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作成者（著者）	Nakamura, Shoko / Jiang, Meizi / Oka, Rena / Yamaguchi, Takashi / Hiruta, Nobuyuki / Ebinuma, Hiroyuki / Schneider, J, Wolfgang / Tatsuno, Ichiro / Bujo, Hideaki
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Original Article

LR11, an LDL Receptor Gene Superfamily Member, Represses the Norepinephrine-Induced Expression of Uncoupling Protein 1 in Primary Cultured Beige Adipocytes

Shoko Nakamura[1, 2] Meizi Jiang[3, 7] Rena Oka[2]
Takashi Yamaguchi[2] Nobuyuki Hiruta[3, 4] Hiroyuki Ebinuma[5]
Wolfgang J Schneider[6] Ichiro Tatsuno[2] and Hideaki Bujo[3, 7]*

[1]Department of Diabetes, Endocrinology and Metabolism, Toho University Graduate School of Medicine, Tokyo, Japan
[2]Center for Diabetes, Endocrinology and Metabolism, Toho University Sakura Medical Center, Sakura, Japan
[3]Department of Clinical-Laboratory and Experimental-Research Medicine, Toho University Sakura Medical Center, Sakura, Japan
[4]Department of Surgical Pathology, Toho University Sakura Medical Center, Sakura, Japan
[5]Tsukuba Research Institute, Sekisui Medical Co Ltd, Ryugasaki, Japan
[6]Department of Medical Biochemistry, Max Perutz Labs, Medical University of Vienna, Vienna, Austria
[7]Department of Genome Research and Clinical Application, Chiba University Graduate School of Medicine, Chiba, Japan

ABSTRACT

Introduction: The soluble form of LR11 (sLR11) inhibits thermogenesis via BMPs/TGFβ signaling pathways in adipocytes. Mice lacking LR11 are protected from diet-induced obesity associated with increased browning of subcutaneous white adipose tissue (WAT) and hypermetabolism. However, the mechanism underlying the recruitment of beige adipocytes has not been elucidated. In this paper, we have investigated the expression of LR11 in the differentiation and norepinephrine-induced activation of cultured beige adipocytes.

Methods: LR11 mRNA levels were analyzed in cultured beige adipocytes differentiated from human immortalized adipose-derived stromal cells or from the subcutaneous WAT of wild-type (WT) and *Lr11*$^{-/-}$ mice. The concentrations of sLR11 were measured by an ELISA specific for murine sLR11.

*Corresponding Author: Hideaki Bujo, 564-1 Shimoshizu, Sakura 285-8741, Japan, tel: +81 43 462 8811
e-mail: hideaki.bujo@med.toho-u.ac.jp
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Results: In the course of browning adipogenesis of human stromal cells, LR11 mRNA levels decreased at the beginning and subsequently increased together with uncoupling protein (UCP) 1, β3-adrenergic receptor, lipoprotein lipase, and adiponectin. The LR11 transcript levels in the differentiated cells were transiently decreased by incubation with norepinephrine, and this change was in clear contrast to the increase of UCP1 mRNA levels. The mRNA levels of UCP1 in murine *Lr11*$^{-/-}$ beige adipocytes were significantly increased compared with those in WT beige adipocytes, regardless of norepinephrine stimulation. Finally, the amounts of sLR11 released from murine WT beige adipocytes were decreased by incubation with norepinephrine.

Conclusions: LR11 potentially represses the norepinephrine-induced expression of UCP1 in differentiated beige adipocytes. The sLR11-mediated repression of the key molecule in thermogenesis may be involved in the sympathetic activation of the recruitment of beige adipocytes in subcutaneous fat.

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KEYWORDS: LR11, adipogenesis, thermogenesis, UCP1, beige

Introduction

The existence of brown adipose tissue (BAT) in adult humans has been reconfirmed using modern imaging techniques and tissue biopsies,[1–3] emphasizing the importance of understanding the pathophysiological significance of the energy-exhausting brown adipocytes as players in the homeostasis of systemic metabolism. Indeed, the levels of detectable BAT have been shown to negatively correlate with age and body mass index (BMI) and to be decreased in patients with type 2 diabetes.[4] Brown adipocyte-like cells, so-called "beige" or "brite" adipocytes, appear in white adipose tissue (WAT) depots under conditions of high thermogenic activity of the mammalian body.[5] These cells share a thermogenic role with "classical" brown adipocytes in BAT depots via the expression of uncoupling protein 1 (UCP1) and possibly other additional mechanisms.[6, 7] Although the mechanism of browning of WAT, or beige adipocyte recruitment, remains elusive, at least three models have been proposed, namely, de novo beige adipogenesis, white-to-beige adipocyte trans-differentiation, and activation of dormant beige adipocytes.[8–10] Although the regulation of browning in response to exposure to a cold environment has not been fully elucidated, sympathetic nervous system activation is a main inducer for this process as well as for the thermogenic activity of BAT.[8–10]

Low-density lipoprotein (LDL) receptor relative with 11 ligand-binding repeats (LR11, also called SorLA or SORL1), an unusually complex and highly conserved member of the family of LDL receptor relatives, has been discovered and molecularly characterized by us and others.[11–13] LR11 is highly expressed in adipose tissues, as well as in brain, kidney and atherosclerotic plaques.[11–14] The shed soluble form of the receptor (sLR11) interacts with the receptors for bone morphogenic proteins (BMPs) on the surface of adipocytes.[15] Thus, treatment of adipocytes with sLR11 inhibits thermogenesis via the BMPs/transforming growth factor beta (TGFβ) signaling pathways in adipocytes, and mice lacking LR11 are protected from diet-induced obesity with an increased browning of subcutaneous WAT and hypermetabolism.[15] Using a sandwich enzyme-linked immunosorbent assay (ELISA) for the exact quantitation of sLR11 in the circulation,[16] sLR11 levels in obese subjects were shown to positively correlate with BMI and adiposity.[15] It could also be demonstrated that the plasma concentrations of sLR11 were increased in patients with type 2 diabetes[15, 17, 18] and/or in relation to markers of glycemic disturbances.[19–21]

Based on the knowledge generated by the above described cell biological, animal, and human studies, the levels of LR11-derived sLR11 have been suggested to regulate the conditions or degrees of beige adipocytes in WAT depots, as a soluble autocrine repressor and/or a released indicator, particularly in association with the pathology of patients with type 2 diabetes.[22, 23] Here, we have investigated the expression of LR11, together with the key genes for thermogenesis, in the differentiation and the norepinephrine (NE)-induced activation of cultured beige adipocytes.[5, 24, 25] Subsequently, the significance of LR11 gene expression for NE-induced UCP1 expression was examined using primary adipocytes from the subcutaneous WAT of *Lr11*$^{-/-}$ mice.[15]

Methods

Culture of immortalized human adipose-derived stromal cells

Immortalized human adipose-derived stromal cells, Bmi-1/hTERT (T0540; Applied Biological Materials, Richmond, Canada) were seeded in 6-well plates (20,000 cells/well) and cultured using Preadipocyte Growth Medium Kit (Basal Medium with Growth Supplements, Cell Applications, San Diego, CA) in a humidified incubator at 37°C and 5% CO_2. The cells were differentiated using PGM-2™ Preadipocyte Growth Medium-2 Bulletkit™ (PGM-2™ Basal Medium with PGM-2™ SingleQuots™ Supplement and Growth Factor Pack, Lonza, Walkersville, MD) with 1 nM 3,3',5-triiodo-thyronine (T_3; Sigma-Aldrich, Tokyo, Japan) at semi-confluent conditions for 14 days.[5, 24, 25] The degrees of differentiation were validated by morphological appearance with multiple lipid droplets in the cells, and the wells containing more than 90% fully differentiated cells were used for further analysis. mRNA was extracted from the differentiating cells on the indicated days. On day 14, the cells were incubated with or without 1 μM norepinephrine L-bitartrate hydrate (392480-1 G, Sigma-Aldrich, Tokyo, Japan) for the indicated times, and the total RNA was extracted from the cells.

Culture of murine primary adipocytes

Murine primary adipocytes were prepared using frozen preadipocytes from the subcutaneous WAT of wild-type (WT) mice or *Lr11*$^{-/-}$ mice,[15] seeded in 6-well plates (20,000 cells/well), and differentiated to brown/beige adipocytes in Dulbecco's Modified Eagle Medium (DMEM; Wako, Osaka, Japan), containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Tokyo, Japan) and 850 nM insulin (Wako) and 1 nM T_3 for 3 days.[15] The cells were further incubated in DMEM containing 10% fetal bovine serum, 1 μM dexamethasone (Wako), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; BioVision, Milpitas, CA), 125 μM indomethacin, 850 nM insulin, and 1 nM T_3 for 2 days, accompanied by subsequent incubation in DMEM containing 10% fetal bovine serum, with 850 nM insulin and 1 nM T_3 for 5 days.[15] The degrees of differentiation were validated by morphological appearance with multiple lipid droplets in the cells, and the wells containing more than 90% fully differentiated cells were used for further analysis. The differentiated cells were incubated for 8 h in DMEM with or without 100 nM norepinephrine L-bitartrate hydrate, and the incubation media were subsequently collected for the measurement of sLR11. The cells were also incubated for 8 h in DMEM containing 10% fetal bovine serum with or without 100 nM NE, and the total RNA was extracted from the cells. All animal procedures were approved by the Special Committee on Animal Welfare, School of Medicine, at the Inohana Campus of Chiba University.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR experiments were performed as described previously.[15, 25] Total RNA was prepared from cultured cells using the Maxwell® 16 LEV Simply RNA purification kit (Promega, Tokyo, Japan) and immediately quantified using the Quantus™ fluorometer (Promega). The reverse transcription reaction was performed with the Affinity Script qPCR cDNA Synthesis Kit (Agilent Technologies Japan, Tokyo, Japan). The target fragments of the cDNA samples were amplified by StepOnePlus™ (Applied Biosystems, Yokohama, Japan) using TaqMan gene expression assay with TaqMan Fast Advanced Master Mix (Applied Biosystems) with the combinations of primers and probes for LR11 (Hs00983770_m1), bone morphogenetic protein 8b (BMP8b; Hs01629120_s1), UCP1 (Hs01084772_m1 or Mm01244861_m1), β3-adrenergic receptor (β3-AR; Hs00609046_m1), lipoprotein lipase (LPL; Hs00173425_m1), adiponectin (Hs00605917_m1), or 18S RNA (1811330). Samples obtained from cells at 0 day or 0 h were used as calibrator to allow comparison of relative mRNA levels in the assay, except where specific descriptions are provided in figure legends.

Measurement of sLR11

A murine soluble LR11 ELISA was performed using two mouse monoclonal antibodies, Mab93222 and Mab 93213, which were established by immunization of *Lr11*$^{-/-}$ mice using synthetic peptides against different epitopes of VPS10p domain of LR11 protein,[26] as reported previously.[16] Briefly, 2.5 μl of incubated medium was diluted 20-fold with 1% BSA in PBST, reacted with the capture Mab 93222 for 2 h, and incubated with the biotinylated Mab 93213 for 1 h. The LR11-antibody complex was quantified with horseradish peroxidase-conjugated streptavidin using purified human and rabbit LR11 protein as a standard.

Statistics

Statistical analysis was performed with SPSS Statistics version 26 (IBM, Chicago, IL). Results are presented as mean ± standard deviations and analyzed using the Friedman test followed by the Bonferroni test or the unpaired t-test. The significance level was set at a p-value < 0.05.

Results

LR11 mRNA levels decreased at the beginning of, and then increased during, browning adipogenesis

We investigated LR11 mRNA levels, together with those of BMP 8b, UCP-1, β3-adrenergic receptor (AR), lipoprotein lipase (LPL), and adiponectin, as key molecules for the functions of thermogenesis and adipogenesis,[8–10] during differentiation after browning stimulation to beige adipocytes, in immortalized human adipose-derived stromal cells, Bmi-1/hTERT (Fig. 1A-F). The expression levels of LR11 tended to decrease for 2 days after stimulation, which was sustained at days 4 and 6, and then gradually increased during days 8, 10, 12, and 14 (Fig. 1A). The BMP8b mRNA levels also showed a tendency for decrease during differentiation, although less than that of LR11, and in fact showed no significant subsequent increase (Fig. 1B). In clear contrast, the mRNA levels of UCP1 (Fig. 1C), β3-AR (Fig. 1D), LPL (Fig. 1E), and adiponectin (Fig. 1F) gradually increased after the stimulation for 12 or 14 days. These results indicated that LR11 mRNA levels decreased at the beginning and then increased, together with UCP1, β3-AR, LPL, and adiponectin, in the course of browning adipogenesis of human stromal cells.

The NE-stimulated transient suppression of LR11 mRNA levels is in clear contrast to the increase of UCP1 mRNA in differentiated beige adipocytes

Next, we examined the response of LR11 mRNA levels to the stimulation with norepinephrine (NE) of immortalized human adipose-derived stromal cells, Bmi-1/hTERT, as sympathetic nervous stimulation has been shown to dramatically induce the recruitment of beige adipocytes in WAT,[8–10, 27] and NE is the major signal from the sympathetic nervous system that stimulates thermogenesis.[28, 29] The LR11 mRNA levels of the differentiated beige adipocytes sharply decreased after 4 h and 6 h of incubation with NE, gradually recovered at 8 h, and then reached original levels at 24 h (note that the LR11 levels without NE at 4 h were 1.21 ± 0.38) (Fig. 2A). The BMP8b mRNA levels remained unchanged except for an increase at 6 h (Fig. 2B). UCP1 mRNA level increased 35-fold within 8 h and then returned to the basal level at 24 h (Fig. 2C). The β3-AR mRNA levels gradually decreased for 8 h and then recovered to the original levels after 24 h (Fig. 2D). These results indicated that the LR11 mRNA levels in the differentiated cells were transiently decreased by NE and that this change was in clear contrast to the increase in UCP1 mRNA levels.

Regardless of NE stimulation, the levels of UCP1 mRNA in murine *Lr11*$^{-/-}$ beige adipocytes were enhanced

In order to examine whether the changing levels of LR11 mRNA were interacting with those of UCP1 mRNA in the differentiated beige adipocytes, we evaluated the effects of incubation with NE on the levels of UCP1 mRNA in primary beige adipocytes prepared from the subcutaneous WAT of WT and *Lr11*$^{-/-}$ mice.[15] The levels of UCP1 mRNA increased 150-fold within 8 h of NE incubation in the beige adipocytes from both *Lr11*$^{-/-}$ and WT mice (Fig. 3). Notably, however, both the basal levels of UCP1 mRNA and those after 8 h of NE stimulation were significantly higher in the beige adipocytes from *Lr11*$^{-/-}$ mice compared to those from WT mice (2.5-fold and 2.6-fold, respectively; Fig. 3). These results indicate that an increase in the LR11 mRNA levels possibly suppresses UCP1 mRNA levels in beige adipocytes regardless of NE stimulation.

The release of sLR11 from murine beige adipocytes is decreased by incubation with NE

Finally, we investigated the effects of NE on the release of sLR11 from primary beige adipocytes prepared from the subcutaneous WAT of WT mice,[15] because most of the translated LR11 protein is known to be shed and released from the cell surface.[30] The concentration of sLR11 in the collected medium after incubation with NE for 8 h was 5.7 ± 3.2 pg/ml, which was significantly less than in the medium from cells incubated without NE (10.2 ± 4.4 pg/ml, $P < 0.05$) (Fig. 4). Thus, the release of sLR11 into the medium of beige adipocytes was reduced by incubation of the cells with NE.

Discussion

We have recently reported that sLR11 inhibits thermogenesis via the BMPs/TGFβ signaling pathways in adipocytes, and mice lacking LR11 are protected from diet-induced obesity associated with the increased browning of subcutaneous WAT and hypermetabolism.[15] However, the mechanism underlying the recruitment of beige adipocytes observed in the WAT depots of *Lr11*$^{-/-}$ mice has not been elucidated. In adult mice, BAT mass is maintained at relatively stable levels by a homeostatic mechanism. In contrast, beige fat is barely detectable at thermoneutrality and is highly induced by cold exposure.[8–10, 31] Such distinct characteristics between brown and beige fat are believed to be caused in part by their completely different progeni-

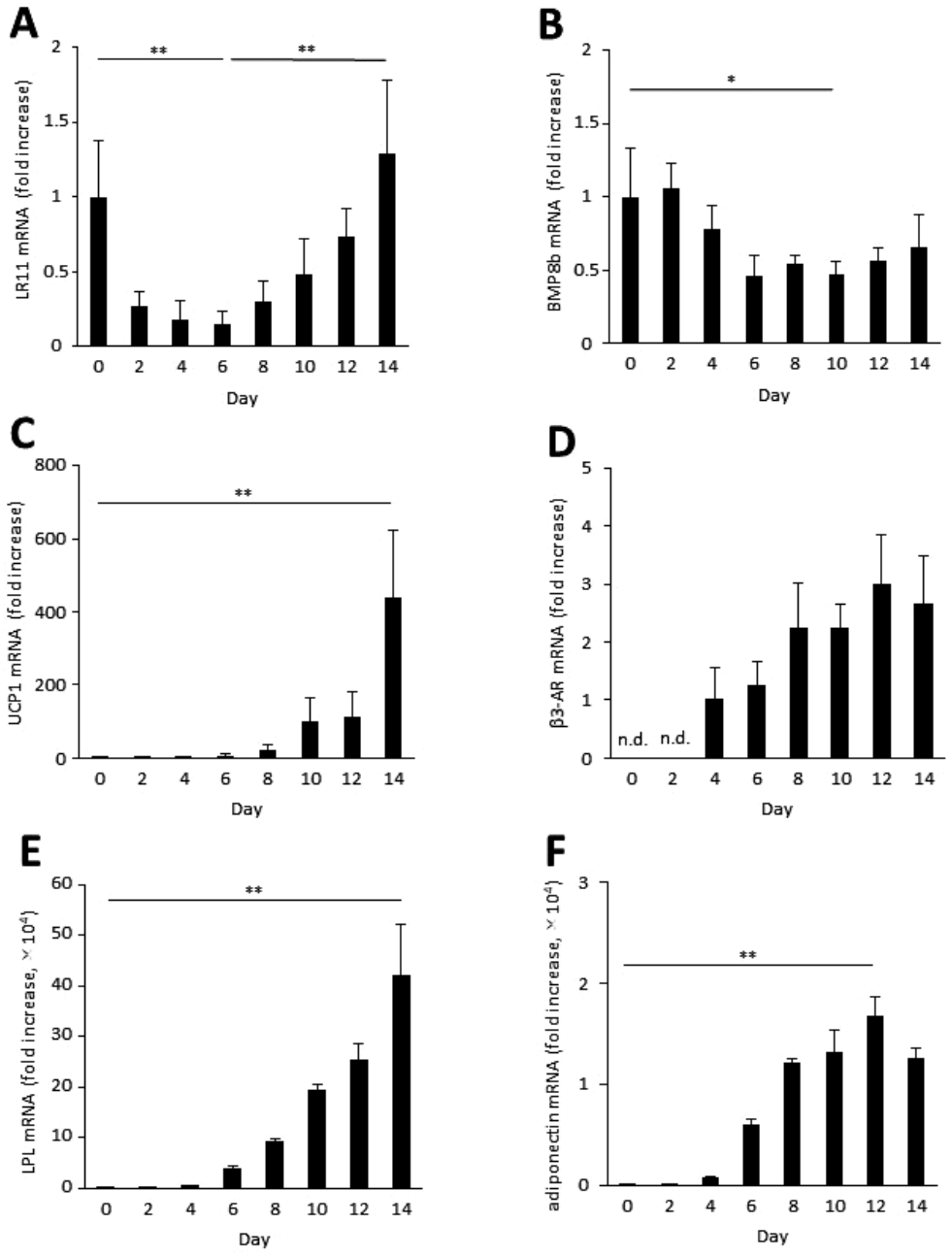

Fig. 1 mRNA levels for LR11 (A), BMP8b (B), UCP1 (C), β3-AR (D), LPL (E), and adiponectin (F), during the browning adipogenesis of human adipose-derived stromal cells. mRNA levels 0, 2, 4, 6, 8, 10, 12, and 14 days after incubation with browning adipogenesis stimulation were analyzed by reverse transcription polymerase chain reaction, as described in Methods. The mRNA levels were calculated as the number of fold increases of the levels on day 0 (A–C, E, and F) or day 4 (D) and represented as means with error bars representing SD (n = 3) for each day point. The statistical differences were analyzed using the Friedman test followed by the Bonferroni test. The figure is a representative of three independent experiments. *$p<0.05$. **$p<0.01$. n.s., not significant; n.d., not detectable. LR11, LDL receptor relative with 11 ligand-binding repeats; BMP8b, bone morphogenetic protein 8b; UCP1, uncoupling protein 1; β3-AR, β3-adrenergic receptor; LPL, lipoprotein lipase.

tor/stem cell origins.[8–10] BAT arises from a progenitor population common for BAT and skeletal muscle, and in adults, BAT homeostasis is maintained by proliferation and differentiation of the progenitors, which appear to re-

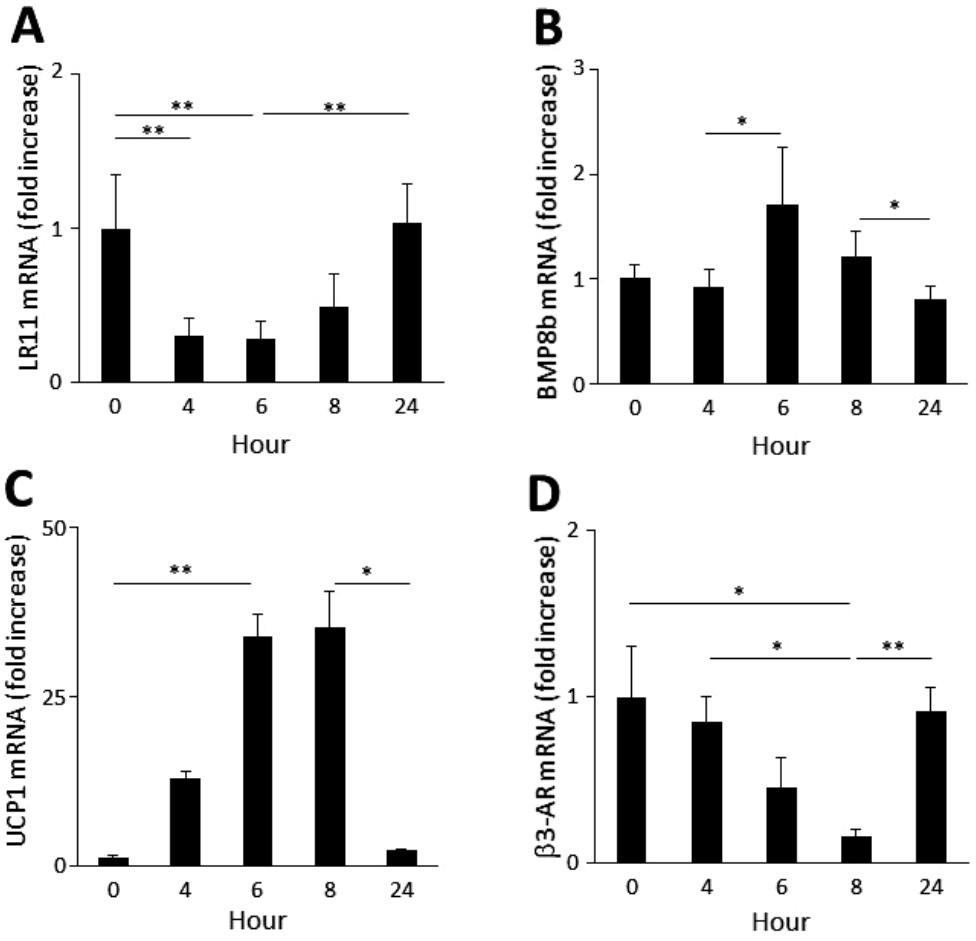

Fig. 2 mRNA levels for LR11 (A), BMP8b (B), UCP1 (C), and β3-AR (D) during the NE stimulation of human adipose-derived stromal cells. mRNA levels 0, 4, 6, 8, and 24 h after incubation with NE were analyzed by reverse transcription polymerase chain reaction, as described in Methods. The mRNA levels were calculated as the number of fold increases of the levels at 0 h and represented as means with error bars representing SD (n = 3) for each time point. The statistical differences were analyzed using the Friedman test followed by the Bonferroni test. The figure is a representative of three independent experiments. *$p < 0.05$. **$p < 0.01$. n.s., not significant. LR11, LDL receptor relative with 11 ligand-binding repeats; BMP8b, bone morphogenetic protein 8b; UCP1, uncoupling protein 1; β3-AR, β3-adrenergic receptor.

side at the dorsal edge of interscapular BAT.[9] On the other hand, beige adipocytes are believed to be heterogeneous populations with regard to their metabolic properties and gene expression profiles,[9] and beige adipocyte heterogeneity is influenced by the nature of its inducers, e.g., β3-AR and PPARγ agonists.[9] Thus, at least three models have been proposed for the mechanism of beige adipocyte recruitments or browning of WAT, namely, (i) de novo beige adipogenesis, (ii) white-to-beige adipocyte trans-differentiation, and (iii) activation of dormant beige adipocytes.[8–10]

In the present study, we have first investigated LR11 mRNA expression in immortalized human adipose-derived stromal cells, Bmi-1/hTERT, during the browning differentiation to "beige" adipocytes.[5, 24, 25] The LR11 mRNA levels were initially reduced upon stimulation for browning adipogenesis and then recovered together with an increase in mRNA levels of UCP1, β3-AR, LPL, and adiponectin in the course of browning adipogenesis of human stromal cells. Thus, the increased expression of LR11 together with other key genes for thermogenesis and adipogenesis supported a potential co-involvement of LR11 in de novo beige adipogenesis.

As a next distinct property of beige adipocytes, cold exposure and sympathetic nervous system activation have been shown to be main inducers of browning of

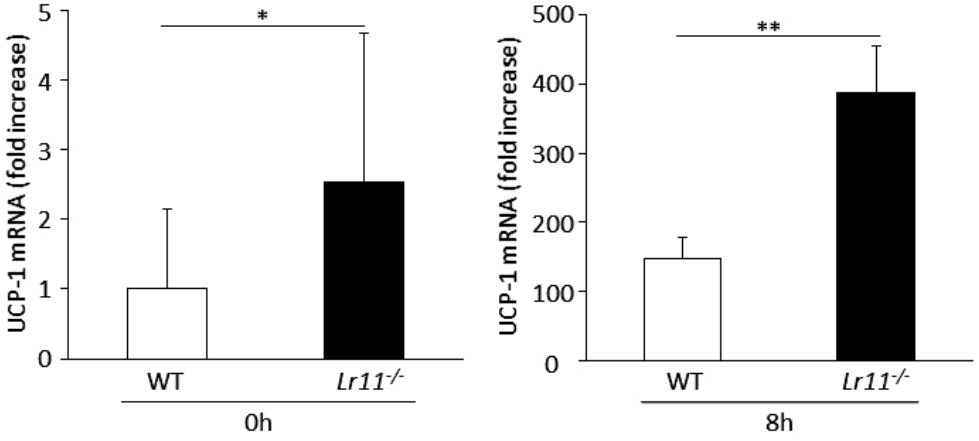

Fig. 3 mRNA levels for UCP1 before or after the NE stimulation in differentiated primary beige adipocytes prepared from the subcutaneous WAT of WT mice or *Lr11*$^{-/-}$ mice. mRNA levels 0 h (left panel) and 8 h (right panel) after incubation with NE were analyzed by reverse transcription polymerase chain reaction, as described in Methods. The mRNA levels were calculated as the number of fold increases of the levels of WT at 0 h and represented as means with error bars representing SD (n = 3) for each time point. Note the different scales for the different time points. The statistical differences were analyzed using the unpaired Student t-test. The figure is a representative of three independent experiments. *p < 0.05. **p < 0.01. LR11, LDL receptor relative with 11 ligand-binding repeats; UCP1, uncoupling protein 1.

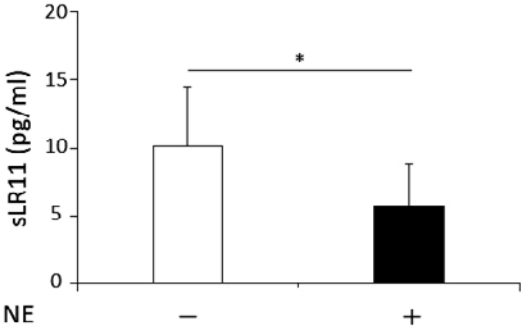

Fig. 4 Concentrations of sLR11 after the incubation with or without NE in the media of differentiated primary beige adipocytes prepared from the subcutaneous WAT of WT mice. The collected media after incubation with or without NE for 6 h were analyzed by ELISA, as described in Methods. The concentrations were represented as means with error bars representing SD (n = 6). The statistical differences were analyzed using the unpaired Student t-test. *p < 0.05. sLR11, soluble form of LDL receptor relative with 11 ligand-binding repeats; NE, norepinephrine.

WAT.[8–10, 27, 29] The differentiated beige adipocytes showed a sharp and transient reduction of LR11 gene expression at the beginning of incubation with NE (Fig. 2), with the pattern showing a clear contrast to the transient increase of UCP1 expression, consistent with the previous studies that have shown the patterns reflecting the downregulation of β3-AR with the recovery after 24 h of NE stimulation.[32–34] The subsequently examined comparison between beige adipocytes from WT mice and those from *Lr11*$^{-/-}$ mice suggests that LR11 suppresses the UCP-1 gene expressions regardless of NE stimulation (Fig. 3). Finally, the release of sLR11, which inhibits the expression levels of UCP-1 and other key genes involved in thermogenesis in adipocytes,[15] was also inhibited by the incubation with NE in the beige adipocytes (Fig. 4). Taken together, the findings of the present study using human and murine cells revealed that the beige adipocyte recruitment observed in the *Lr11*$^{-/-}$ mice[15] is likely caused by the lack of inhibition of de novo beige adipogenesis and the activation of dormant beige adipocytes by sympathetic nervous system activation.[34] Although the mechanism of NE-induced downregulation of LR11 is not fully elucidated, the strict regulation in response to the gene regulations of β3-AR and UCP-1 may suggest that LR11 is also regulated through the common signaling pathway as one of key molecules for thermogenesis. Finally, an interesting finding from the view of diabetes pathology is that LR11 expression suppresses the expression of UCP-1 regardless of NE stimulation in beige adipocytes (Fig. 3). Considering that the con-

centrations of sLR11 are increased in patients with type 2 diabetes[15, 17, 18] and/or in relation to markers of glycemic disturbances,[19–21] the poor sensitivity of diabetic beige adipocytes to NE-mediated induction of key thermogenesis genes may cause the decreased energy expenditure in response to cold exposure, or to sympathetic nervous system activation, in patients. Further human studies on the correlations of circulating thermogenesis repressor levels with the extent and activities of beige fat are needed to increase our knowledge about the role of sLR11 as a soluble autocrine repressor and/or a released indicator for beige fat.

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Authors' contributions: SN, MJ, WJS, and HB are responsible for the work described in this paper. SN, MJ, RO, IT, and HB were involved in the conception, design, and planning of the study. SN, MJ, HE, and HB were involved in the analysis of data. SN, MJ, TY, NH, WJS, IT, and HB were involved in the interpretation of results. SN, MJ, WJS, IT, and HB substantially contributed to the drafting of the manuscript.

Ethics approval and consent to participate: This study does not include human or animal study.

Conflicts of interest: SN, MJ, RO, TY, NH, WJS, IT, and HB declare no relevant conflict of interest. HE is an employee of Sekisui Medical Co., Ltd.

Availability of data and materials: All data generated or analyzed during this study are included in this published article.

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