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ORIGINAL PAPER

Leptin is a dose-dependent marker of caloric restriction in adipose tissues located in different parts of the mouse body

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Abstract We investigated leptin expression in four fat tissues located in different body parts of male mice. In the absence of caloric restriction (CR), the highest relative levels of leptin mRNA were detected in epididymal fat (EF). Lateral abdominal fat (LAF) and subcutaneous fat (SF) had intermediate leptin expression levels, while the lowest leptin mRNA content was revealed in brown adipose tissue (BAT). We also investigated differential regulation of leptin expression in these adipose tissues following diets that were based on various levels of CR for 10 weeks. The largest reduction of leptin mRNA levels by CR was observed in adipose tissue where maximal leptin mRNA expression was detected in the absence of CR. Leptin protein expression was also decreased by CR as revealed by immunohistochemistry. The changes in leptin mRNA and protein expression positively correlated with adipocyte size that was also diminished with the increase in the strength of CR.

Keywords: Adipocyte, Adipose tissue, Caloric restriction, Leptin, Mouse

Introduction

Mammals have two types of adipose tissue. White adipose tissue (WAT) is the major source of free fatty acids (FFA), while brown adipose tissue (BAT) produces heat. Furthermore, adipocytes secret bioactive molecules, such as leptin, into blood-stream[1]. Leptin is an adipocyte-derived hormone produced mainly in WAT[2]. The leptin gene was identified in obese mice homozygous for the *ob* mutation. Historically, the leptin gene was therefore known as obese (*ob*) gene, whereas the leptin receptor gene was known as diabetic (*db*) gene. Homozygous mutations of either *ob* or *db* led to a common obesity phenotype, which was also concomitant with other physiological disturbances such as insulin resistance and diabetes, respectively[3]. Administration of leptin to *ob*/*ob* mice had a significant weight-reducing effect[3].

Leptin plays an important role in energy homeostasis[4], lipid synthesis[5], insulin sensitivity[6], and stimulation of the sympathetic nervous system[7]. In addition, leptin increased caveolin-1 expression in adipose tissue thereby causing a subsequent attenuation of the leptin-dependent intracellular lipid accumulation[4]. Furthermore, leptin can induce lipolysis by directly stimulating the expression of FFA oxidation enzymes carnitine palmitoyl transferase-1 (CPT-1) and acyl CoA oxidase (ACO) as well as by inducing the peroxisome proliferator-activated receptor (PPAR)-α[5]. In addition, leptin can directly down-regulate the expression of the fatty acid synthase (FAS).

Caloric restriction (CR) has been identified as the most effective intervention to prolong lifespan so far[8]. Various mechanisms for lifespan extension by CR have been proposed including the modulation of lipolysis. According to a recent proteomic study of epididymal WAT and interscapular BAT of 9-month-old male rats that utilized two-dimensional gel electrophoresis combined with matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS)[9], 6-month long CR increased the expression of proteins implicated in lipolysis and mitochondrial energy metabolism in WAT and up-regulated proteins involved in fatty acid biosynthesis in BAT. Importance of leptin

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for the anti-aging effect induced by CR has been suggested previously[10]. It is possible that leptin takes part in some adaptive mechanism that mediates the anti-aging effect of CR. Decreased plasma leptin by CR could suppress the gonadal, somatotropic, and thyroid signaling axes, while simultaneously activating the adrenal axis. These signaling changes due to altered leptin expression could regulate energy utilization to maximize survival.

Adipose tissues are differentially distributed in the body[11]. Principal roles of adipose tissues could vary depending on their location[9,12]. Here, we analyzed leptin expression in several fat tissues located in different parts of the mouse body in order to gain a better understanding of CR influence on leptin signaling.

Results

To investigate effects of caloric restriction (CR) on leptin expression in adipose tissues located in different parts of the mouse body, we measured leptin mRNA and protein. Leptin mRNA was analyzed by reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR) (Figure 1A). We found that leptin mRNA content was decreased proportionally to the strength of CR in all adipose tissues. The difference between each CR diet and control diet of leptin mRNA was displayed by difference of their C_t values (Table 1). We also computed the fold change by converting the C_t values to $\log_2$ scale (Table 1). In particular, leptin mRNA levels in epididymal fat (EF) were decreased by approximately 50-fold after 45% CR. In other two white adipose tissues, leptin mRNA levels were decreased by approximately 31-fold in lateral abdominal fat (LAF) and 12-fold in subcutaneous fat (SF). In brown adipose tissue (BAT), 45% CR caused an 11-fold decrease in leptin mRNA (Table 1). We also determined the expression of the leptin protein in adipose tissue by immunohistochemistry in control and 45% CR conditions (Figure 1B). We revealed that leptin protein levels were also decreased by CR in all adipose tissues studied.

In the next set of experiments, we performed hematoxylin and eosin (HE) staining of paraffin-embedded adipose tissues (Figure 2A). HE staining demonstrated a particular structural modulation of adipose tissues by CR. White adipocyte cell sizes were decreased proportionally to the increase in CR strength. BAT adipocytes were the only exception, as they showed a specific enlargement of cell size after 15% CR. Nonetheless, 30% and 45% CR caused a consistent reduction of BAT adipocyte cell size (Figure 2B). A clear negative relationship between adipocyte size and CR strength was noted in the remaining three fat tissues examined (EF, LAF, and SF) and the steepest reciprocal dependency was observed for SF (Table 2). The greatest change was observed in EF (approximately 8.7-fold decrease at 45% CR). In LAF and SF, we observed approximately 6.6-fold and 6.4-fold decrease, respectively. The smallest change was observed in BAT (approximately 4.0-fold decrease) (Table 2). We have not detected any quantitative relationship between cell size and leptin mRNA level.

Discussion

We found that caloric restriction (CR) dynamically altered transcriptional expression of leptin mRNA in adipose tissues located in different parts of the mouse body. Interestingly, the extent of leptin mRNA expression decrease by CR varied depending on the location of adipose tissues (Figure 1A). Basal leptin mRNA levels are also varied in adipose tissues from different parts of the body. EF and brown adipose tissue (BAT) exhibited the highest and the lowest levels of basal leptin mRNA expression, respectively (Figure 1B). We also revealed a difference between CR-induced changes in leptin expression in adipose tissues located outside and inside the body cavity. EF and LAF situated inside the body cavity showed more dramatic changes in leptin expression after CR, compared to alterations seen in SF and BAT that are located outside the body cavity (summarized in Figure 3).

Adipocyte cell size in EF and LAF demonstrated a gradual reciprocal dependency on the level of CR. In SF, the negative relationship between the cell size and the strength of CR was the steepest, while in BAT it fluctuated between an increase (at 15% CR) and a decrease (at 30% and 45% CR) (Figure 2). We conclude that fats inside the body cavity are more sensitive to the levels of CR than adipose tissues located outside the body cavity. Notably, brown fat adipocytes increased in size following 15% CR, although stronger CR conditions led to a decrease in cell size, as in other fat tissues examined. The increase in the size of adipocytes revealed by us in BAT is superficially consistent with a recently reported observation in rats that received CR treatment at 70% of *ad libitum* consumption levels[9]. Authors interpreted that unusual phenotype as a result of infiltration of white adipose tissue (WAT) into BAT. However, our data clearly demonstrated a decrease in cell size under stronger CR regimens except the 15% CR condition, which were more similar to the level of CR used by Okita *et al*. (2012). Therefore, the modulation of BAT adipocytes at this particular level of CR is probably a transient response.

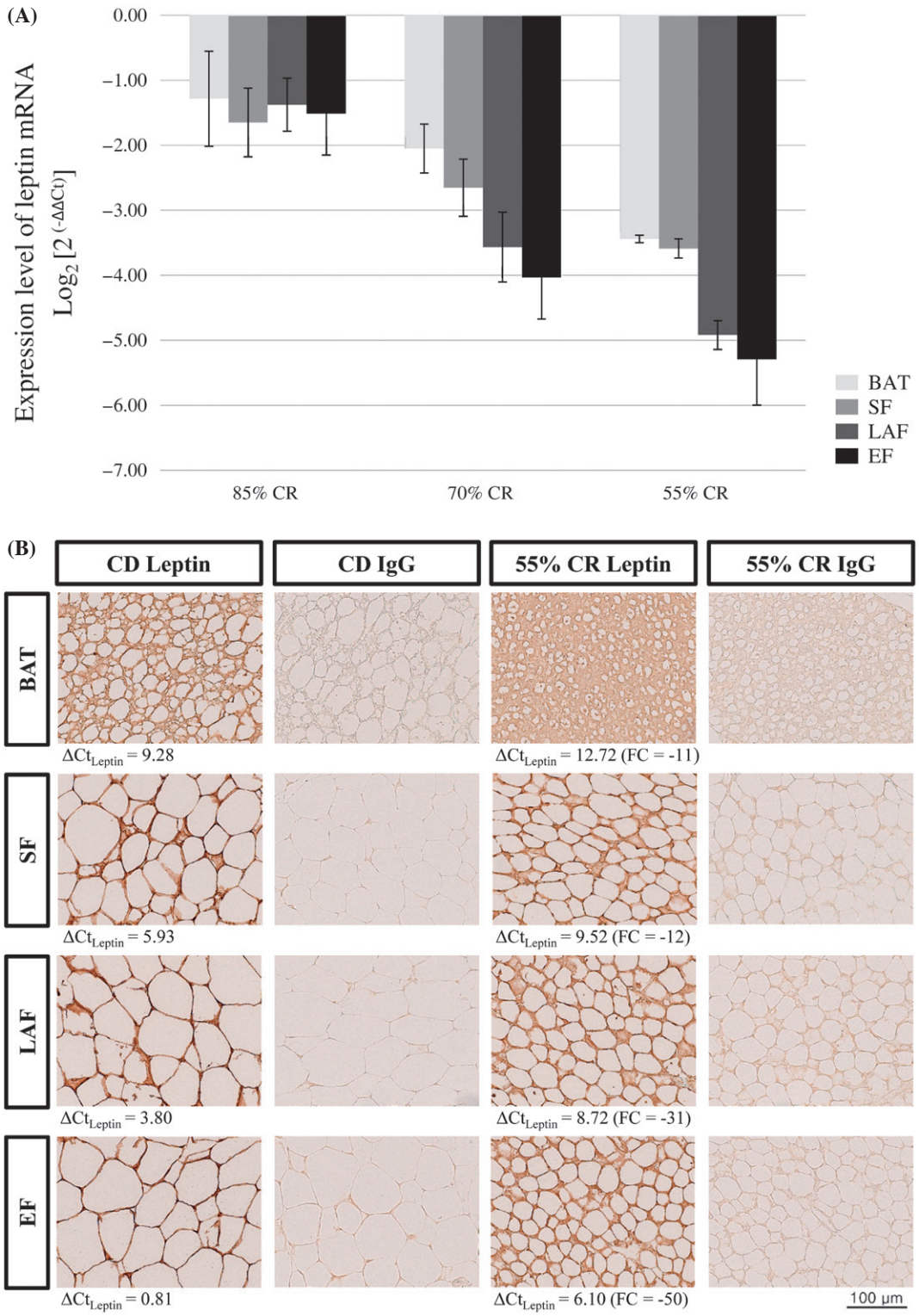

Figure 1. Comparative expression level of leptin mRNA and protein in caloric restriction (CR) and control diet (CD) groups. A, Quantification of leptin mRNA content in epididymal fat (EF), lateral abdominal fat (LAF), subcutaneous fat (SF), and brown adipose tissue (BAT). Data represent the mean ± SE, where $\log_2$ scale of fold change (FC) is based on the ratio between expression levels in CR and CD groups. ΔC_t values indicate relative expression levels of leptin mRNA. B, Immunohistochemistry of leptin protein (scale bar 100 μm) in adipose tissues of mice that received either the CD or the strongest CR treatment (55% CR). Images were obtained at 40x objective magnification. FC illustrates the fold difference in mRNA expression between 55% CR and CD diets.

Table 1. Real-time polymerase chain reaction results for relative leptin expression levels ($\Delta\Delta C_t$) and fold changes (FCs). The representative C_t value was determined by averaging two technical replicates for each sample and three biological replicates for each group. The difference between average C_t values of Gapdh and leptin (ΔC_t) was used to calculate the difference ($\Delta\Delta C_t$) between ΔC_t values obtained in the control diet (CD) and each caloric restriction (CR) group. Finally, fold change values were calculated using $2^{-\Delta\Delta C_t}$ method.

Items	EF			LAF			SF			BAT		
	85%	70%	55%	85%	70%	55%	85%	70%	55%	85%	70%	55%
$\Delta\Delta C_t$	1.51	4.03	5.29	1.38	3.45	4.92	1.65	2.65	3.59	1.29	2.05	3.44
$\Delta\Delta C_t$ SE	0.64	0.64	0.70	0.41	0.54	0.22	0.53	0.44	0.15	0.73	0.38	0.06
FC	−3.49	−19.46	−50.08	−2.82	−13.21	−30.96	−3.55	−6.92	−12.15	−3.17	−4.40	−10.88
FC SE	1.61	7.00	24.94	0.84	5.65	4.45	1.16	2.18	1.18	1.63	0.97	0.42

EF: Epididymal fat, LAF: Lateral abdominal fat, SF: subcutaneous fat, BAT: Brown adipose tissue, SE: Standard error

Table 2. Quantitative data of Hematoxylin and eosin (H&E) staining and calculated cell size.

Items	EF				LAF				SF				BAT			
	CD	85%	70%	55%	CD	85%	70%	55%	CD	85%	70%	55%	CD	85%	70%	55%
Average cell size (μm^2)	3013.9	1907.6	698.6	348.3	3671.9	2821.0	788.0	552.9	3206.0	1019.5	768.3	501.8	312.8	446.4	191.5	77.9
(SE)	(731.7)	(534.6)	(223.9)	(97.2)	(1329.0)	(753.7)	(294.1)	(162.1)	(1045.4)	(370.5)	(237.7)	(151.0)	(133.5)	(208.4)	(59.8)	(24.2)
Cell count	23	38	105	162	21	27	96	126	23	75	94	137	180	139	209	257

EF: Epididymal fat, LAF: Lateral abdominal fat, SF: subcutaneous fat, BAT: Brown adipose tissue, CD: Control diet, SE: Standard error.

There have been many studies that documented differential responses between WAT and BAT to biological or environmental changes, including CR and cold acclimatization[9,13], but very few studies addressed variability in physiological characteristics between different fat masses in the body[14]. Our data demonstrate that basal leptin expression level and the degree of its decrease by CR vary depending on the location of adipose tissues in the body. Therefore, we conclude that the relative importance of leptin gene activity may be differ according to the localization and type of adipose tissues.

Materials & Methods

Animals and tissue collection

Three male C57BL/6N mice per each dietary condition were used in this study. The mice were raised under identical conditions except for differences in dietary caloric consumption. CR mice were fed with calorically reduced diets of 15%, 30%, and 45% of control values for 10 weeks and sacrificed at the age of 18 weeks. Because the estrous cycle could affect body composition and might create variability, we eliminated female mice for this study. We collected adipose tissues from different parts of the mouse body including epididymal fat (EF), lateral abdominal fat (LAF), subcutaneous fat (SF), and brown adipose tissue (BAT)[15].

RNA isolation and reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was isolated from 100-200 mg fat tissue using 1-2 mL of TRIzol Reagent (Invitrogen Inc, CA, USA) and a Power Gen 125 homogenizer (Fisher Scientific, HA, USA). Purification of RNA was confirmed by measuring A260/A280 values (>1.8) using a Nanodrop Spectrophotometer, ND-1000 (Thermo Scientific, DE, USA). RNA integrity was checked by electrophoresis in 1.2% (w/v) agarose gel supplemented with formaldehyde. To synthesize cDNA template, 1 μg total RNA was reverse transcribed with 4 μL of 5x iScript reaction mix and 1 μL of iScript reverse transcriptase (Bio-Rad Inc., HE, USA). One microliter of the cDNA reaction mixture was used as a template for each qPCR reaction. qPCR was conducted using SYBR Green reagent (Bio-Rad) in a real-time CFX-96 PCR analyzer (Bio-Rad). Leptin primer sequences were designed using NCBI reference sequence for leptin (NM_008493.3) and IDT Primer Quest program (USA) (forward primer sequence: 5′-GGT TGA TCT CAC AAT GCG TTT C-3′ and reverse primer sequence: 5′-TGG GAG ACA GGG TTC TAC TT-3′). The glycer-

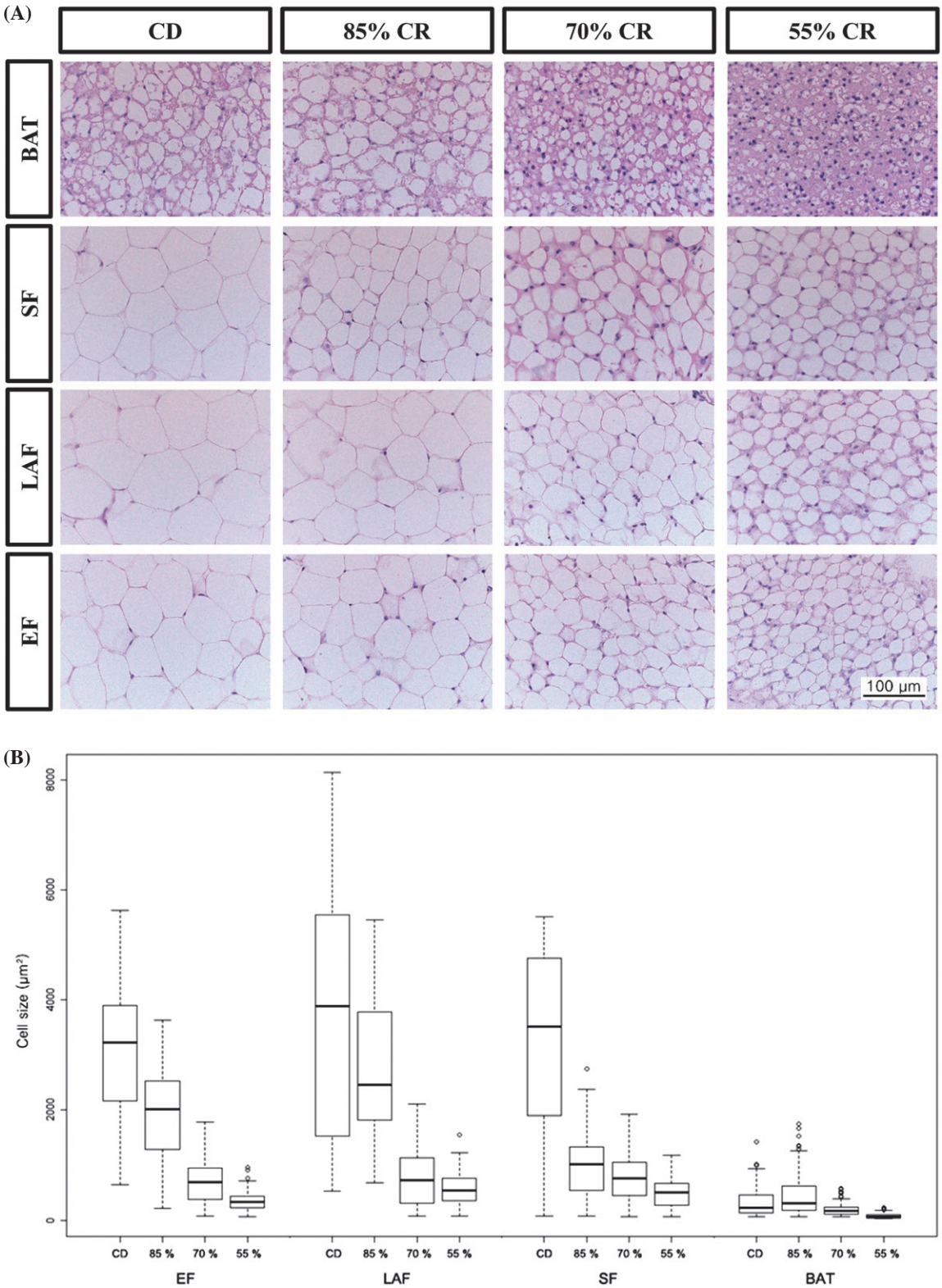

Figure 2. Analysis of adipocyte cell size by hematoxylin and eosin (H&E) staining. A, H&E staining (scale bar 100 μm) demonstrates alteration of cell size as a result of exposure to CR conditions of different strengths. Images were captured at 40x objective magnification. B, Box plots illustrate distribution of adipocyte cell sizes depending on the strength of CR conditions. With the exception of BAT, all other adipose tissues show a constant negative relationship between adipocyte cell size and the extent of CR.

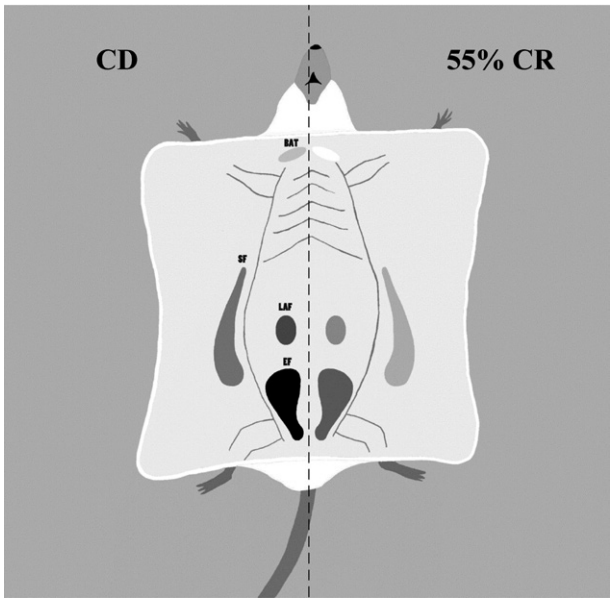

Figure 3. Summary of leptin mRNA expression data in adipose tissues examined in this study. Color density correlates with the relative expression level of leptin during CD and 55% CR condition.

aldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous control to normalize leptin qPCR results. Representative C_t value was determined by averaging two technical replicates for each sample and three biological replicates for each group. The difference between average C_t values of *Gapdh* and *Leptin* (ΔC_t) was used to calculate the difference ($\Delta\Delta C_t$) between ΔC_t values obtained for control diet (CD) samples and samples from each CR group. Finally, fold change values were calculated using the $2^{\Delta\Delta C_t}$ method.

Immunohistochemistry

Leptin protein was detected by immunohistochemistry using an anti-leptin antibody (ab9749; Abcam, CB, UK) and a rabbit IgG (Sigma-Aldrich Co. LLC., MO, USA) as a negative control. To detect antibody reactions, we used a VECTASTAIN® ABC Kit (Vector Laboratories Inc., CA, USA) for immunoperoxidase detection reagents. For immunohistochemistry, paraffin tissue sections were deparaffinized in xylene and rehydrated to PBS through a series of alcohol gradients. Deparaffinized sections were incubated in boiling citrate buffer (Na citrate + Citric acid + dH_2O) for 10 min. Endogenous peroxidase activity was quenched by incubating rehydrated tissue sections in 1% (v/v) hydrogen peroxide-containing methanol at room temperature. After protein blocking using the VECTASTAIN® ABC Kit protein blocker (Vector Laboratories), the sections were incubated overnight at 4°C with a rabbit anti-mouse leptin antibody (1 μg/mL in PBS containing 1% (w/v) BSA; Abcam) or rabbit IgG (1 μg/mL in PBS-BSA; Sigma-Aldrich) as a negative control. Tissue section slides were incubated with a biotinylated secondary antibody and a protein blocker in PBS for 1 h at 37°C. After diluted detector reagent was added to the slides, they were incubated in a humidified chamber at 37°C for 30 min. Immunoreactive protein was visualized using 3,3′-diaminobenzidine (DAB) solution (Sigma-Aldrich) according to the manufacturer's instruction. The slides were dehydrated by immersion into 70, 90, and 100% (v/v) ethanol, and finally in xylene for the subsequent microscopic observation.

Hematoxylin and eosin (HE) staining and cell size measurements

HE staining procedures were carried out according to the manufacturer's instruction (Sigma-Aldrich). HE staining products were quantified using ImageJ (NIH, MD, USA) to assess cell size.

Statistical analysis

To stabilize variation of qPCR data, we transformed $-2^{\Delta\Delta C_t}$ fold change values into $\log_2$ scale. Data are presented as the mean ± standard error (SE) of measurement. Distribution of cell sizes was illustrated by a box plot.

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Conflict of Interest Seok-Jin Hong, Seung-Soo Kim, Whasun Lim, Gwonhwa Song and Cheol-Koo Lee declare that they have no conflict of interest.

Human and animal rights All the experimental procedures followed the law of animal protection that was approved by the Committee on Animal Experimentation and Ethics of Korea University (KUIACUC-2010-104), Korea.

Author's contributions Hong, S.-J. carried most experiments and drafted the manuscript. Kim, S.-S. carried mouse handling and tissue experiments. Lim, W. performed immunohistochemistry. Song, G. designed experiments and finalized the manuscript. Lee, C.-K. designed experiments, analyzed data, and drafted and finalized the manuscript.

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