Hypothalamic TTF-1 orchestrates the sensitivity of leptin

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ABSTRACT

Objective: Thyroid transcription factor-1 (TTF-1), a homeodomain-containing transcription factor, is predominantly expressed in discrete areas of the hypothalamus, which acts as the central unit for the regulation of whole-body energy homeostasis. Current study designed to identify the roles of TTF-1 on the responsiveness of the hypothalamic circuit activity to circulating leptin and the development of obesity linked to the insensitivity of leptin.

Methods: We generated conditional knock-out mice by crossing TTF-1fl/fl mice with leptin receptor (ObRb)Cre or proopiomelanocortin (POMC)Cre transgenic mice to interrogate the contributions of TTF-1 in leptin signaling and activity. Changes of food intake, body weight and energy expenditure were evaluated in standard or high fat diet-treated transgenic mice by using an indirect calorimetry instrument. Molecular mechanism was elucidated with immunohistochemistry, immunoblotting, quantitative PCR, and promoter assays.

Results: The selective deletion of TTF-1 gene expression in cells expressing the ObRb or POMC enhanced the anorexigenic effects of leptin as well as the leptin-induced phosphorylation of STAT3. We further determined that TTF-1 inhibited the transcriptional activity of the ObRb gene. In line with these findings, the selective deletion of the TTF-1 gene in ObRb-positive cells led to protective effects against diet-induced obesity via the amelioration of leptin resistance.

Conclusions: Collectively, these results suggest that hypothalamic TTF-1 participates in the development of obesity as a molecular component involved in the regulation of cellular leptin signaling and activity. Thus, TTF-1 may represent a therapeutic target for the treatment, prevention, and control of obesity.

Keywords Thyroid transcription factor-1; Leptin receptor; Leptin sensitivity; Leptin resistance; Obesity

1. INTRODUCTION

Obesity is a predisposing condition for several serious metabolic syndromes, including type 2 diabetes and various cardiovascular diseases, and is highly associated with complex abnormalities originating from disturbance of the delicate balance between caloric excess and body weight homeostasis [1]. The hypothalamus is the central unit that orchestrates homeostatic controls for feeding, physical activity, and energy expenditure by mediating the afferent inputs derived from metabolically active peripheral organs such as the adipose tissues, pancreas, and intestines [2]. In particular, leptin, an adipocyte-derived hormone, triggers catabolic responses by targeting hypothalamic neurons expressing leptin receptors [3]. Since the circulating level of leptin is proportional to the level of adiposity, leptin directly participates in the operation of a negative feedback loop to maintain energy balance in the hypothalamic neurons that control energy intake and expenditure [4]. Leptin action in the hypothalamic neurons mainly includes the regulation of two opposing neuronal populations: the inhibition of orexigenic agouti-related peptide (AgRP) neurons and the stimulation of anorexigenic proopiomelanocortin (POMC) neurons [5]. Over the last two decades, intensive studies have attempted to determine the usefulness of leptin in treating obesity. However, thus far, the application of leptin has not been effective in improving obesity. A major pathological event in the unsuccessful therapeutic usefulness of leptin is a perturbed responsiveness of the target cells to leptin.

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known as leptin resistance. A great deal of attention has been paid to unmasking the cellular and molecular causes in the development of leptin resistance. Nonetheless, strategies that have tried to mitigate leptin insensitivity have so far been unsuccessful.

Thyroid transcription factor-1 (TTF-1) is a transcription factor first found in the thyroid gland and is also known as homeodomain-containing transcription factor Nkx2.1. TTF-1 plays a critical role in the development of the diencephalic brain regions. Mice carrying the null mutation of the TTF-1 gene are stillborn due to lung failure, along with severe malformation or absence of some hypothalamic structures such as the ventral third ventricle and periventricular nuclei. In line with this notion, we have found the presence of TTF-1 expression in several discrete hypothalamic nuclei, where it is involved in neural or neuroendocrine physiological mechanisms important in the control of body homeostasis. Intriguingly, a previous study has reported that the brain-specific homeobox transcription factor (Bsx) was functionally linked to the whole-body energy metabolism. In addition, our previous studies identified physiological roles of TTF-1 in the regulation of energy homeostasis. Notably, TTF-1 was strongly expressed in the arcuate nucleus (ARC), where it stimulated the transcription of AgRP but inhibited the transcription of POMC. The resulting intensive appetite-driving effect of TTF-1 opposes the well-known function of leptin. These previous results implicate the potential involvement of TTF-1 in leptin signaling pathways for the regulation of body energy homeostasis in the hypothalamic ARC.

In the present study, using transgenic mice harboring selective TTF-1 deletion in the cells expressing the long-form leptin receptor ObRb or in the POMC cells, we aim to provide experimental evidence suggesting that TTF-1 is a crucial molecular component involved in the leptin signaling pathway for the regulation of whole-body energy homeostasis and is involved in the responsiveness and resistance to leptin in obesity pathogenesis.

2. MATERIALS AND METHODS

2.1. Animals

All animals were housed individually in a temperature-controlled room with a 12-hour light–dark cycle and had ad libitum access to a standard chow diet (Feedlab, Gyeonggi-Do, Korea) and water unless otherwise stated. Transgenic mice (POMC-EGFP [stock no. 009593], ObRb-Cre [stock no. 008320], POMC-Cre [stock no. 005965] and Ai14-Cre [stock no. 007914]) were obtained from Jackson Laboratory (Bar Harbor, ME, USA). TTF-1f/f mice were crossbred with ObRb-Cre or POMC-Cre mice to generate TTF-1 conditional knockout mice specifically in the cells expressing ObRb or POMC. The resulting mouse lines that bore the TTF-1 deletion in the POMC cells (POMC-TTF-1−/−) and in the cells expressing ObRb (ObRb-TTF-1−/−) were first subjected to IHC observations to identify the specific deletion of TTF-1 in each cell (Supplementary Figures 2 and 3). All animals and procedures used were in accordance with the guidelines and approval of the Institutional Animal Care and Use Committee at the University of Ulsan (permission numbers: BJL-14-050, BJL-14-052 and BJL-17-030).

2.2. Leptin administration and food intake assay

Animals were intraperitoneally injected with saline or recombinant mouse leptin (0.5, 1 or 3 mg/kg; Q544U0, R&D Systems, Minneapolis, MN, USA) after overnight fasting. Food intake of the individually caged animals was monitored for 2–24 h after injection. Their body weights were measured 24 h after administration of leptin.

2.3. Immunohistochemistry

Mice were first anesthetized with isoflurane, followed by transcardiac perfusion with 0.1 M phosphate buffer (PB, pH 7.4) and 3% paraformaldehyde in PB. Brains were dissected and incubated in 3% paraformaldehyde overnight at 4 °C. Coronal sections (thickness, 50 μm) were prepared using a vibratome (5100mz, Campden Instruments Ltd., Leicestershire, UK). Coronal sections containing the hypothalamic arcuate nucleus were preincubated with 0.2% Triton X-100 (78787, Sigma-Aldrich, St. Louis, MO, USA) in PB for 30 min at room temperature (RT). After further washing with PB, the sections were incubated overnight at 4 °C with rabbit anti-TTF-1 (1:1000, SC-13040, Santa Cruz Biotechnology, Dallas, Texas, USA), sheep anti-α-MSH (1:10,000, ABS087, Millipore, Darmstadt, Germany), rabbit anti-POMC (1:1000, H-029-30, Phoenix Pharmaceuticals, Burlingame, CA, USA) and rabbit anti-c-Fos (1:1000, SC-52, Santa Cruz Biotechnology). For pSTAT3 staining, sections were incubated with 0.01 mol/L citrate buffer for 10 min at 80 °C and washed in PB at RT. The sections were then incubated with 2 N HCl for 30 min and incubated with 0.2% Triton X-100 in PB for 30 min at RT. Afterward, sections were incubated with rabbit anti-pSTAT3 (1:1000, #9148, Cell Signaling Technology, Danvers, MA, USA) overnight at RT. The next day, the sections were washed in PB for 30 min and incubated with the following secondary antibodies at RT: goat anti-rabbit Alexa Fluor 488 (1:500, A11008, Invitrogen, Carlsbad, CA, USA), goat anti-rabbit Alexa Fluor 594 (1:500, A11012, Invitrogen), and donkey anti-sheep Alexa Fluor 594 (1:500, A21209, Invitrogen). For diaminobenzidine (DAB)-based IHC analysis to detect pSTAT3, the sections were washed thoroughly and incubated with biotinylated anti-rabbit secondary antibodies, Avidin–Biotin Complex (ABC) reagent (PK-6102, Vector Laboratories, Burlingame, CA, USA), and the DAB substrate (SK-4100, Vector Laboratories). The sections were then mounted onto glass slides and coverslips were then placed on these slides with a drop of mounting medium (Dako North America Inc., Carpinteria, CA, USA). The coverslips were sealed with nail polish to prevent the drying and movement of the samples under the microscope.

2.4. Microscopy and quantification

The stained brain sections were imaged using an Olympus FV-1200 laser scanning confocal microscope (Shinjuku, Tokyo, Japan) and a fluorescence microscope (Axioplan2 Imaging, Carl Zeiss MicroImaging Inc., Thornwood, NY, USA). All parameters were kept constant for quantification purposes. Measurements of fluorescence-positive cell counts and density were performed using ImageJ software v. 1.47 (National Institute of Health, Bethesda, MD, USA).

2.5. Electrophysiology

Whole-cell patch-clamp recordings were conducted in the POMC-EGFP neurons of hypothalamic slices. The brains were removed and immediately submerged in ice-cold, carbogen-saturated (95% O2, 5% CO2) sucrose solution containing 75 mM sucrose, 2.5 mM KCl, 7 mM MgCl2, 0.5 mM CaCl2, 1.3 mM NaH2PO4, 25 mM NaHCO3, and 25 mM glucose at pH 7.3. Hypothalamic coronal slices (300 μm) were made with a Leica VT1200 (Leica Biosystems, Wetzlar, Germany) vibratome in sucrose solution. The slices were then placed in a holding chamber filled with gassed artificial cerebrospinal fluid (ACSF) (95% O2, 5% CO2) that contained 125 mM NaCl, 2.5 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 1.3 mM NaH2PO4, 25 mM NaHCO3, and 25 mM glucose at pH 7.3. They were then incubated at RT for at least 1 h before recording. Slices were transferred to a recording chamber and bathed with continuously bubbled ACSF (30–32 °C) at a flow rate of ~3 ml/min. Whole-cell patch-clamp recordings were performed using pipettes
with 4–6 MΩ resistance after they were filled with internal solution. The recording pipettes were made of borosilicate glass (World Precision Instruments, Sarasota, FL, USA) using a PC-10 vertical puller (Narishige, Tokyo, Japan). The internal solution contained 135 mM potassium gluconate, 0.5 mM EGTA, 10 mM HEPES, 2 mM Mg-ATP, 0.3 mM NaN₂-GTP, and 10 mM phosphocreatine. POMC neurons in the arcuate nucleus were identified under fluorescence, and then, differential interference contrast was used to obtain a seal in these cells. After a gigahm seal was obtained, a gentle negative pressure was applied to break through to the whole-cell configuration. Leptin (10 nM) was added to ACSF and perfused for 5 min into the recording chamber. All recordings were performed with a Multiclamp 700 B amplifier (Molecular Devices, Sunnyvale, CA, USA). Data were collected with pCLAMP software (Molecular Devices). Mean firing activity and membrane potential were calculated using Clampfit (Axon Instruments Inc., Union City, CA, USA) software.

### 2.6. Measurements of O₂ consumption, CO₂ production, and respiratory exchange ratio

Metabolic parameters of O₂ consumption (VO₂), CO₂ production (VCO₂), and respiratory exchange ratio (RER) were analyzed with a Promethion Metabolic Measurement System (Sable System, Las Vegas, NV, USA) using control TTF-1−/− and ObRb-TTF-1+/− mice. The mice were fed a high-fat diet containing 60% calories from lard and 20% calories from sugar (D12492, Research Diets, New Brunswick, NJ, USA) for 16 weeks. Food and water were provided ad libitum. Mice were acclimated to the metabolic cages for 48 h prior to data collection under 12 h light/dark cycle conditions. VO₂ and VCO₂ were measured in each mouse at 10 min intervals. The RER was calculated as the ratio of VCO₂ to VO₂. Data acquisition and instrument control were coordinated by the MetaScreen software (version 2.3.12, Sable Systems), and the obtained raw data were processed using Expe-Data (version 1.9.14, Sable Systems).

### 2.7. Transfection and western blotting

Mouse hypothalamic mHypoA cells purchased from CELLutions Biosystems (CELLutions Biosystems Inc., Toronto, Ontario, Canada) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone) and 1% penicillin/streptomycin (Hyclone). The cells were maintained at 37 °C under 5% CO₂ conditions. The mHypoA cells were seeded at 3 × 10⁵ cells/well in 6-well plates and attached overnight. After starvation for 1 h, cells were transfected with a plKO.1-Puro vector (Sigma-Aldrich) carrying shRNA that targets the 30th to 50th nucleotides (5′-GTTCGTCATGCTGAATCTTT-3′) in the TTF-1 mRNA sequence [14] using Lipofectamine/PLUS (Invitrogen) for 4 h and were incubated in DMEM + 10% FBS for 24 h. Cells were then treated with leptin (500 ng/ml) and incubated for 30 min. Next, the cells were collected for total protein extraction. Extracted proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and were transferred to a polyvinylidene fluoride membrane (Millipore). Membranes were probed with primary antibodies against pSTAT3 (1:1000, #9145, Cell Signaling) and β-actin (1:1000, A5441, Sigma-Aldrich).

### 2.8. Transfection and quantitative real-time PCR

mHypoA-POMC cells (CELLutions) had grown to 70% confluence and were transiently transfected for 24 h with expression vectors (pcDNA3.1 containing the TTF-1 coding region or control pcDNA3) using jetPRIME® reagent (Polyplus, New York, NY, USA). The cells were then serum-starved (6 h) and treated with leptin (100 ng/ml) or vehicle (Tris–HCl). Mediobasal hypothalamic samples were collected from mice, flash-frozen in liquid nitrogen, and stored at −80 °C until analysis. Total RNA was extracted from the hypothalami and cultured mHypoA-POMC cells using Trizol Reagent (Invitrogen) according to the manufacturer’s protocol. First-strand cDNA was synthesized with 2 mg total RNA using a high-capacity cDNA reverse transcription kit (Intron Biotechnology, Seoul, Korea). Quantitative real-time PCR was performed using the SYBR Green Real-time PCR Master Mix Kit (Takara Bio Inc., Foster, CA, USA), and mRNA expression levels were measured using a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Data were normalized to the housekeeping gene β-actin. The primers used were as follows: AgRP forward: 5′-TGG AGA CCG AGC AGA AG-3′, reverse: 5′-ACT GGT GCA GCC TTA CAC AG-3′; POMC forward: 5′-TCC TAC TCC ATG AGC GAG TAC TTC C-3′, reverse: 5′-ACT GTG TCT CAG CAA CTG TG-3′; TTF-1 forward: 5′-GAT CTT GCT AGC GGC ATA GG-3′, reverse: 5′-AGC TGG AGG TGT TTG GTG CAT CT-3′; SOCS3 forward: 5′-GCT CAA AGA GCC CTA GAG G-3′, reverse: 5′-TGA CGC TCA AGC TGA AGA AGG-3′; PTP1B forward: 5′-GAC TGC TCA GTG CAG GAT CA-3′, reverse: 5′-CTG TCT TTC ATC CCC ACA GAT G-3′; ObRb forward: 5′-GCA TGC AGA ATA AGT ATT TGG-3′, reverse: 5′-CAA GCT GTA TCG ACA CTG ATG TTC TC-3′; β-actin forward: 5′-TAA AAC GCA GCT CAG TAA CAG TCC AAC AAA TAA C-3′. The ObRb primer was specifically designed to detect long-form leptin receptor [16].

### 2.9. Promoter assay

To determine whether TTF-1 regulates ObR transcription, mHypoA-POMC cells were co-transfected with an ObR promoter-luciferase reporter vector (500–1000 ng) and a TTF-1 expression vector (100–500 ng) using jetPRIME® (Polyplus). The 5′ flanking region of the mouse ObR gene (−1715 to +40 bp) that we used for this promoter analysis was cloned by PCR from mouse genomic DNA based on sequence information [17]. The 1755-nucleotide-long PCR product was inserted into a luciferase reporter plasmid (pGL4-promoter vector, Promega, Madison, WI, USA), and its sequence was confirmed by DNA sequencing. This 5′ flanking sequence of the mouse ObR gene includes 9 conserved core domains (CAAG) for the binding of TTF-1 [14]. Transfection efficiency was normalized by co-transfecting a Renilla reporter plasmid (pRL-SV40 vector; Promega) at 100 ng/well. Transfected cells were harvested 24 h after transfection, and luciferase activity was measured using a luciferase reporter assay system (Promega) according to the manufacturer’s protocols.

### 2.10. ChIP assay

mHypoA-POMC cells were transiently transfected using jetPRIME® reagent (Polyplus) with expression vector pcDNA3.1 containing the TTF-1 coding region or control pcDNA3.1. To determine the effect of leptin on TTF-1 binding to the ObR 5′ flanking region the cells were treated with leptin (100 ng/ml) for 24 h after serum starvation for 6 h. For cross-linking of the DNA and protein binding, cells were treated with formaldehyde to a final concentration of 1% and the cross-link reaction was stopped by adding glycerine to a final concentration of 125 mM. The cells were lysed with cell lysis buffer (5 mM PIPES (pH 8.0), 85 mM KCl, 0.5% NP40, and protease inhibitors) and nuclei were extracted and resuspended with nuclear lysis buffer (50 mM Tris, 10 mM EDTA, 1% SDS, and protease inhibitors). Chromatin was sheared by sonication; then, the mixture was diluted 5-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris, 134 mM NaCl and protease inhibitors). The reactions were incubated with 5 μg of rabbit anti-TTF-1 antibody (Santa Cruz Biotechnology) or normal rabbit IgG (Santa Cruz Biotechnology) at 4 °C.
for two days. Immune complexes were collected by reaction with 60 μl of protein G agarose Resin 4 B (Lugon Sci Inc., Gyeonggi-do, Korea) and then washed with washing buffer containing different concentrations of salts (150 mM—500 mM) and 0.25 M LiCl. DNA from the protein—DNA cross-links was extracted by incubating the reactions with extraction solution (1% SDS and 0.1 M NaHCO3), and formaldehyde cross-links were reversed by adding NaCl to a final concentration of 0.3 M and incubation at 65°C for 5 h. The samples were precipitated by treatment with 100% ethanol and incubated overnight at −20°C. DNA was purified using spin columns (Geneall Biotechnology, Seoul, Korea) and was eluted with 10 mM Tris. PCR amplification was performed using 35 cycles of 94°C for 30 s, 57–63°C for 30 s, and 72°C for 30 s, preceded by 94°C for 30 s and followed by 72°C for 10 min using the following primer sets: TTF-1 at −1567, forward, 5′-CCA GCC ATT TTT CTC TGC C-3′; TTF-1 at −1567, reverse, 5′-GAT CCA TCT CCC CCA TAC C-3′; TTF-1 at −1357, forward, 5′-CTC AGA ACC GAT AAG CTA GGT G-3′; TTF-1 at −1357, reverse, 5′-GTC CAA TGG ACA TGA AGC AC-3′; TTF-1 at −1238, forward, 5′-GTC TTT GAT GTC CAT TGG C-3′; TTF-1 at −1238, reverse, 5′-GGT TGG TGT TCT TTC-3′; TTF-1 at −1181/−1201, forward, 5′-GGG AGA ACA CCA ACC AGC-3′; TTF-1 at −1181/−1201, reverse, 5′-ACC TGT TAG TGG AGT TGT AC-3′; TTF-1 at −807, forward, 5′-GTC CTA CAG AGT GCC CGT-3′; TTF-1 at −807, reverse, 5′-GCA GAA TTA CCA GC-3′; TTF-1 at −728, forward, 5′-GGT CGT TAA GAG AGC TGG-3′; TTF-1 at −728, reverse, 5′-GGG ACC AAA GGG TTT ATC TC-3′; TTF-1 at −669, forward, 5′-GAT GAA TGG CTA AGT TGG ATT GTG TGT G-3′; TTF-1 at −669, reverse, 5′-GTC TGT GTG TGT GTG TGT G-3′.

2.11. Statistical analysis
The number of mice in each experimental group is indicated in the figure legends. All values are expressed as the mean ± standard error of the mean (SEM), and a two-tailed Student's t-test was used for pairwise comparisons. One-way and two-way analysis of variance (ANOVA) tests, followed by the Bonferroni post hoc test, were used to compare the data from more than two groups. Statistical evaluation was performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA).

3. RESULTS

3.1. Hypothalamic TTF-1 responds to leptin
Leptin, an adipose-derived hormone, regulates whole-body energy homeostasis via governing of the hypothalamic neurons expressing ObRb to reduce appetite and enhance energy expenditure [18–20]. To investigate whether TTF-1 is involved in leptin action on energy metabolism, we determined the presence of TTF-1 in ObRb-positive cells by performing immunohistochemistry (IHC) with an antibody against the TTF-1 protein in brain sections from a transgenic mouse line retaining reporter signals in ObRb-positive cells (ObRb cre:Ai14-ttdTomato). Double positive signals of TTF-1 and ObRb were found in the ARC, dorsomedial hypothalamic nucleus (DMH), and lateral hypothalamic area (LH), but not in the ventromedial hypothalamic nucleus (VMH) (Figure 1A). To verify whether hypothalamic TTF-1 responds to leptin, we next tested the number of TTF-1-positive cells in the hypothalamus of mice systemically administrated with leptin by performing IHC with an antibody against TTF-1 protein. Administration

Figure 1: Leptin regulates the hypothalamic expression of TTF-1. (A) Representative images show TTF-1 protein (green) in the ObRb-positive cells (red) in hypothalamic regions including the arcuate nucleus (ARC), dorsomedial hypothalamic nucleus (DMH), and lateral hypothalamic nucleus (LH), but not in the ventromedial hypothalamic nucleus (VMH), and was eluted with 10 mM Tris. PCR amplification was performed using 35 cycles of 94°C for 30 s, 57–63°C for 30 s, and 72°C for 30 s, preceded by 94°C for 30 s and followed by 72°C for 10 min using the following primer sets: TTF-1 at −1567, forward, 5′-CCA GCC ATT TTT CTC TGC C-3′; TTF-1 at −1567, reverse, 5′-GAT CCA TCT CCC CCA TAC C-3′; TTF-1 at −1357, forward, 5′-CTC AGA ACC GAT AAG CTA GGT G-3′; TTF-1 at −1357, reverse, 5′-GTC CAA TGG ACA TGA AGC AC-3′; TTF-1 at −1238, forward, 5′-GTC TTT GAT GTC CAT TGG C-3′; TTF-1 at −1238, reverse, 5′-GGT TGG TGT TCT TTC-3′; TTF-1 at −1181/−1201, forward, 5′-GGG AGA ACA CCA ACC AGC-3′; TTF-1 at −1181/−1201, reverse, 5′-ACC TGT TAG TGG AGT TGT AC-3′; TTF-1 at −807, forward, 5′-GTC CTA CAG AGT GCC CGT-3′; TTF-1 at −807, reverse, 5′-GCA GAA TTA CCA GC-3′; TTF-1 at −728, forward, 5′-GGT CGT TAA GAG AGC TGG-3′; TTF-1 at −728, reverse, 5′-GGG ACC AAA GGG TTT ATC TC-3′; TTF-1 at −669, forward, 5′-GAT GAA TGG CTA AGT TGG ATT GTG TGT G-3′; TTF-1 at −669, reverse, 5′-GTC TGT GTG TGT GTG TGT G-3′.
of leptin effectively suppressed the elevation of TTF-1 immunosignals induced by overnight food deprivation in the ARC (Figure 1B and C). Similarly, elevated TTF-1 immunosignals in response to fasting were almost completely rescued by leptin treatment in the DMH. However, no alteration of TTF-1 immunosignals was observed in the paraventricular nucleus (PVN) in response to the fasting and leptin treatment (Supplementary Figure 1). Additionally, we found that leptin suppressed the overnight fasting-induced increase of TTF-1 mRNA levels in the mediobasal hypothalamus (Figure 1D). The effectiveness of systemic administration of leptin was confirmed by changes in hypothalamic AgRP and POMC mRNA levels (Figure 1E and F). These findings suggest that TTF-1 is negatively controlled by leptin in energy homeostasis.

3.2. Deletion of TTF-1 in ObRb-positive cells leads to enhanced sensitivity to leptin with regard to feeding behavior

Hypothalamic TTF-1 expression was altered according to the whole-body energy state and was under the control of circulating leptin. Thus, we next observed the participation of TTF-1 in regulating leptin control of energy homeostasis using a mouse model (ObRb-TTF-1 Knock-down) bearing a selective deficiency of TTF-1 expression in ObRb-positive cells. The model was generated by crossing a TTF-1 floxed mouse with a mouse expressing Cre recombinase in ObRb-positive cells. Specific knock-down of TTF-1 expression was verified by IHC in the ARCs of ObRb-TTF-1−/− mice (Supplementary Figure 2). The body weights of control and ObRb-TTF-1−/− mice were similar before leptin treatment (29.38 ± 0.75 for control mice versus 28.53 ± 0.67 for ObRb-TTF-1−/− mice). Reduced food intake and weight loss induced by the systemic administration of leptin (1 mg/kg) were significantly enhanced in the ObRb-TTF-1−/− mice compared to the control mice (Figure 2A and B). Intriguingly, ObRb-TTF-1−/− mice displayed significantly decreased food intake in response to a low concentration of leptin (0.5 mg/kg) that normally did not induce a reduction of food intake in the control group (Figure 2C). To confirm the enhanced responsiveness to leptin seen in the ObRb-TTF-1−/− mice, we determined the food intake of individual mice in both groups of experimental animals before and after systemic administration of leptin (3 mg/kg). The leptin-induced reduction of food intake seen in each individual mouse was much greater in the ObRb-TTF-1−/− mice than in the control mice (Figure 2D). Similarly, significantly elevated immunosignals of c-Fos, a molecular marker for neuronal activity, induced by leptin treatment (3 mg/kg) was observed in the ARCs of ObRb-TTF-1−/− mice compared to the control mice (Figure 2E and F). In addition, leptin-induced c-Fos activation was sustained until 6 h after treatment in the ObRb-TTF-1−/− mice, while it disappeared after 3 h in the control mice (Figure 2F).

![Figure 2](image_url)

**Figure 2:** Selective deletion of TTF-1 in ObRb-positive cells enhances sensitivity to leptin. (A) Reduced cumulative food intake for 24 h was greater in ObRb-TTF-1−/− mice than in the control mice after the i.p. injection of leptin (1 mg/kg) (n = 5/group). (B) Weight loss for 24 h in response to i.p. leptin injection (1 mg/kg) was significantly greater in ObRb-TTF-1−/− mice than in control mice (n = 5/group). (C) I.p. injection with a low dose of leptin (0.5 mg/kg) was associated with a decrease in food intake for 24 h in the ObRb-TTF-1−/− mice but not in the control mice (CTL, n = 5; ObRb-TTF-1−/−, n = 7). (D) Anorexogenic effects of leptin were significantly more prominent in the individual ObRb-TTF-1−/− mouse than in the control mouse, based on the measurement of food intake for 24 h after the i.p. administration of leptin (3 mg/kg; CTL, n = 4; ObRb-TTF-1−/−, n = 5). (E, F) Leptin-induced activation of neurons was determined by assessing the change of c-Fos immunoreactivity in the ARCs of mice that were treated with i.p. leptin (1 mg/kg) 90 min before sacrifice. (E) Representative photographs and (F) calculated graphs show an elevation of leptin-induced c-Fos activity in the ARCs of ObRb-TTF-1−/− mice compared to that observed in case of the control mice (n = 4–9 sections/3–5 mice/group). Data are presented as the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 vs. vehicle-treated control group (A–D) or control wild mice (F). #p < 0.05, ##p < 0.01 vs. change in food intake by control wild mice. NS, not significant. P-values for unpaired comparisons were analyzed by two-tailed Student’s t-tests. Two-way repeated-measured ANOVA tests were performed to detect significant interactions between genotypes.
Thus, the selective blockade of TTF-1 expression in ObRb-positive cells may result in the increased responsiveness of these neurons to leptin.

3.3. TTF-1 regulates responsiveness to leptin in POMC neurons

Since TTF-1 is a negative regulator for the expression of POMC [14], we further analyzed the contribution of TTF-1 to the leptin responsiveness of POMC neurons. We generated a mouse model that bears the POMC neuron-specific deletion of TTF-1 (POMC-TTF-1−/−) and analyzed TTF-1’s function in mediating leptin’s action on POMC neurons. The selective knock-down of TTF-1 expression in ObRb-positive cells (Supplementary Figure 3). The body weights of control and POMC-TTF-1+/− mice were similar before leptin treatment (29.03 g ± 0.52 for control mice versus 28.86 g ± 0.76 for POMC-TTF-1−/− mice). Although systemic administration of leptin at a low concentration (1 mg/kg) did not induce a significant decrease in food intake in the control mice, the POMC-TTF-1−/− mice responded to this low dose of leptin treatment with significantly reduced food intake (Figure 3A). In addition, this elevated leptin sensitivity seen in the POMC-TTF-1−/− mice was observed until 24 h after the leptin injection, indicating a prolonged responsiveness to leptin. Accordingly, this low dose of leptin induced a much greater decrease in body weight in

Figure 3: POMC neuron-specific deletion of TTF-1 leads to increased sensitivity to leptin. (A, B) Leptin (i.p., 1 mg/kg) induced a significant reduction of (A) food intake for 6 h and 24 h and (B) body weight for 24 h in the POMC-TTF-1−/− mice, but not in the control mice (n = 4–5/group). (C, D) Leptin-induced activation of POMC neurons was determined by measuring the change in c-Fos immunoreactivity (red) in the POMC-GFP cells (green) of overnight-fasted control and POMC-TTF-1−/− mice that were treated with i.p. leptin (1 mg/kg) 90 min before sacrifice. (E) Representative photographs and (D) calculated data show a greater increase in leptin-induced c-Fos activation in the POMC cells of POMC-TTF-1−/− mice than in those of the control mice (n = 4 sections/4 mice/group). (E–G) To identify changes in the membrane potential of the POMC neurons of the POMC-TTF-1−/− mice, the electrical property was measured by whole cell patch-clamp recording. (E) Bright-field and fluorescent illumination of POMC-GFP neurons attached to a whole-cell recording pipette (the arrowhead indicates the targeted cell). (F) Treatment with leptin (10 nM) produced high-frequency spikes in the POMC neurons of the POMC-TTF-1−/− mice, but not in those of the control (POMC-TTF-1+/+) mice. (G) Leptin depolarized the POMC neurons of the POMC-TTF-1−/− mice to a greater extent than that observed in case of the control mice (n = 7 cells/4 mice/group). (H–J) Changes in α-MSH innervation were determined in the PVN of POMC-TTF-1−/− mice. (H) Representative images show α-MSH immunosignals in the PVN. Leptin (1 mg/kg)-induced increases in (I) relative density and (J) particle numbers of α-MSH fiber signals were significantly higher in POMC-TTF-1−/− mice than those observed in case of the control mice (n = 4 sections/4 mice/group). Data are presented as the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 vs. vehicle-treated control group (A, B, G) or fasted group of each genotype (D, I, J). ²p < 0.05, ³p < 0.01 vs. change in control mice. NS, not significant. Scale bar = 50 μm. P-values for unpaired comparisons were analyzed by two-tailed Student’s t-tests. Two-way repeated-measured ANOVA tests were performed to detect significant interactions between genotypes.
the POMC-TTF-1−/− mice than in the control mice (Figure 3B). As stronger neuronal activity was induced by leptin in the ARCs of the ObRb-TTF-1−/− mice than in the control mice, we observed that there were more c-Fos-positive POMC cells following leptin administration (1 mg/kg) in POMC-TTF-1−/− mice compared with the control mice (Figure 3C and D). To verify whether the specific ablation of TTF-1 in the POMC neurons caused enhanced activation of POMC neurons in response to leptin, the electrical activity of POMC neurons was measured by whole-cell recording (Figure 3E). The POMC neurons of the POMC-TTF-1−/− mice were strongly depolarized in response to a low dose of leptin (10 nm), whereas those of the control mice were not (Figure 3F). The resting membrane potential was more depolarized in the POMC neurons of POMC-TTF-1−/− mice than in control mice following leptin administration (Figure 3G). Since α-melanocyte stimulating hormone (α-MSH) is produced by a proteolytic cleavage process from a POMC gene product and targets the PVN, where it triggers satiety signals, we next evaluated the pattern of α-MSH innervation into the PVN after intraperitoneal (i.p.) injection of leptin (1 mg/kg). Leptin induced much greater increases in the density and number of α-MSH fibers into the PVN of POMC-TTF-1−/− mice than those observed in the control mice (Figure 3H–J). Thus, TTF-1 is a critical molecular orchestrator for leptin responsiveness in POMC neurons, thus affecting leptin-induced melanocortin inputs to the PVN neurons.

3.4. TTF-1 controls cellular sensitivity to leptin by regulation of phosphorylation of STAT3

Leptin receptor signaling in target cells induces a series of tyrosine phosphorylations: the binding of leptin induces phosphorylation of the receptor itself and of Janus kinase, which results in STAT3 phosphorylation. Thus, phosphorylated STAT3 (pSTAT3) can be used as a molecular indicator to evaluate the activity of the leptin signaling pathway [21]. We examined pSTAT3 in hypothalamic sections from control and ObRb-TTF-1−/− mice at 1.5 h, 3 h, and 6 h after systemic injection of leptin (1 mg/kg). Levels of pSTAT3 were below that of a detectable level in both control and ObRb-TTF-1−/− mice when they were fed normally or fasted overnight (Figure 4B). However, pSTAT3 signals were dramatically elevated by leptin treatment after overnight fasting (Figure 4A and B). Leptin-induced levels of pSTAT3 were significantly higher in the ObRb-TTF-1−/− mice than in the control mice at every observation time. We next found that increased pSTAT3 levels in response to leptin were also observed in the POMC-TTF-1−/− mice compared to the control mice (Figure 4C and D). Small hairpin RNA (shRNA)-mediated blockade of TTF-1 expression consistently enhanced leptin-induced pSTAT3 levels in the mouse hypothalamic mHypoA cell line (Figure 4E).

In parallel with these findings, we observed a significant increase in the expression of ObRb, a long-form leptin receptor, in the hypothalamus of ObRb-TTF-1−/− mice (Figure 4F), suggesting that the increased ObRb mRNA expression could be linked to the enhanced STAT3 phosphorylation and increased sensitivity to leptin in ObRb-TTF-1−/− mice. Interestingly, we observed a marked increase in the mRNA levels of molecular switches for the intracellular feedback loop of leptin signaling, such as suppressor of cytokine signaling 3 (SOCS3) and protein-tyrosine phosphatase 1B (PTP1B), in ObRb-TTF-1−/− mice (Figure 4G and H). These paradoxical results likely reflect that a sustained increase in cellular leptin sensitivity may cause activation of the

![Figure 4: TTF-1 is involved in STAT3 phosphorylation and ObRb expression.](image-url)
negative feedback loop of leptin receptor signaling. Thus, TTF-1 may affect cellular sensitivity to leptin, at least in part, by regulating ObR expression and STAT3 phosphorylation.

3.5. TTF-1 controls the transcription of the ObRb gene

To better understand the enhanced leptin sensitivity seen in TTF-1-deficient mice, we investigated the transcriptional regulation of the ObRb gene by TTF-1 in an adult mouse hypothalamic cell line (mHypoA-POMC) originally generated and immortalized from the hypothalamic cells that express POMC and secrete α-MSH. We first confirmed the responsiveness of these cells to leptin with an observation of change in pSTAT3 levels after leptin treatment (Supplementary Figure 4). In support of findings showing enhanced leptin sensitivity in ObRb-TTF-1-/- and POMC-TTF-1-/- mice, overexpression of TTF-1 led to a decrease in ObRb mRNA levels, and moreover almost completely blunted the increased ObRb mRNA expression that was induced by leptin treatment in the mHypoA-POMC cells (Figure 5A and B). We next performed promoter assays using a luciferase construct containing the 5’-flanking region of the mouse ObR gene (-1755 to +40) in mHypoA-POMC cells. The ObR promoter was active over a wide range of concentrations, indicating that this promoter construct is transcriptionally active in mHypoA-POMC cells (Figure 5C). When the ObR promoter was co-transfected with a TTF-1 overexpression vector, the promoter activity of ObRb was significantly reduced in a dose-dependent manner in mHypoA-POMC cells (Figure 5D). To identify the functionally active TTF-1 binding sites in the ObR promoter region, ChIP assays were performed using TTF-1 antibodies and PCR primer sets specific to the promoter regions recognized by TTF-1. Promoter sequences at −669, −728, −1181, −1201, −1238, −1357, and −1567 were detected by PCR and their band intensities were markedly decreased in DNA samples isolated from the leptin-treated cells (Figure 5E). No detectable band was observed in the control IgG precipitations and the PCR performing using the primer sets for the −807 and −1317 binding sites (Figure 5E). These results suggest a molecular mechanism that supports the inhibitory function of TTF-1 on the actions of leptin through regulation of ObRb expression.

3.6. Deletion of TTF-1 in ObRb-positive cells protects against diet-induced obesity by improving leptin resistance

To determine whether the enhanced leptin sensitivity of ObRb-TTF-1-/- mice affects leptin resistance and development of obesity, we fed mice a high-fat diet (HFD) for 16 weeks to generate a diet-induced obesity (DIO) model and analyzed their metabolic phenotypes. ObRb-TTF-1-/- mice gained significantly less body weight than the control mice during HFD feeding (Figure 6A and B). To confirm whether alteration of energy expenditure was responsible for the lower weight gain observed in ObRb-TTF-1-/- mice, we assessed carbon dioxide generation (VCO2) and oxygen consumption (VO2), determined by indirect calorimetry, during HFD feeding. VCO2 and VO2 were...
significantly higher in the ObRb-TTF-1^{−/−} mice than in the control mice (Figure 6C and D). The respiratory exchange ratio (RER), calculated based on the collected VO₂ and VCO₂, was also higher in the ObRb-TTF-1^{−/−} mice during the dark and light periods (n = 4/group). (E, F) ObRb-TTF-1^{−/−} mice showed increased RER compared to the control mice during the dark and light periods (n = 4/group). (G, H) ObRb-TTF-1^{−/−} mice showed a significant reduction in (G) food intake and (H) body weight for 24 h in response to systemic administration of leptin (3 mg/kg, i.p.), while the control HFD-fed mice were unresponsive to leptin with respect to anorexia and weight loss (n = 5–6/group). (I) Hypothalamic sections from the ObRb-TTF-1^{−/−} mice, but not those from the control mice, showed increased pSTAT3 levels in response to the i.p. injection of leptin (3 mg/kg). Data are presented as the mean ± SEM. *p < 0.05, **p < 0.01 ***p < 0.001 vs. control group (A–D, F) or fasted group of each genotype (G, H). #p < 0.05, ##p < 0.01, ###p < 0.001 vs. change in the control mice. Scale bar = 50 μm. P-values for unpaired comparisons were analyzed by two-tailed Student’s t-tests. Two-way repeated-measured ANOVA tests were performed to detect significant interactions between genotypes.
**4. DISCUSSION**

The present study highlights the contribution of TTF-1 to the leptin signaling pathway in the hypothalamic neuronal circuit and its resulting impact on the development of obesity in association with leptin resistance. The current observations suggest that TTF-1 is an important molecular cue in the leptin signaling pathway during development of leptin resistance.

Whole body energy metabolism is intimately related to the communication between the peripheral organs that propagate signals reflecting the energy state and central compartments involved in signal integration for the triggering of proper behavioral outputs. In particular, it has long been recognized that the hypothalamic neuronal circuit is essential for interpreting afferent chemical messengers such as adiposity signals and gut-derived hormones [22]. Since leptin is one of the most effective hormones for controlling energy balance, and establishment of multiple leptin secretory- or receptor signaling-impaired genetic lines have led to the development of obesity and metabolic disorders, leptin has been referred to as an attractive therapeutic target for anti-obesity drugs [23,24]. Despite its strong effects on appetite reduction and enhanced energy expenditure, using leptin therapeutically has not been feasible thus far as it has not been shown to be effective for the severely obese [1]. Indeed, the disruption of leptin signaling in hypothalamic cells gives rise to insensitivity to leptin’s actions, or so-called leptin resistance, and this could be a critical pathogenic element in the development of obesity [25,26]. In line with these findings, numerous studies have explored the primary cellular events and molecular mediators for the actions of leptin in order to interpret the development of leptin resistance. Until now, the most acceptable explanation has been that the development of leptin resistance induced by over-nutrition is closely linked to a variety of cellular stresses such as endoplasmic reticulum stress, oxidative stress, and inflammation [27–29].

Transcriptional regulation is a crucial molecular action in the mechanism of cellular stress-induced leptin resistance. STAT3 is a pivotal transcriptional modulator in the leptin signaling pathway. Phosphorylation of STAT3 gives rise to an increase in POMC transcription and a decrease in AgRP transcription via its specific binding motif, known as the leptin response element [30]. A mouse line in which STAT3 is specifically impaired in neural cells displayed obesity accompanied by hyperphagia [31]. TTF-1 has recently been considered a key component in the hypothalamic complex of regulatory systems for energy homeostasis as it has been found that alteration of TTF-1 expression triggers a marked change in metabolic phenotypes including food intake [14] and thermogenesis [32] through the transcriptional regulation of several downstream targets such as the POMC and AgRP genes. These previous studies have raised further questions as to whether TTF-1 is responsive to hormonal inputs for the triggering of feeding or satiety. At the beginning of this study, we studied localization of TTF-1 in the entire brain and found that TTF-1 is predominantly expressed in leptin receptor-expressing cells and the hypothalamic areas that are critical in the control of energy metabolism. These histological findings and previous reports together led us to design the current study exploring the molecular contribution of TTF-1 to the leptin signaling pathway. We found that mice with selective deletion of TTF-1 in ObRb- or POMC-positive cells responded to leptin with more sensitivity, which promoted the development of anorexia and body weight loss. This suggests that these mice may display a lower leptin-responsiveness threshold. These genetically modified mice also displayed prolonged responsiveness to leptin, suggesting that TTF-1 is an important molecular modulator for the action of leptin in the control of energy balance. Since TTF-1 actively participates in the development of the neural tube ventral midline and the diencephalic brain region [7,33], we interrogated whether altered number of POMC cells could be a cellular event for the enhanced leptin sensitivity seen in ObRb-TTF-1−/− or POMC-TTF-1−/− mice. However, no difference in number of POMC cells was observed between control and TTF-1-deficient mice (Supplementary Figure 5), suggesting that enhanced leptin sensitivity induced by TTF-1 ablation is coupled to the activity of POMC neurons rather than the density of POMC cells in hypothalamic ARC. Moreover, these mice displayed changes in the intracellular molecules involved in leptin signaling in the hypothalamus: for example, the mRNA levels of ObRb were increased in the hypothalamic of mutant mice, and phosphorylation of STAT3 was greatly increased by leptin treatment in the mutant mice compared to the control mice. These molecular findings suggest a potential explanation as to how TTF-1 is involved in cellular responsiveness to leptin. In this way, the TTF-1 knockout-induced increase in ObRb expression may cause an increase in the phosphorylation of STAT3, and the increased ObRb and pSTAT3 together, in turn, render the cells more sensitive to leptin [34,35]. Intriguingly, ObRb-TTF-1−/− mice showed higher expression levels of both SOCS3 and PTPIB, both of which are involved in the intracellular feedback loop of leptin receptor signaling. These increases in negative feedback activity might be due to the homeostatic cellular response to compensate for the enhanced activation of leptin receptor signaling in the mutant cells. We verified the transcriptional regulation of the ObR gene by TTF-1 in cultured hypothalamic cells. These observations further confirmed that enhanced leptin sensitivity is associated with increased activity of leptin signaling triggered by TTF-1 deficiency. Although deficiency in leptin can lead to obesity, a high level of circulating leptin in diet-induced obesity can neither reduce food intake nor activate energy expenditure [36]. Thus, the majority of studies have been conducted to understand the mechanism of leptin resistance and to manipulate the sensitivity of leptin action. It has been well established that mice with long-term exposure to an HFD develop leptin resistance that accounts for the development of obesity and its related pathogenesis [1,37]. In accordance with the aforementioned cellular and molecular changes, ObRb-TTF-1−/− mice also displayed metabolic phenotypes indicating higher sensitivity to leptin after a long-term HFD-fed condition that caused leptin resistance in the control mice.

In terms of molecular event, leptin resistance was effectively reversed by modulating the molecules involved in leptin signaling pathway such as SOCS3 and pSTAT3 [21]. Moreover, an elevation of ObRb expression also improved the leptin resistance and obesity pathogenesis [38]. From these evidences, we thought that increased ObRb expression could be a molecular clue to interpret the enhanced leptin sensitivity seen in TTF-1-deficient mice models. However, further studies are required to better understand the contributions of TTF-1 in the regulation of obesity pathogenesis coupled to the leptin resistance. Particularly, it is valuable to uncover detailed molecular mechanisms of TTF-1 functions in the leptin signaling pathway, especially its role with respect to molecules such as SOCS3 and PTPIB in the negative regulation of cellular leptin action. It is also important to clarify whether TTF-1 affects intracellular factors in other neuronal populations beyond POMC neurons in the hypothalamus. In this study, we identified the changes in number of TTF-1-positive cells in response to fasting and leptin treatment in hypothalamic DMH where ObRb is also expressed. These data suggest that enhanced leptin sensitivity seen in TTF-1-deficient mice models used in this study might be also linked to the functions of TTF-1 in ObRb-positive cells in hypothalamic DMH. Although we highlighted the functions of TTF-1 in hypothalamic ARC,
further studies in other hypothalamic nuclei are required for better understating the physiological and pathological functions of TTF-1 in the regulation of leptin sensitivity.

In summary, the present study identified TTF-1 as a key regulator in the leptin signaling pathway and highlighted its contribution to the leptin sensitivity of the hypothalamic neuronal circuit controlling energy homeostasis. Thus, TTF-1 may serve as a novel therapeutic target for the treatment of obesity.

AUTHOR CONTRIBUTIONS

Byung Seo Park: Data curation, Formal analysis, Investigation, Methodology, Funding acquisition, Writing - original draft; Dasol Kang: Formal analysis, Investigation, Methodology, Writing - original draft; Kwang Kon Kim: Investigation, Methodology; Bora Jeong: Investigation, Methodology; Tae Hwan Lee: Investigation, Methodology; Jeong Woo Kang: Investigation, Methodology; Tae Hwan Lee: Investigation, Methodology; Jeong Woo Kang: Investigation, Methodology; Bora Jeong: Investigation, Formal analysis, Investigation, Methodology, Writing - original draft; Byung Ju Lee, Conceptualization, Formal analysis, Supervision, Funding acquisition, Writing - original draft, Writing - review and editing; Sungchil Yang: Writing - review and editing; Jae Geun Yang: Formal analysis, Writing - review and editing; Byung Ju Lee, Conceptualization, Formal analysis, Supervision, Funding acquisition, Writing - original draft, Writing - review and editing.

DATA AVAILABILITY

Data will be made available on request.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest, financial or otherwise.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2022.101636.

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