Overexpression of the adiponectin gene mimics the metabolic and stress resistance effects of calorie restriction, but not the anti-tumor effect

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Highlights

- Adiponectin (Adipoq) ameliorates cellular senescence in the white adipose tissue.
- Adipoq increases insulin sensitivety.
- Adipoq increases survival over endotoxin shock.
- These effects of Adipoq are similar to those induced by calorie restriction (CR).
- Adipoq does not inhibit tumor growth, incontrast to CR.

Abstract

Adiponectin (Adipoq), a peptide hormone secreted from the white adipose tissue, may play a role in the anti-aging and/or anti-tumor effects of calorie restriction (CR). We analyzed metabolic traits in *Adipoq* gene-overexpressing mice fed ad libitum with a regular diet (RD) or a high-fat diet (HFD), or fed 30% CR of RD initiated at 12 weeks of age. Adipoq-RD and -HFD mice at 6 months of age showed reduced blood glucose and insulin concentrations, and thus increased insulin sensitivity, compared with WT mice fed a RD or a HFD. In the epididymal white adipose tissue in Adipoq mice, senescence-like changes such as upregulation of p53 protein and of biomarkers of inflammation, *Cd68* and *Ccl2* mRNA, were ameliorated compared with WT-RD and WT-HFD mouse tissues. Resistance to stress induced by lipopolysaccharide was also strengthened in Adipoq mice compared with WT mice. These metabolic changes and stress resistance were also noted in the WT-CR mice, suggesting that Adipoq plays a part in the effect of CR. In contrast, in an allograft tumor growth model, tumor growth was not inhibited in Adipoq mice. The present findings suggest that Adipoq plays a part in the anti-aging, but not in the anti-tumor, effects of CR.

Keywords: Adiponectin, Calorie restriction, p53, Insulin, ER stress,
Tumor growth

1. Introduction

Modest restriction of dietary calorie intake (with sufficient supply of essential nutrients) extends lifespan and inhibits diseases including cancers in aging animals (Weindurch and Walford 1988). This experimental paradigm has been a tool to investigate signaling pathways that regulate lifespan and aging. Although many researchers have investigated this phenomenon, our knowledge on the pathways involved remains incomplete.

Long-term calorie restriction (CR) is thought to promote metabolic adaptation in animals for survival, i.e., increase energy efficiency to maintain basic physiological functions with limited energy intake. This adaptive process, which copes with aging-related insulin resistance and glucose intolerance, may play a part in the effect of CR (Masoro, 2003). Among candidates that promote the metabolic adaptation, we have focused on adiponectin (Adipoq), which is secreted from white adipose tissues (WAT: Hui, 2012). The plasma concentration of Adipoq is increased in CR rodents in parallel with reduction of fat mass (Berg, 2001; Zhu, 2004). Increased plasma Adipoq is known to be associated with insulin-sensitizing, anti-diabetic, and anti-atherosclerotic effects (Hui, 2012). Finally, overexpression of the human *Adipoq* gene is reported to extend the lifespan of mice; not only those fed a high-fat diet (HFD) but also those fed a regular diet (RD: Otabe, 2007). Therefore, we can speculate that CR exerts a life-extending effect through the metabolic adaptation process, which upregulates Adipoq in the WAT and thus

in the circulation.

Excess calorie intake in mice, either genetically-induced or resulting from dietary changes, leads to the accumulation of oxidative stress in the WAT and promotes senescence-like changes including increased expression of p53 and production of proinflammatory cytokines in the WAT (Minamino, 2009). Concomitantly, whole body insulin resistance and glucose intolerance occur in mice; these impairments are ameliorated by inhibition of p53 in the WAT (Minamino, 2009). In the present study, to understand the molecular mechanisms underlying the effect of CR on insulin and glucose homeostasis, we first tested whether or not *Adipoq* overexpression ameliorates the senescence-like phenotypes in the WAT in parallel with insulin sensitization.

We then assessed stress resistance in Adipoq mice, because CR is known to enhance resistance to a variety of stressors (Masoro, 1998). In the present study, survival rates and endoplasmic reticulum (ER) stress were monitored in an endotoxin shock mouse model.

Inhibition of tumor growth is another hallmark of the effects of CR (Weindruch, 1988). The plasma Adipoq concentration is reported to be inversely associated with a variety of human cancers (Barb, 2007), and these findings correlate with the increased risk of cancers in obese people. We therefore also tested whether or not overexpression of Adipoq inhibits tumor growth in an allograft tumor growth model (Yaker, 2006).

2. Materials and methods

2.1. Experimental Animals

The animal care and all experiments were performed in accordance with the guidelines approved by the Ethics Review Committee for Animal Experimentation at Nagasaki University. Pairs of human *Adipoq* gene overexpressing homozygotic mice and the appropriate wild-type (WT) mice (C57BL/6N) were transferred to the Laboratory animal center at Nagasaki University from Kurume University, where the original SPF colony of Adipoq and WT mice is maintained (Otabe, 2007).

The details of animal husbandry were as described elsewhere (Yamaza, 2010). Briefly, two or three (typically three) mice were housed in a cage in a barrier facility (temperature, 22–25°C; 12-h light/dark cycle) under specific pathogen-free conditions. Male Adipoq and WT mice were fed a regular diet (RD, Charles River-LPF (CR-LPF) diet, Oriental Yeast Co. Ltd., Tsukuba, Japan) until 12 weeks of age. The composition of the CR-LPF diet, which is based on the formula of the Charles-River formula (CRF)-1 diet but with reduced protein content and reduced total energy for long-term experiments to minimize aging-related disorders (Shumiya, 1993), was as follows (per 100 g; 347 kcal): 16.5-g protein, 3.9-g fat, 5.9-g mineral mix, 4.4-g fiber, 61.3-g nitrogen-free water-soluble substance, and 8.0-g water.

Thereafter, mice were divided into two groups, a RD group and a

high-fat diet (HFD, i.e., F2HFD1 diet, Oriental Yeast Co. Ltd.: 414 kcal/100 g) group. The composition of the HFD was as follows (per 100 g): 7.5-g Cacao butter, 1.25-g cholesterol, 0.5-g cholic acid sodium salt, 7.5-g milk casein, 1.25-g cellulose, 1.0-g vitamin mix, 1.0-g mineral mix, 1.625-g sucrose, 1.625-g glucose, 1.625-g dextrin, 0.125-g choline chloride, 72-g CRF-1, 3-g lard). In summary, the macronutrient content of the HFD diet was as follows (per 100 g; 414 kcal); 22.6-g protein, 16.5-g fat, and 45.1-g carbohydrates. These mice were fed ad libitum. A calorie-restricted (CR) group, in which WT mice were fed the RD diet at a level 30% less than the ad libitum level of mice in the WT-RD group, was also included. At 12 weeks of age, the CR regimen was initiated by providing the CR animals with a daily food allocation consisting of 70% of the mean daily ad libitum food intake of the WT-RD mice, 30 min before lights were turned off; on Saturday, two portions of food (for Saturday and Sunday) were provided. The food allocation for the WT-CR group was adjusted every 4 weeks depending on the amount of food consumed by the WT-RD group. The daily amount of food consumed by a single mouse was estimated by the total amount of food consumed in each cage, in which three mice were housed, divided by the number of mice in the cage.

Body weight (BW) and food intake were monitored every 4 weeks from 6 weeks of age. Mice were humanely euthanized after anesthesia with isoflurane between 24 and 28 weeks for the experiments.

ER stress-responsive alkaline phosphatase (ESTRAP) mice

(Kitamura, 2011) were used to monitor ER stress in vivo. Pairs of ESTRAP heterozygotic mice, in which secreted alkaline phosphatase (SEAP) is constitutively expressed, were transferred from the University of Yamanashi. The genetic background was C57BL/6. Male and female ESTRAP mice were mated to expand the experimental mouse colony. To determine the genotype of the offspring, genomic DNA was extracted from the tail using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and PCR was performed with SEAP-specific primer as follows: forward: 5′-AACATGGACATTGACGTGATCCTAG-3′; reverse: 5′-TCTCGTATTTCATGTCTCCAGGCTC-3′ (Hiramatsu, 2006). Male ESTRAP heterozygous mice and female Adipoq homozygous or heterozygous mice were mated to produce double transgenic (Adipoq:ESTRAP) as well as wild type (WT:ESTRAP) mice. The 30% CR regimen was also initiated in these mice at 12 weeks of age.

2.2. Hormone and glucose assays

Plasma and serum samples were prepared from blood samples collected via cardiac puncture after anesthesia with isoflurane. These samples were collected 3 hours prior to feeding (thus in the fasting phase) for the CR group; for the ad libitum feeding groups, mice were fasted overnight (approximately 17 hours fasting) prior to sample collection. The plasma concentrations of human and mouse adiponectin were measured with specific

ELISA kits for human and mouse/rat adiponectin (Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan), respectively, without detectable cross-reactivity. The mouse plasma leptin and serum insulin levels were also determined using ELISA kits (Shibayagi Co. Ltd., Gumma, Japan). Blood glucose levels were measured using the glucose dehydrogenase method. The number of mice in each group was 8, except for WT-CR (n = 5).

To estimate insulin sensitivity, the quantitative insulin-sensitivity check index (Quicki; Muniyappa, 2008) was calculated using fasting serum insulin (I0) and glucose (G0) according to the following formula; Quicki = 1/[log(I0) + log(G0)]. Thus, larger values of Quicki represent greater insulin sensitivities. The Quicki has been validated extensively against the reference standard glucose clamp method (Muniyappa, 2008).

2.3. Western blotting for p53 and p21

Approximately 200 mg of frozen fat tissues were homogenized in 800 mL T-PER buffer (PIERCE Biotechnology, Rockford, IL, USA) with a protease-inhibitor cocktail (P8340 Sigma-Aldrich, St. Louis, MO, USA) and a phosphatase-inhibitor cocktail (Nacalai Tesque Inc., Kyoto, Japan). The homogenates were centrifuged at 10000 × g for 15 min at 4°C, and the supernatant was collected. Protein concentrations were measured using the BCA assay kit (PIERCE Biotechnology). For detection of p53, immunoglobulins were removed from total protein lysates with protein A/G

agarose (PIERCE Biotechnology), following the method published by Minamino et al. (2009). All samples were mixed with Laemmli's sample buffer and heated at 95°C for 5 min. Proteins (10 μg) were separated by 10 or 15% SDS-PAGE and transferred to PVDF membranes. The membranes were immediately placed in blocking solution (PVDF Blocking Reagent for Can Get Signal®: TOYOBO, Osaka, Japan) for 60 min. The membranes were incubated with anti-p53 mouse monoclonal antibody (Cell Signaling Technology Denvars, MA, #2524) diluted at 1:500 in immunoreaction enhancer solution (Can Get Signal® solution 1, TOYOBO) for 16 h at 4°C with gentle shaking, and washed 3 times in TBS-T. The membranes were then incubated for 1 h with HRP-conjugated anti-mouse IgG (GE Health Care) diluted 1:10,000 in immunoreaction enhancer solution. Immunoreactive proteins were visualized using ECL (Amersham Biosciences, Tokyo, Japan) and quantified using a densitometer (LAS-3000: Fuji Film, Tokyo, Japan) and MultiGauge software (Fuji Film). To minimize variations in signal intensity, a standard sample of p53 was included in each blot. The number of mice in each group was 3.

2.4. Real-time PCR for determination of mRNA levels

Total RNA was purified from the epididymal adipose tissue using an RNeasy lipid tissue mini kit and RNase-Free DNase Set (QIAGEN) and quantified by spectrophotometry (NanoDrop, Wilmington, DE). The quality of extracted RNA was evaluated as the densitometric ratio of 28S and 16S

ribosomal RNA. The extracted RNA was reverse-transcribed using a Bio-Rad iScript™ cDNA Synthesis kit (Hercules, CA, USA) according to the manufacturer's instructions. The real-time PCR reaction was performed using Platinum® Quantitative PCR SuperMix-UDG with ROX (Invitrogen) and primers and fluorescent probes sets. Primers and fluorescent probes sets (TaqMan Gene Expression Assays) were purchased from Applied Biosystems: human adiponectin (*ADIPOQ*; Hs00605917_m1), mouse adiponectin (*Adipoq*; Mm01343606_m1), *Adipor1* (Mn01291334_mH), *Adipor2* (Mn01184032_m1), *Cd68* (Mm03047340_m1), *Ccl2* (Mn00441242_m1), Leptin (*Lep*; Mm00440181_m1), *Ppara* (Mm00440939_m1), *Pparg* (Mm01184322_m1), *Ppargc1a* (Mm01208835_m1), *Glut4* (Mm01245502_m1), *Fatp1* (Mm00449511_m1), actin beta (*Actb*; Mm02619580_g1), and Eukaryotic 18S rRNA (Hs99999901_s1).

The amount of mRNA for the gene of interest in each sample was normalized to that of the housekeeping gene [actin beta (*Actb*) or 18S rRNA]. All samples ware run in duplicate on the ABI PRISM 7900HT Sequence Detector system (Applied Biosystems). The number of mice in each group was 8, except for WT-CR (n = 5). Tissues were collected without fasting; in CR mice, tissues were collected within 3 hours of the provision of food, i.e., during the fed phase.

2.5. Endotoxin shock and SEAP assay

Between the period of 24 and 28 weeks of age, endotoxin shock was induced in (Adipoq:ESTRAP)-RD mice and control (WT:ESTRAP) mice, fed RD ad libitum or at 30% CR, by intraperitoneal injection of a single dose (10 mg/kg BW) of lipopolysaccharide (LPS, *Escherichia coli* O111:B4, Product Number L2630, Sigma). After LPS injection, mice were inspected twice daily for 5 days. Blood samples were collected from retro-orbital veins at 0, 2, 4, 8, 24, and 48 h after LPS injection to measure SEAP activity. The SEAP detection assay was performed using the phospha-Light-EXP SEAP reporter gene assay system (Life Technologies) according to the manufacturer's instructions. In brief, an aliquot of 5 μL serum was mixed with 50 μL of assay buffer in a white 96-well assay plate and incubated at 65°C for 5 min. Then, 50 μL of reaction buffer was added and the mixture was incubated at room temperature for 20 min. The chemiluminescence was determined using a Synergy 2 Microplate Reader (BioTek, Tokyo, Japan). The number of mice in each group was 7.

2.6. Allograft tumor growth model

To evaluate the effect of Adipoq on tumor growth, 24-week-old mice were injected subcutaneously with 0.5×10^6 tumor cells (H-59 Lewis lung carcinoma cells), and monitored daily for 16 days. We followed the experimental procedure reported elsewhere (Yakar, 2006). The numbers of mice in the experimental groups were as follows; n = 9 for WT-RD and Adipoq-

RD; n = 10 for WT-HFD; and n = 8 for Adipoq-HFD.

2.7. Statistics

Data are presented as means ± standard error. One- or two-factor analysis of variance (ANOVA) and post hoc tests (Hsu-Dunnett or Tukey's honestly significant difference (HSD) tests) were applied for data analyses. Survival curves were estimated using the Kaplan-Meier method and were compared with the log-rank test. A p value of less than 0.05 was considered to be statistically significant. All statistical tests were performed using JMP® Pro 10.0.2 statistical software (SAS Institute Inc., Cary, NC, USA).

3. Results and Discussion

3.1. General properties of experimental mice

Mean BW of Adipoq-RD mice did not significantly differ from that of WT-RD mice (Fig. 1A). Mean food consumption (FC) of Adipoq-RD mice was also not significantly different from that of WT-RD mice (Fig. 1B). Mean BW of mice in the WT-HFD group was significantly greater than that in the WT-RD (Fig. 1A), i.e., WT mice showed a diet-induced obesity phenotype. However, Adipoq-HFD mice did not show any significant increase in BW compared with Adipoq-RD mice (Fig. 1A), although the FC of Adipoq-HFD mice was similar to that of WT-HFD mice (Fig. 1C). These findings have already been noted in

the original report describing these mice (Otabe, 2007). Adipoq mice are known to increase oxygen consumption rate and decrease body fat content, particularly in response to high calorie diet feeding (Otabe, 2007). In the present study, we found that even when fed the RD, the fat content (peri-renal and epididymal fat) of Adipoq mice was less than that of WT mice (0.011 ± 0.001 g/ g BW in Adipoq mice versus 0.044 ± 0.004 g/ g BW in WT mice, $p < 0.0001$). Thus, Adipoq overexpression increased energy expenditure even in mice fed the RD, although the effect was minor compared with that seen in response to HFD feeding. In the genetic background of ESTRAP mice, Adipoq:ESTRAP-RD mice displayed slightly lower BW between 12 and 24 weeks of age, compared with WT:ESTRAP-RD mice (2f ANOVA, $p < 0.0001$; Fig. 1D), although there was no statistical difference in BW at individual time points. Mean BW of WT:ESTRAP-CR mice was also significantly lower than that of WT:ESTRAP-RD mice in response to the 30% reduction of food intake (Fig. 1D).

Human Adipoq was detected in Adipoq mice (447 ± 52 μg/mL), but not in WT mice. Mouse Adipoq was increased in Adipoq mice compared with WT mice (92.7 ± 7.1 μg/mL in Adipoq mice versus 55.7 ± 2.5 μg/mL in WT mice, $p < 0.0001$).

3.2. Insulin and glucose homeostasis

We also confirmed that Adipoq-RD and Adipoq-HFD mice have lower

insulin concentrations compared with the respective WT mice ($p = 0.0303$ and $p = 0.0004$, respectively, Table 1). WT-CR mice also exhibited 56% lower levels of insulin compared with WT-RD mice ($p = 0.006$). The fasting blood glucose concentration of Adipoq-RD mice did not differ from that of WT-RD mice (Table 1). Blood glucose was lower in Adipoq-HFD than in WT-HFD mice ($p < 0.0001$), although blood glucose was increased in WT-HFD mice compared with WT-RD mice ($p < 0.0001$). CR reduced the blood glucose concentration in WT mice (152 ± 6 mg/dL in WT-RD versus 110 ± 5 mg/dL in WT-CR, $p < 0.0001$). Quicki analysis (Muniyappa, 2007) indicated that insulin sensitivity was significantly increased in Adipoq-RD and Adipoq-HFD mice compared with the respective WT mice ($p = 0.0084$ and $p < 0.0001$), as was the case in WT-CR mice ($p < 0.0001$; Table 1). These findings indicate that Adipoq overexpression mimics the effect of CR on insulin and glucose homeostasis.

3.3. Cellular senescence-like changes in the WAT

Excessive calorie intake has been reported to induce cellular senescence-like changes such as expression of p53 and inflammation in the WAT (Minamino, 2009). Inhibition of p53 activity in the WAT markedly ameliorates the senescence-related changes and inflammation, and improves insulin resistance and glucose intolerance (Minamino, 2009). In the present study, p53 protein abundance and the downstream p21 protein levels in the epididymal adipose tissue were significantly reduced in Adipoq-RD and

Adipoq-HFD mice, compared with WT-RD and WT-HFD mice (p53; $p = 0.0004$, $p < 0.0001$, p21; $p < 0.0001$, $p = 0.0004$); there was no difference between WT-RD and WT-HFD mice (Fig. 2A, B, & C). The expression levels of p53 and p21 were also lower in WT-CR mice than in WT-RD mice (p53; $p = 0.0002$, p21; $p < 0.0001$). We also confirmed that mRNA levels of the proinflammatory genes *Cd68* and *Ccl2* were reduced in the WAT of Adiopoq-RD and Adipoq-HFD mice, compared with WT-RD and WT-HFD mice (*Cd68*; $p = 0.0062$, $p = 0.0003$, *Ccl2*; $p = 0.0006$, $p = 0.0180$; Fig. 2D & E); there was no difference between WT-RD and WT-HFD or between Adipoq-RD and Adipoq-HFD mice. There was a significant reduction in the expression of these proinflammatory genes in WT-CR mice compared with WT-RD mice ($p = 0.0002$, $p < 0.0001$). Therefore, the present findings indicate that Adipoq and CR ameliorate senescence-like changes in the WAT, leading to increased insulin sensitivity and glucose tolerance.

3.4. Gene expression in WAT associated with energy metabolism

To further characterize the properties of WAT in Adipoq mice, we analyzed the expression of mRNA encoded by genes associated with energy metabolism, adipokines, and adipogenesis (Fig. 3). Expression of these genes has been reported to be significantly altered by CR (Higami, 2004; Park, 2009). The mRNA expression level of mouse *Adipoq* was increased by 30% in Adipoq-RD and Adipoq-HFD mice, as well as in WT-CR mice, compared with WT-RD

and WT-HFD mice (Fig. 3A, $p = 0.0029$, Adipoq-RD versus (vs) WT-RD; $p = 0.0260$, Adipoq-HFD vs WT-HFD; $p = 0.0081$, WT-CR vs WT-RD, respectively). There was no difference between WT-RD and WT-HFD or between Adipoq-RD and Adipoq-HFD mice. Thus, overexpression of human Adipoq mimics the effect of CR on WAT with respect to endogenous adiponectin expression, probably through reduction of fat mass. In fact, not only the reduction of net amounts of fat pads but also fat cell size was smaller in Adipoq-RD and Adipoq-HFD mice than in the respective WT mice (Supplemental Fig. 2). One of the specific receptors for Adipoq, *Adipor2* (but not *Adipor1*), showed significantly increased mRNA expression levels in Adipoq-RD and Adipoq-HFD mice, compared with WT-RD and WT-HFD mice ($p = 0.0036$, $p < 0.0001$; Fig. 3B & C). The *Adipor2*-mRNA level was also increased in WT-CR mice compared with WT-RD mice ($p < 0.0001$). There was no difference between WT-RD and WT-HFD or between Adipoq-RD and Adipoq-HFD mice. Adipoq mice and WT-CR mice showed reduced mRNA levels of mouse leptin ($p < 0.0001$; Fig. 3D). These changes of Adipoq and leptin coincide with protection of the animals from metabolic disorders such as diabetes (Cao, 2014).

Although many of the published data indicate an increase of Adipoq by CR in the peripheral blood, it should be noted that the effect of CR on plasma Adipoq and/or *Adipoq* mRNA expression in the WAT depends on experimental settings. In the epididymal fat tissue of control mice for long-lived Ames dwarf mice, *Adipoq* mRNA expression levels are significantly (2.3-

fold) increased by 8-month-long 30% CR initiated at 3 months of age (Wiesenborn, 2014). The plasma adiponectin concentration in CR mice was also significantly elevated, compared with control mice fed ad libitum. However, 16-months' CR does not significantly increase the plasma concentration or the mRNA level of Adipoq in the epididymal fat tissue (Wang, 2006). A 25% CR regimen using a diet formulated to be isocaloric with the control diet (American Institute Nutrition (AIN)-93M diet) with 25% increases in protein, vitamin, mineral and fat content did not elevate the plasma Adipoq concentration in mice of a mammary tumor model during a 64-week-long experiment (Dogan, 2010). In that experiment, the body weight and visceral fat pads in female mice were decreased by the CR regimen, compared with mice fed the control diet ad libitum. We presume that CR mice in that experiment received relatively greater amounts of protein compared with WT-CR mice in the present study. Therefore, the age of mice and/or duration of the CR regimen, as well as diet composition, may affect the expression level of *Adipoq* mRNA in the WAT, and thus plasma concentrations of Adipoq, under CR conditions.

In contrast, in the present experiment, HFD feeding did not affect the expression level of mouse *Adipoq* mRNA in the WAT in WT mice, when compared with the RD feeding, although the HFD feeding induced obesity. The present findings are in accord with those reported by Qiao et al. (Qiao, 2011). Although fasting glucose levels were significantly elevated in WT-HFD

compared with WT-RD mice, the insulin sensitivity assessed by Quicki did not significantly differ between the two groups. An experiment using male C57BL/6J mice in experimental settings (age of mice and duration of the experiment) similar to the present study demonstrated that a high fat diet induced obesity with a significant reduction of *Adipoq* mRNA in the WAT while not affecting the plasma Adipoq (Barnea, 2006). The calories from fat in the HFD in the present study were 36%, whereas this was 42% in the study reported by Barnea. The sources of fat were also different; cacao butter and lard in the present study but soybean oil and palm stearin in Barnea's study. Therefore, the composition of the HFD may also affect the expression of *Adipoq* in the WAT, although HFD feeding induces obesity regardless of the fat composition.

Adipogenesis is driven by a cascade of transcription factors including Ppara, Pparg, and Ppargc1a (or PGC-1a). During adipogenesis, Ppara and Ppargc1a drive a thermogenic brown or beige phenotype (Peirce V, 2014). *Ppara* mRNA levels were elevated in Adipoq-RD and Adipoq-HFD mice compared with WT-RD and WT-HFD mice ($p = 0.0007$, $p < 0.0001$; Fig. 3E); the levels in WT-CR mice did not statistically differ from those in WT-RD mice ($p = 0.1265$). The level of *Pparg* mRNA was increased in WT-CR mice compared with WT-RD mice; there was no such increase in Adipoq mice (Fig. 3F). The expression level of *Ppargc1a* mRNA was significantly elevated in Adipoq-RD and Adipoq-HFD and WT-CR mice compared with the respective

control groups ($p = 0.0068$, $p < 0.0001$, $p = 0.0114$, respectively; Fig. 3G).

In CR mice, fatty acid synthesis is elevated in the WAT within 6 hours following provision of food, relative to that in the fasting phase, which enables the animals to utilize fatty acids as an energy source in the fasting phase prior to the next feeding (Bruss, 2010). CR augmented mRNA expression levels of a glucose transporter (*Glut4*, $p < 0.0001$; Fig. 3H) and a fatty acid transporter (*Fatp/Slc27a*, $p < 0.0001$; Fig. 3I). In Adipoq mice, although there could be an increase in the level of *Glut4* mRNA in Adipoq-HFD compared with that in WT-HFD mice ($p = 0.050$), this was not significant in Adipoq-WT mice (Fig. 3H). *Fatp*/*Slc27a1* mRNA was upregulated in Adipoq mice compared with WT mice, particularly in the HFD group ($p = 0.0011$; Fig. 3I). Taken together, these results show that some of the genes associated with adipogenesis and/or energy metabolism which are regulated by Adipoq change in the same direction as is seen in response to CR in mice.

Because many studies have reported that CR increases the plasma concentration of Adipoq in mice and rats (Berg, 2001; Zhu, 2004; Qiao, 2011), the present findings emphasize a CR-related protective role for Adipoq in insulin resistance and glucose intolerance occurring in high-calorie-induced obese animals, as well as in aging animals fed RD. To summarize briefly, the present study demonstrated that Adipoq overexpression mimics the metabolic effects of CR through down-regulation of p53 in the WAT. The findings also suggest that p53 and Adipoq are important factors in the effect

of CR on energy metabolism.

3.5. Survival and ER stress in response to endotoxin

Resistance to a variety of stressors is a hallmark of CR in animals (Masoro, 1998). We assessed the survival rate after an inflammatory challenge induced by injection of a high dose of lipopolysaccharide (LPS). In this experiment, heterozygous Adipoq mice were used. These were generated by mating ESTRAP mice with Adipoq mice, and thus enabled us to monitor ER stress *in vivo*. In these mice, the plasma concentration of human Adipoq was less than 50% of that seen in mice homozygous for the human *ADIPOQ* gene (Supplemental Table 1). However, the plasma concentration of mouse Adipoq was also significantly greater in Adipoq:ESTRAP-RD mice than in WT:ESTRAP-RD mice. All WT:ESTRAP-RD mice were found dead within 2.5 days after LPS injection (Fig. 4A). The survival rates were significantly greater in Adipoq:ESTRAP-RD and WT:ESTRAP-CR mice than that in WT:ESTRAP-RD mice ($p = 0.0009$, $p = 0.0043$ by log-rank test respectively: Fig. 4A). The survival rates showed a tendency to be greater in WT:ESTRAP-CR mice than in Adipoq:ESRTAP-RD mice ($p = 0.0628$ by log-rank test).

We also monitored ER stress during the LPS experiment using ESTRAP mice (Kitamura M, 2011), because Adipoq is reported to lower ER stress and inflammation (Boddu, 2014). In the WT:ESTRAP-RD mice, ER stress is known to reduce SEAP activity in serum (Kitamura, 2011). The

SEAP activity was sharply reduced between 0 and 8 h after LPS administration, at which point it started to increase again, in WT:ESTRAP-CR and Adipoq:ESTRAP-RD mice (Fig. 4B). However, in WT:ESTRAP-RD mice, there was no evidence of a recovery of SEAP activity even after 24 h. The SEAP activity was significantly greater in WT:ESTRAP-CR mice than in Adipoq:ESTRAP-RD and WT:ESTRAP-RD mice ($p = 0.0017$, $p = 0.0002$), indicating that ER stress was significantly reduced by CR; the impact of Adipoq was minor compared with that of CR. Because the plasma level of mouse Adipoq was lower in WT:ESTRAP-CR mice than in Adipoq:ESTRAP-RD mice (Supplemental Table 1), factors other than Adipoq could be attributable to the stress resistance induced by CR. Even though the contribution of Adipoq to the stress resistance resulting from CR could be minor, the present findings regarding survival rate and SEAP levels suggest that Adipoq could play a part in stress resistance in mice.

3.6. Tumor growth

To test the anti-tumor effect of Adipoq, Lewis lung carcinoma cells were subcutaneously implanted in six-month-old mice fed a RD or HFD ad libitum. Tumor size and weight were not significantly reduced in Adipoq-RD or Adipoq-HFD mice compared with the respective groups of WT mice (Fig. 5A & B); in contrast, the tumor size and weight appeared to be increased in Adipoq-RD mice, compared with WT-RD mice, although this was not

statistically significant ($p = 0.5273$ for tumor size and $p = 0.3392$ for tumor weight; the total number of mice needed to reduce the variance of the tumor weights sufficiently to achieve a significant result when $\alpha = 0.05$ with 50% power is estimated to be 84 (thus $n = 21$ for each group) using a power analysis). Thus, we conclude that Adipoq overexpression does not exert the anti-tumor effect seen in CR, at least in this allograft model. In the present study, the anti-tumor effect of CR was not assessed because of limitations on animal use. However, in the allograft model of Lewis lung carcinoma cells, reduced calorie intake has been reported to inhibit tumor growth significantly (Yakar S, 2006). In tumors formed in the subcutaneous tissue, we measured specific receptors for Adipoq, Adipor1 and Adipor2 (Fig. 5C & D). HFD decreased *Adipor2* mRNA levels in WT and Adipoq mice ($p = 0.0367$, 0.0158; Fig. 5D), but not those of *Adipor1* (Fig. 5C). However, there was no difference in the expression levels of *Adipor1* and *Adipor2* between Adipoq and WT mice. We also analyzed mRNA expression levels of p16 and p21, downstream effectors of the tumor suppressor p53, in the tumor tissues (Fig. 5E & F). Adipoq significantly reduced the p16 mRNA expression level in HFD mice ($p = 0.0152$; Fig. 5E) but not in RD mice. Thus, it is unlikely that Adipoq affects tumor growth through modulation of Adipor signaling and p16 and/or p21. Although genetic variations in the Adipoq gene are reported to be associated with increased risk of breast and prostate cancer and decreased risk for colorectal cancer, the involvement of Adipoq in lung cancer pathogenesis

remains unclear (Ntikoudi, 2014). The present study is limited to an allograft model. Thus, the anti-tumor effect of Adipoq should be further investigated.

3.7. Summary

The present study indicates that Adipoq overexpression mimics the metabolic effects and stress resistance induced by CR at least in part, if not completely (Table 2). In the present experimental setting, HFD feeding did not confound most of the beneficial effects of Adipoq, even compared with RD feeding. Regarding the metabolic effects, the present findings suggest the sequence of Adipoq, p53, and senescence-like phenotypes in the WAT and their involvement in the underlying mechanisms of the anti-aging effect of CR. The experiment investigating endotoxin shock also suggests a role for Adipoq in the enhancement of stress resistance by CR with amelioration of ER stress. Whether Adipoq has any role in the neoplastic process, however, remains unclear.

Figure Legends

Figure 1. Body weight and food consumption of adiponectin (Adipoq) and wild type (WT) mice fed a regular diet (RD) or high-fat diet (HFD) ad libitum. (A) The body weight of Adipoq mice and WT mice. The data represent means and standard errors (n = 8 or 9). ***, $p < 0.001$ versus WT-RD as determined by 2f ANOVA with Tukey's HSD test. (B) The average amount of food consumed by a single mouse fed a RD. The values were estimated by the total amount of food consumed in each cage divided by the number of mice in that cage. The data represent means and standard errors (n = 3 or 4 cages). (C) The average amount of food consumed by a single mouse fed a HFD. The values were estimated by the total amount of food consumed in each cage divided by the number of mice in that cage. The data represent means and standard errors (n = 3 or 4 cages). (D) The body weight of endoplasmic reticulum (ER) stress-responsive alkaline phosphatase (ESTRAP) mice. Adipoq:ESTRAP-RD represents a group of double transgenic mice for Adipoq and ESTRAP genes fed a RD ad libitum. WT:ESTRAP-RD represents a group of wild type mice for the Adipoq gene in the ESTRAP background fed a RD ad libitum. WT-ESTRAP-CR mice were fed a RD at a level 30% less than the average amount of food intake in WT:ESTRAP-RD mice. ***, $p < 0.001$ versus ESTRAP:WT-RD, as determined by 2f ANOVA with Tukey's HSD test at individual time points. Adipoq:ESTRAP-RD mice weighed slightly less compared with

WT:ESTRPA-RD mice (2f ANOVA, $p < 0.0001$), although there was no significant difference between the two groups at individual time points.

Figure 2. Adipoq and CR reduce p53 and p21 expression in the epididymal fat tissue as does CR. (A) A representative image of western blotting for p53, p21, and control (β-actin) in the epididymal adipose tissue. All blots for the quantitative analysis are shown in Supplemental Figure 1. (B) Quantitative data of p53 normalized to β-actin. (C) Quantitative data of p21 normalized to β-actin. Bars and lines in (B) and (C) represent means and standard errors ($n = 3$). *** $p < 0.001$ compared with WT-RD, as determined by 1f ANOVA with Tukey's HSD test. ###, $p < 0.001$ compared with WT-HFD. (D) mRNA expression levels of *Cd68* normalized to *18S* mRNA. (E) mRNA expression levels of *Ccl2* normalized to *18S* mRNA. Bars and lines in (D) and (E) represent means and standard errors ($n = 8$ except for WT-CR ($n = 5$)). ** $p < 0.01$, *** $p < 0.001$ compared with WT-RD, as determined by 1f ANOVA with Tukey's HSD test. # $p < 0.05$, ### $p < 0.001$ compared with WT-HFD.

Figure 3. Adipoq regulates mRNA levels of adipokine, adipogenesis and/or metabolism genes in the same directions as changes induced by CR. Data represent means and standard errors ($n = 8$ except for WT-CR ($n = 5$)). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$ versus WT-RD, as determined by 1f ANOVA with Tukey's HSD test. & $p = 0.05$, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$

compared with WT-HFD. (A) *Adipor1*, Adiponectin receptor 1. (B) *Adipor2*, Adiponectin receptor 2. (C) *Adipoq*, mouse adipinectin. (D) *Lep*, Leptin. (E) *Ppara*, Peroxisome proliferator activated receptor alpha. (F) *Pparg*, Peroxisome proliferator activated receptor gamma. (G) *Ppargc1a*, Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha. (H) *Glut4*, Solute carrier family 2 (facilitated glucose transporter), member 4, (I) Fatp/*Slc27a1*, Solute carrier family 27 (fatty acid transporter), member 1.

Figure 4. Adipoq and CR mice exhibit stress resistance. (A) Survival curves after lipopolysaccharide (LPS) injection. Adipoq:ESTRAP-RD and WT:ESTRAP-RD mice fed a RD ad libitum. WT:ESTRAP-CR mice fed a 30% calorie-restricted RD compared with ad libitum feeding. The initial number of mice in each group was 7. ** $p < 0.01$, *** $p < 0.001$ versus WT:ESTRAP-RD, as determined using the log-rank test. Note, $p = 0.0628$, Adipoq:ESTRAP-RD vs WT:ESTRAP-CR by log-rank test. (B) Reduction in the rate of secreted alkaline phosphatase (SEAP) activity after LPS injection. Data represent mean ± SE (n = 7 for each group at 0, 2, 4, 8 h: n = 6 for WT:ESTRAP-RD & WT:ESTRAP-CR at 24 h: n = 6 for WT:ESTRAP-CR, n = 7 for Adipoq:ESTRAP-RD at 48 h). *** $p < 0.001$ versus WT: ESTRAP-RD, as determined by 2f ANOVA with Tukey's HSD test using the data between 2 and 24 h.

Figure 5. Adiponectin (Adipoq) does not inhibit tumor growth in the allograft model of Lewis lung carcinoma cells. The data represent means and standard errors. (A) The wet weight of tumors grown in the subcutaneous tissue (n = 9 for WT-RD and Adipoq-RD; WT-HFD for n = 10 and n = 8 for Adipoq-HFD). (B) Tumor sizes were estimated by the following formula, length × width × height × 0.52 (n = 9 for WT-RD and Adipoq-RD; WT-HFD for n = 10 and n = 8 for Adipoq-HFD). (C) mRNA expression levels of adiponectin receptor 1 (*Adipor1*) (n = 6). (D) mRNA expression levels of adiponectin receptor 2 (*Adipor2*) (n = 6). * $p < 0.05$ versus (vs) respective RD groups. (E) mRNA expression levels of p16 (n = 6). # $p < 0.05$ vs WT-HFD group. (F) mRNA expression levels of p21 (n = 6).

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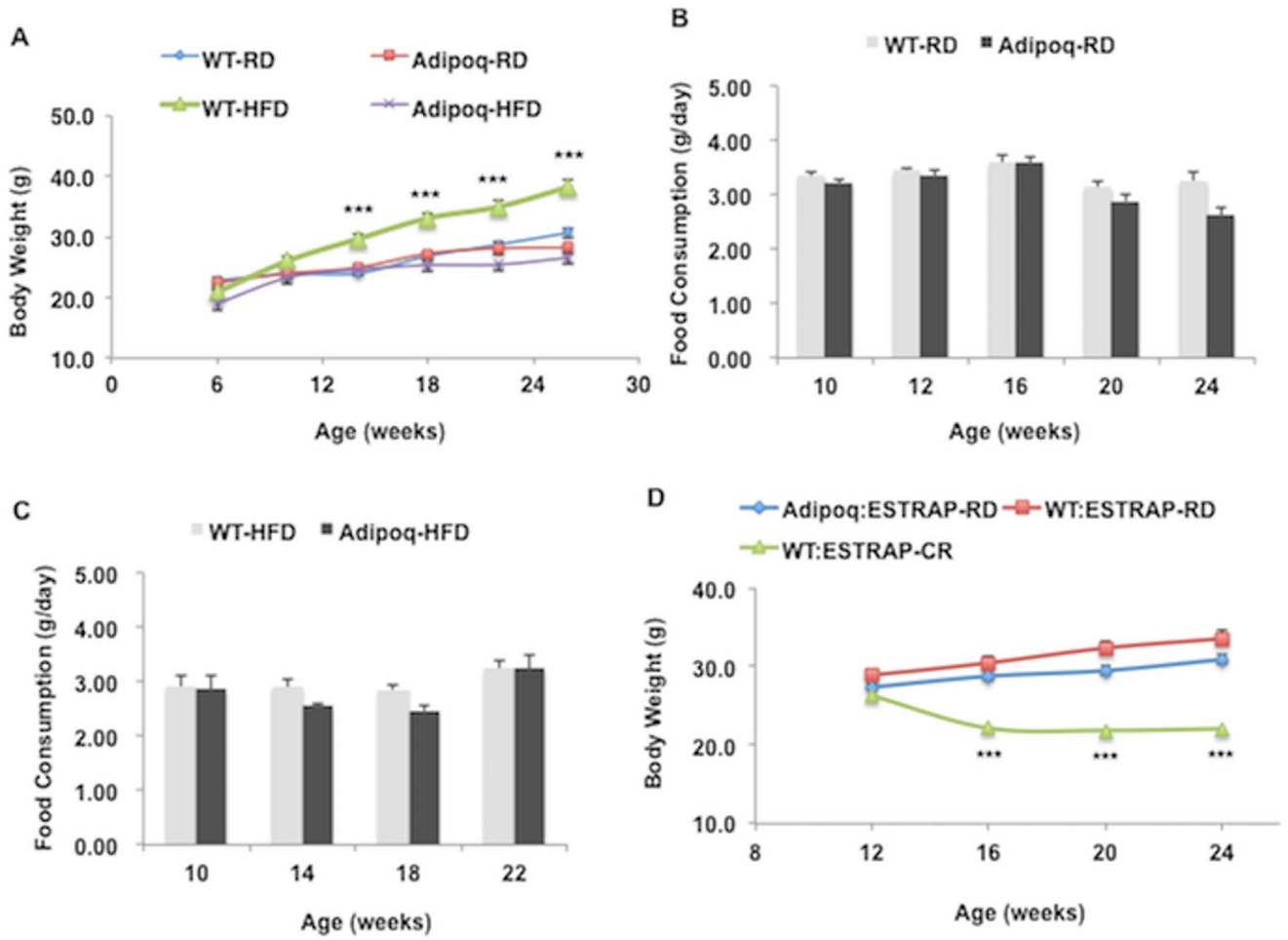
Fig. 1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WT-RD
Adipoq-RD
WT-HFD
Adipoq-HFD
Body Weight (g)
50.0
40.0
30.0
20.0
10.0
0
6
12
18
24
30
Age (weeks)
***
B
WT-RD
Adipoq-RD
Food Consumption (g/day)
5.00
4.00
3.00
2.00
1.00
0.00
10
12
16
20
24
Age (weeks)
C
WT-HFD
Adipoq-HFD
Food Consumption (g/day)
5.00
4.00
3.00
2.00
1.00
0.00
10
14
18
22
Age (weeks)
D
Adipoq:ESTRAP-RD
WT:ESTRAP-RD
WT:ESTRAP-CR
Body Weight (g)
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30.0
20.0
10.0
8
12
16
20
24
Age (weeks)
***

Fig. 2

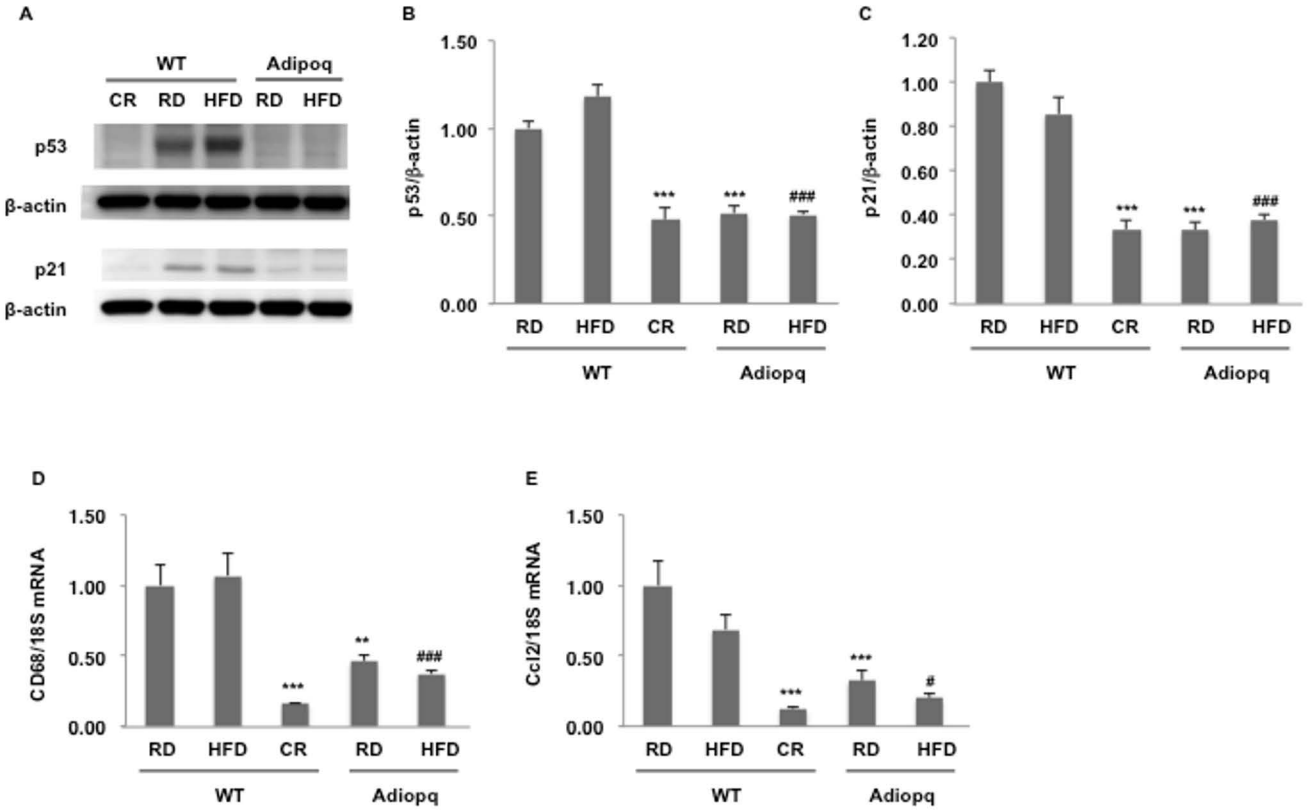
A
WT
Adipoq
CR
RD
HFD
RD
HFD
p53
β-actin
p21
β-actin
B
p53/β-actin
1.50
1.00
0.50
0.00
***
***
###
RD
HFD
CR
RD
HFD
WT
Adiopq
C
p21/β-actin
1.20
1.00
0.80
0.60
0.40
0.20
0.00
***
***
###
RD
HFD
CR
RD
HFD
WT
Adiopq
D
CD68/18S mRNA
1.50
1.00
0.50
0.00
***
**
###
RD
HFD
CR
RD
HFD
WT
Adiopq
E
Ccl2/18S mRNA
1.50
1.00
0.50
0.00
***
***
#
RD
HFD
CR
RD
HFD
WT
Adiopq

Fig. 3

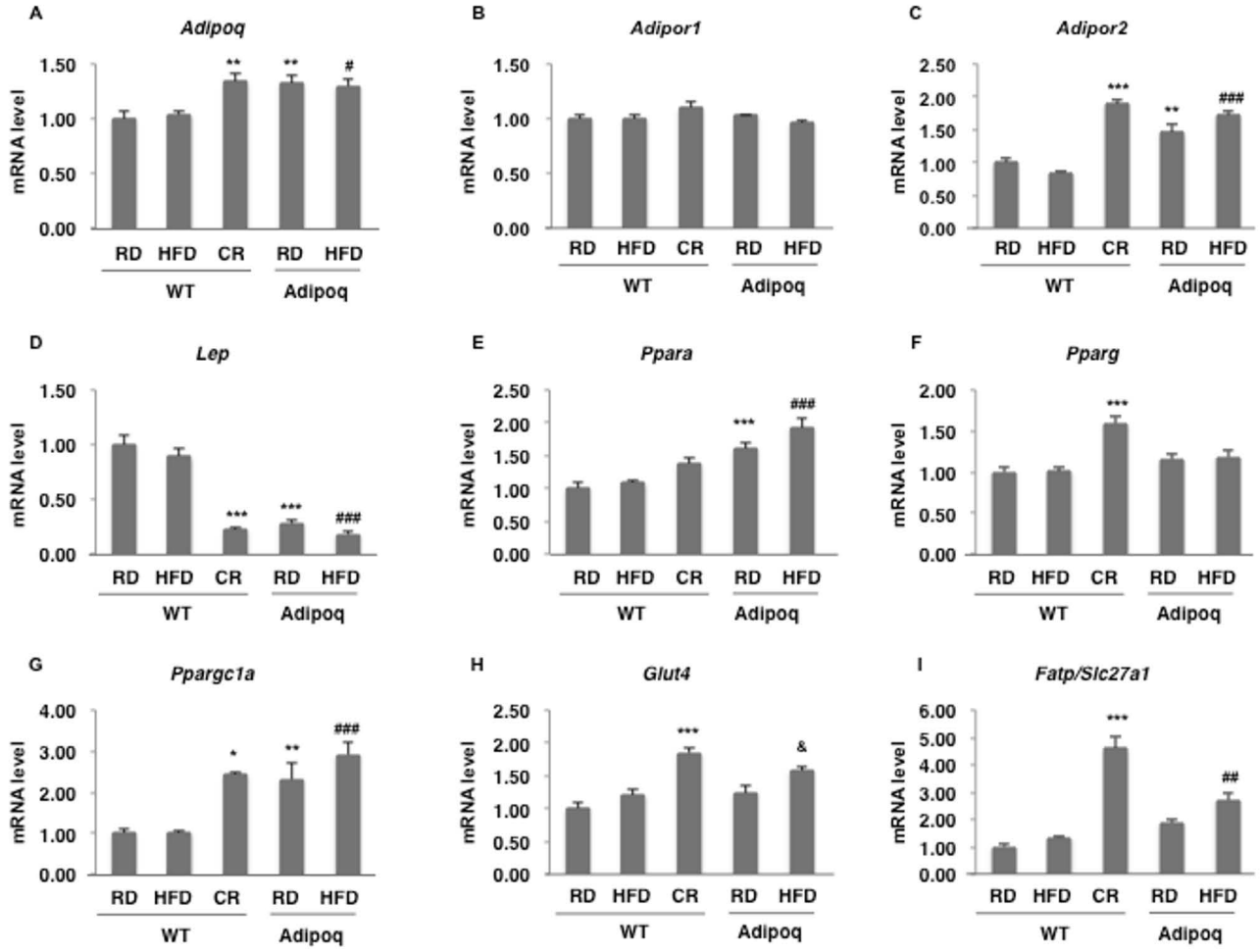
A
Adipoq
mRNA level
1.50
1.00
0.50
0.00
**
**
#
RD HFD CR RD HFD
WT
Adipoq
B
Adipor1
mRNA level
1.50
1.00
0.50
0.00
RD HFD CR RD HFD
WT
Adipoq
C
Adipor2
mRNA level
2.50
2.00
1.50
1.00
0.50
0.00
***
**
###
RD HFD CR RD HFD
WT
Adipoq
D
Lep
mRNA level
1.50
1.00
0.50
0.00
***
***
###
RD HFD CR RD HFD
WT
Adipoq
E
Ppara
mRNA level
2.50
2.00
1.50
1.00
0.50
0.00
***
###
RD HFD CR RD HFD
WT
Adipoq
F
Pparg
mRNA level
2.00
1.50
1.00
0.50
0.00
***
RD HFD CR RD HFD
WT
Adipoq
G
Ppargc1a
mRNA level
4.00
3.00
2.00
1.00
0.00
*
**
###
RD HFD CR RD HFD
WT
Adipoq
H
Glut4
mRNA level
2.50
2.00
1.50
1.00
0.50
0.00
***
&
RD HFD CR RD HFD
WT
Adipoq
I
Fatp/Slc27a1
mRNA level
6.00
5.00
4.00
3.00
2.00
1.00
0.00
***
##
RD HFD CR RD HFD
WT
Adipoq

Fig. 4

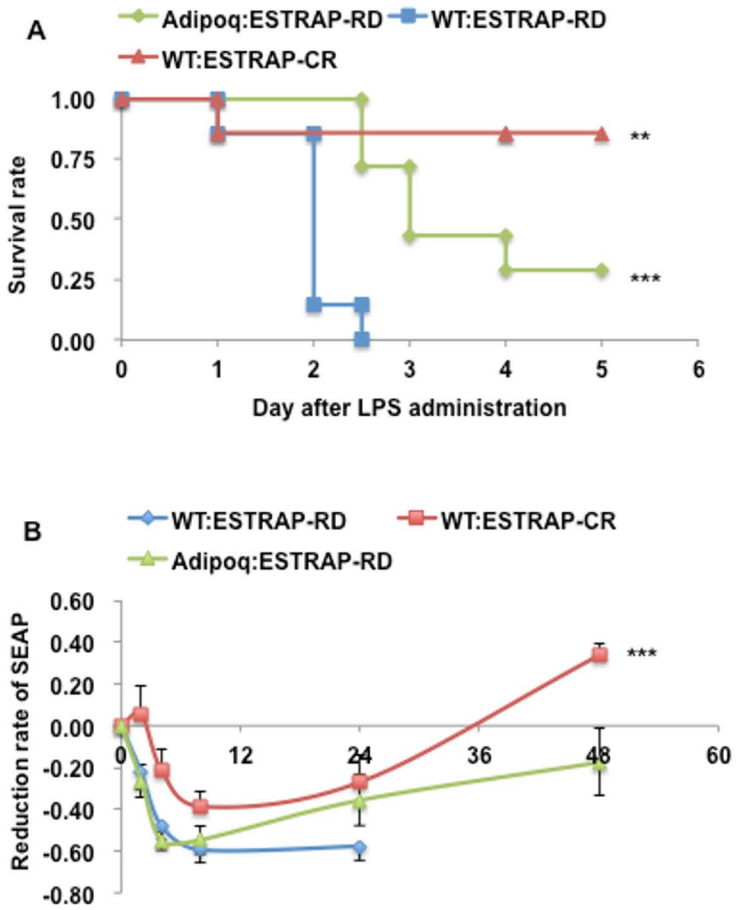
A
Adipoq:ESTRAP-RD
WT:ESTRAP-RD
WT:ESTRAP-CR
Survival rate
1.00
0.75
0.50
0.25
0.00
0
1
2
3
4
5
6
Day after LPS administration
**
***
B
WT:ESTRAP-RD
WT:ESTRAP-CR
Adipoq:ESTRAP-RD
Reduction rate of SEAP
0.60
0.40
0.20
0.00
-0.20
-0.40
-0.60
-0.80
0
12
24
36
48
60
***

Fig. 5

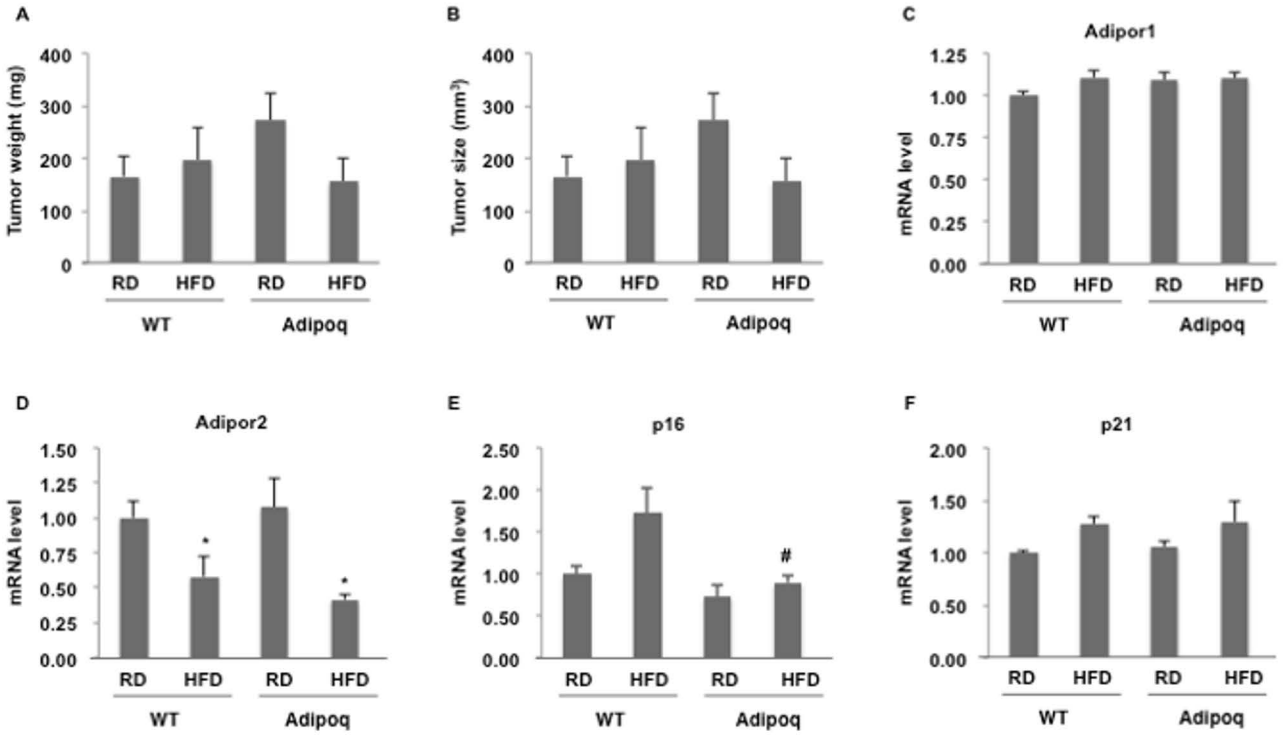
A
Tumor weight (mg)
400
300
200
100
0
RD
HFD
RD
HFD
WT
Adipoq
B
Tumor size (mm³)
400
300
200
100
0
RD
HFD
RD
HFD
WT
Adipoq
C
Adipor1
mRNA level
1.25
1.00
0.75
0.50
0.25
0.00
RD
HFD
RD
HFD
WT
Adipoq
D
Adipor2
mRNA level
1.50
1.25
1.00
0.75
0.50
0.25
0.00
*
*
RD
HFD
RD
HFD
WT
Adipoq
E
p16
mRNA level
2.50
2.00
1.50
1.00
0.50
0.00
#
RD
HFD
RD
HFD
WT
Adipoq
F
p21
mRNA level
2.00
1.50
1.00
0.50
0.00
RD
HFD
RD
HFD
WT
Adipoq

Table 1. Blood glucose and insulin concentrations and Quicki in Adipoq and WT mice.

	WT-RD	**WT-HFD**	**WT-CR**	**Adipoq-RD**	**Adipoq-HFD**
Glucose (mg/dL)	152 ± 6	193 ± 5***	110 ± 5***	145 ± 6	135 ± 5$^{\#\#\#}$
Insulin (ng/mL)	1.31 ± 0.13	1.59 ± 0.27	0.58 ± 0.06**	0.70 ± 0.01*	0.39 ± 0.07$^{\#\#\#}$
Quicki	0.271 ± 0.003	0.260 ± 0.005	0.313 ± 0.004***	0.293 ± 0.002**	0.323 ± 0.007$^{\#\#\#}$

Data represent means ± standard errors (n = 8). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with WT-RD as determined by Tukey's HSD test. ### $p < 0.001$, compared with WT-HFD. Quicki, quantitative insulin-sensitivity check index (Muniyappa, 2008). Adiponectin (Adipoq) and wild type (WT) mice fed a regular diet (RD) or a high-fat diet (HFD) ad libitum. WT-CR, WT mice fed a RD diet at a level 30% less than the ad libitum level consumed by WT-RD mice. Blood sampling was performed during the fasting phase (refer to the methods section for details).

Table 2. Summary of study findings; similarities between the effects of calorie restriction (CR) and overexpression of adiponectin (Adipoq)

	CR	Adipoq
Insulin sensitivity	increase	increase
Cellular senescence-like changes		
p53	decrease	decrease
p21	decrease	decrease
proinflammatory cytokines	decrease	decrease
Genes related to energy metabolism in WAT		
Adipoq	increase	increase
Adipor1	no change	no change
Adipor2	increase	increase
Lep	decrease	decrease
Ppara	no change	increase
Pparg	increase	no change
Ppargc1a	increase	increase
Glut4	increase	increase (HFD)
Fatp/Slc27a1	increase	increase (HFD)
Stress resistance		
survival	increase	increase
ER stress	decrease	NS
Tumor growth	ND	no effect

WAT, white adipose tissue. Increase (HFD), the mRNA was increased in the Adipoq-HFD group, but not in the Adipoq-RD group. NS, not significant. ND, not determined in the present study.