA as reported [14], but it had no effect on the expression of TUSC5 (BEC-1) mRNA, a result entirely consistent with the data from the primary cultured brown adipocytes, which indicated that the regulation of TUSC5 (BEC-1) mRNA expression in BAT is mediated via β-adrenergic stimulation-independent pathway in vivo and in vitro.

The question to be answered then is what system is involved in the regulation of BEC-1 mRNA by cold exposure? Interestingly, it was reported that cold exposure elevates plasma corticosterone in rats [21,22]. Although the action of corticosterone for BAT under cold exposure has not been completely clarified, RU-486, an antagonist of glucocorticoid receptor, was reported to stimulate oxygen consumption with the increment of thermogenesis in BAT. Such thermogenic effects appear to be due to central stimulation of sympathetic outflow and may involve CRF release [23,24], and a single intracerebroventricular (i.c.v.) injection of dexamethasone decreased GDP binding to BAT mitochondria in adrectomized ob/ob mice in a dose-dependent manner [25], indicating the importance of the pituitary–adrenal axis in the regulation of thermogenesis in BAT.

In the present study, a single i.p. injection of dexamethasone decreased UCP-1 mRNA of BAT in ZL in a dose-dependent manner as reported previously [26]. Glucocorticoids were reported to inhibit UCP-1 gene transcription in brown adipocytes in vitro [27]. However, there was an apparent discrepancy in UCP-1, as cold exposure stimulated, not suppressed, the mRNA expression of UCP-1. Because the mRNA expression of UCP-1 was up-regulated by the β-adrenergic pathway that was stimulated by cold exposure, its glucocorticoid-induced suppression was overcome by the β-adrenergic-dependent increase during cold exposure. Interestingly, the mRNA expression of TUSC5 (BEC-1) was suppressed by dexamethasone in a dose-dependent manner in vivo, but not in RAPA. Thus, glucocorticoid suppressed the mRNA expression of TUSC5 (BEC-1) in BAT of ZL in an indirect manner. From these findings, it is clear that further studies will be needed, focusing on its regulation and role in adipose tissues.

In summary, we demonstrated that the decreasing expression of BEC-1 mRNA by cold exposure was regulated through a β-adrenoceptor-independent pathway, and that glucocorticoid inhibited BEC-1 expression in BAT. These findings indicate that BEC-1 expression in BAT might be involved in stress response, and that BEC-1 mRNA decrement for dexamethasone might be necessary for other factors in vivo.

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