



Published in final edited form as:

*Int J Obes (Lond)*. 2015 June ; 39(6): 967–976. doi:10.1038/ijo.2015.23.

## Resveratrol induces brown-like adipocyte formation in white fat through activation of AMP-activated protein kinase (AMPK) $\alpha$ 1

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### Abstract

**Objective**—Development of brown-like/beige adipocytes in white adipose tissue (WAT) helps to reduce obesity. Thus, we investigated the effects of resveratrol, a dietary polyphenol capable of preventing obesity and related complications in humans and animal models, on brown-like adipocyte formation in inguinal WAT (iWAT).

**Methods**—CD1 female mice (5-month-old) were fed a high-fat diet with/without 0.1% resveratrol. In addition, primary stromal vascular cells separated from iWAT were subjected to resveratrol treatment. Markers of brown-like (beige) adipogenesis were measured and the involvement of AMP-activated protein kinase (AMPK)  $\alpha$ 1 was assessed using conditional knockout.

**Results**—Resveratrol significantly increased mRNA and/or protein expression of brown adipocyte markers including uncoupling protein 1 (UCP1), PR domain-containing 16 (PRDM16), Cell death-inducing DFFA-like effector A (Cidea), elongation of very long chain fatty acids protein 3 (Elovl3), peroxisome proliferator-activated receptor- $\gamma$  coactivator 1 $\alpha$  (PGC1 $\alpha$ ), cytochrome C and pyruvate dehydrogenase (PDH) in differentiated iWAT stromal vascular cells (SVC), suggesting that resveratrol induced brown-like adipocyte formation *in vitro*. Concomitantly, resveratrol markedly enhanced AMPK $\alpha$ 1 phosphorylation and differentiated SVC oxygen consumption. Such changes were absent in cells lacking AMPK $\alpha$ 1, showing that AMPK $\alpha$ 1 is a critical mediator of resveratrol action. Resveratrol also induced beige adipogenesis *in vivo* along with the appearance of multiocular adipocytes, increased UCP1 expression and enhanced fatty acid oxidation.

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The authors declare no conflicts of interest.

**Conclusion**—Resveratrol induces brown-like adipocyte formation in iWAT via AMPK $\alpha$ 1 activation and suggest that its beneficial anti-obesity effects may be partly due to the browning of WAT and as a consequence, increased oxygen consumption.

### Keywords

beige adipocytes; obesity; resveratrol; stromal vascular cells; uncoupling protein 1; white adipose tissue

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### Introduction

Mammals have two morphologically and functionally distinct types of adipose tissue, white adipose tissue (WAT) and brown adipose tissue (BAT), both of which are involved in energy homeostasis. WAT mainly stores energy in the form of lipids (triglycerides) in unilocular white adipocytes and secretes a number of adipokines and other factors, such as leptin, adiponectin, tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), and interleukin-6 (IL-6), to regulate energy metabolism and immune function<sup>1,2</sup>. Excessive WAT accumulation that occurs in obesity is a major risk factor for developing insulin resistance, type 2 diabetes mellitus and cardiovascular diseases<sup>3,4</sup>. In contrast, BAT specializes in dissipating energy as heat due to its high mitochondrial content and expression of uncoupling protein 1 (UCP1)<sup>5,6</sup>. However, the amount of BAT found in adults is typically quite low<sup>7</sup>. Recently, brown-like adipocytes were discovered in WAT, so called beige adipocytes<sup>8,9</sup>. Similar to brown adipocytes, beige adipocytes express UCP1 to dissipate energy. Thus, stimulating the development of beige adipocytes in WAT, so called ‘browning’, might reduce adverse effects of WAT and could help to improve metabolic health<sup>10-12</sup>.

There are many transcriptional regulators including PRDM16, PGC1 $\alpha$ , C/EBP $\alpha$  and PPAR $\gamma$ , as well as various secreted mediators, such as bone morphogenetic protein 7 (BMP7), Irisin, fibroblast growth factor 21 (FGF21), atrial and brain natriuretic peptides, that can induce the formation of brown-like adipocytes<sup>6,10,11,13,14</sup>. Meanwhile, certain pharmacological and nutritional agents, are also involved in promoting WAT browning<sup>15,16</sup> by activating transcription factors or related regulatory signaling pathways<sup>17</sup>. As a nutritional or dietary supplement, resveratrol, a natural polyphenol present in the skin of grapes and other plants, has remarkable beneficial effects on energy metabolism and related disorders in mammals<sup>18,19</sup>. It has been reported that resveratrol protects against high-fat diet induced obesity in mice<sup>20,21</sup> and elicits beneficial effects on obese persons<sup>22,23</sup>. Resveratrol also inhibits adipogenesis<sup>24-26</sup> and enhances fat mobilization<sup>27-29</sup>. Resveratrol increased UCP1 expression in 3T3-L1 cells<sup>25</sup>, and enhanced the mitochondrial DNA content and UCP1 expression in primary mouse embryonic fibroblasts (MEF)-derived adipocytes<sup>30</sup>. To date, studies regarding resveratrol in adipose tissue mainly focus on the white adipogenesis and lipid metabolism, and the effects of resveratrol on the formation of brown-like or beige adipocytes remains sparsely studied. To our knowledge, there is no report about mechanisms in which resveratrol induces the formation of brown-like adipocytes.

The effects of resveratrol on metabolic health are due, at least in part, to its ability to activate the AMP-activated protein kinase (AMPK), a master regulator of energy metabolism<sup>15, 19</sup>. AMPK consists of one  $\alpha$ -catalytic subunit and two regulatory subunits,  $\beta$  and  $\gamma$ . The catalytic subunit of AMPK has two isoforms,  $\alpha 1$  and  $\alpha 2$ , which have different tissue expression patterns. In adipose tissue, the  $\alpha 1$  catalytic subunit is the predominant isoform expressed<sup>31, 32</sup>, while the  $\alpha 2$  isoform is highly expressed in muscle and liver but at a low level in adipose tissue<sup>33, 34</sup>. The measurement of AMPK activity indicates that the  $\alpha 1$  isoform accounts for the majority of the total activity of this kinase in adipose tissue<sup>32, 35</sup>. Although there are a number of studies on the role of AMPK in adipose tissue metabolism<sup>21, 36, 37</sup>, it is unclear whether AMPK is involved in the browning of white adipocytes.

In the present study, we sought to elucidate the role of resveratrol in brown-like adipocyte formation in WAT and to explore the mechanism underlying this process. Our data show that resveratrol induces browning of white fat, a process mediated by AMPK $\alpha 1$ .

## Materials and methods

### Animals

Twelve adult CD1 female mice (5-month-old) were randomly divided into 2 groups: a control group, which was fed a high-fat diet (HFD; 45% energy from fat, D12451, Research Diet, New Brunswick, NJ), a resveratrol (Resv) group, which was fed a HFD containing 0.1% (w/w) resveratrol. Mice were housed in environmentally controlled rooms on a 12-h light-dark cycle with free access to food and water. Before and after the treatment, we measured the basal metabolic rate (BMR) (oxygen consumption ( $VO_2$ ),  $CO_2$  production ( $VCO_2$ ) and respiratory exchange ratio (RER)) of mice during the day (quiescent phase) using a CLAMS (Columbus Instruments, Columbus, OH) indirect open circuit calorimetry system. We deprived the mice of food for 4 h prior to measurement and continuously measured for 3 h (with water provided), taking a measurement every 30 s<sup>38, 39</sup>. We used the lowest 10 consecutive measures (5 min) as the estimate of BMR.

Body weight and food intake were measured weekly. At the end of 4 weeks of treatment, mice were sacrificed by carbon dioxide anesthesia. Inguinal WAT (iWAT) was rapidly isolated and weighed. One side of the adipose tissues were frozen in liquid nitrogen and stored at  $-80^\circ C$  until further analyses. A middle portion of the other side was fixed in 4% paraformaldehyde for sectioning and staining. Another portion of the other side was cultured in DMEM/F12 medium for tissue oxygen consumption measurement. Wild type and Rosa<sup>Cre</sup>/AMPK $\alpha 1$ <sup>flox/flox</sup> C57BL/6 mice (Jackson Lab, Bar Harbor, Maine) were housed in environmentally controlled rooms on a 12-h light-dark cycle with free access to food and water. All animal experiments and care procedures were performed according to protocols pre-approved by the Institutional Animal Care and Use Committees (IACUC) at Washington State University.

## Antibodies and chemicals

Antibodies against AMPK $\alpha$  (#2532), phospho-AMPK $\alpha$  at Thr172 (#2535), pyruvate dehydrogenase (PDH) (#2784), cytochrome c (#4280),  $\beta$ -tubulin (#2146) and goat anti-rat antibody Alexa Fluor 488 (#4416) were purchased from Cell Signaling (Danvers, MA). Anti-PRDM16 polyclonal antibody (#ABD130) was purchased from Millipore (Billerica, MA). Anti-UCP1 polyclonal antibody (#sc28766) was bought from Santa Cruz Biotechnology (Dallas, TX). Goat anti-rabbit IRDye 800CW (#926-32211) and goat anti-rabbit IRDye 680RD (#926-68070) secondary antibodies for western blot were purchased from LI-COR (Lincoln, NE). Fluoro-Gel II with 40, 6-diamidino-2-phenylindole (DAPI) (#17985-50) was purchased from Electron Microscopy Sciences (Hatfield, PA). Insulin, dexamethasone, indomethacin, 3-isobutyl-1-methylxanthine (IBMX), Triiodothyronine (T3), Oil-Red O, and compound C were purchased from Sigma (St. Louis, MO). Collagenase D and dispase II were purchased from Roche Diagnostics (Indianapolis, IN). DMEM/F12 and fetal bovine serum (FBS) were purchased from Life Technologies (Grand Island, NY).

## Stromal vascular cell (SVC) isolation and *in vitro* differentiation

SVC were isolated from iWAT as previously described<sup>40</sup>. The medium was changed every other day<sup>41</sup>. To induce brown adipogenic differentiation of SVC, confluent SVC were cultured in DMEM/F12 containing 10% FBS, 1% penicillin-streptomycin solution with 5  $\mu$ g/ml insulin, 1 nM T<sub>3</sub>, 1  $\mu$ M dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 0.125 mM indomethacin for 2 days. The cells were then switched to DMEM/F12 supplemented with 10% FBS and 5  $\mu$ g/ml insulin for 5 more days and the medium was changed every other day<sup>42</sup>. For SVC from iWAT of weaning Rosa26<sup>Cre</sup>/AMPK $\alpha$ 1<sup>flox/flox</sup> mice, confluent SVC were treated with 250 nM 4-hydroxytamoxifen (4-OHT) for 2 days to delete AMPK $\alpha$ 1 before being induced to undergo brown adipogenic differentiation<sup>43</sup>.

## *In vitro* O<sub>2</sub> consumption assay

*In vitro* O<sub>2</sub> consumption measurement was performed with Thermo Scientific Orion 3-Star Dissolved Oxygen meter and probe (Thermo Electron Corporation, Madison, WI)<sup>44</sup>. Equal numbers of iWAT SVC were seeded and treated with vehicle (control) or 10  $\mu$ M resveratrol (Resv) to induce differentiation. On day 7, the differentiated SVC were changed to fresh DMEM/12 for 30 min. The dissolved oxygen (DO) in the medium were measured at the start and end of incubation. For the tissues, a thin slice (50 mg) of iWAT from control and resveratrol fed mice were cultured in medium for 1 h, and DO was measured before and after incubation. O<sub>2</sub> consumption of differentiated SVC or iWAT were calculated as the rate of decrease in DO<sup>45</sup>.

## Oil-Red O staining

Differentiated cells were subjected to Oil-Red O staining as previously described<sup>46</sup>.

## Immunostaining of cells and tissue sections

Immunofluorescence staining of cells was conducted as previously described<sup>43</sup>. Fluorescence was examined and images were acquired using an EVOS fl fluorescence microscope (Advanced Microscopy Group, Bothell, WA). As for iWAT, paraffin-embedded

iWAT sections (5  $\mu\text{m}$  thick) were either stained with hematoxylin and eosin (H&E)<sup>47</sup> or used for UCP1 immunohistochemical (IHC) staining<sup>48</sup>. Adipocyte diameters were analyzed by Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD).

### Real-time quantitative PCR

Total RNA was extracted from differentiated iWAT SVC cells using TRIzol reagent (Sigma, St. Louis, MO) according to the manufacturer's protocol and cDNA was synthesized from 0.5  $\mu\text{g}$  of total RNA using a reverse transcription kit (Bio-Rad, Hercules, CA). Real-time quantitative PCR was carried out in the final 10  $\mu\text{l}$  volume of the amplification mixture containing 2x Qprecise Green Master Mix (EarthOx, LLC, San Francisco, CA), primers, and cDNA using a CFX RT-PCR detection system (Bio-Rad). cycle threshold (CT) was used to calculate the differences between the target CT value and the control (18S) for each sample:  $\Delta\text{CT} = \text{CT}(\text{target}) - \text{CT}(\text{control})$ . The relative expression level was calculated using  $2^{-\Delta\text{CT}}$ . The following cycle parameters were used: 40 two-step cycles of 95°C for 15 s, 58°C for 60 s<sup>46</sup>. Primer sequences (with their respective PCR fragment lengths) were shown in Table 1.

### Western blot analysis

Western blot was conducted as previously described<sup>46</sup>. Immunoreactive proteins in the membrane were scanned and analyzed by Odyssey Infrared Imaging System (LI-COR, Inc., Lincoln, NE). Band density was normalized according to the  $\beta$ -tubulin content.

### Statistical analysis

The *in vitro* data were generated from three independent experiments and 3 parallels were used in each experiment. The *in vivo* data were obtained from one experiment, with six mice in each treatment. Data are presented as means  $\pm$  standard error of the means (SEM). Statistical analysis was performed using Sigmaplot 12.5 (Systat Software, Inc., San Jose, CA). Differences between means were determined using Student's *t*-test or one way analysis of variance (ANOVA) followed by Duncan's multiple test when appropriate and a confidence level of  $P < 0.05$  was considered to be statistically significant.

## RESULTS

### Resveratrol exerts dose dependent effects on brown adipogenic differentiation of iWAT SVC

First, we investigated the effects of resveratrol on brown adipogenic differentiation of iWAT SVC. The result of Oil Red O staining demonstrated that the higher concentrations (20  $\mu\text{M}$  or 40  $\mu\text{M}$ ) of resveratrol significantly ( $P < 0.001$ ) inhibited lipid accumulation in the differentiated iWAT SVC after 7-day brown adipogenic differentiation (Fig. 1A, B) and suppressed the expression of adipogenic markers PPAR $\gamma$  and aP2 (Fig. 1C, D). Similar inhibitory effects of resveratrol on white adipogenesis were observed previously<sup>24, 25</sup>. On the other hand, at the concentrations 10  $\mu\text{M}$  or lower, resveratrol had no effect on lipid accumulation.

### Resveratrol promotes formation of brown-like adipocytes in differentiated iWAT SVC cells

Although low concentrations of resveratrol had no effect on lipid accumulation during brown adipogenic differentiation of iWAT SVC, we further determined whether resveratrol stimulated the generation of brown-like adipocytes by analyzing the mRNA expression of brown adipocyte specific genes. As shown in Fig. 2A, resveratrol increased the mRNA level of PRDM16 (3.6-fold versus control,  $P < 0.05$ ), a key transcription factor regulating brown adipogenesis. UCP1 expression, which is specific to brown adipocytes and does not occur in white adipocytes, was also markedly elevated (2.2-fold versus control,  $P < 0.05$ ). In addition, expression of Cidea, a gene predominantly expressed in brown adipocytes, and Elovl3, a very long chain fatty acid elongase that is expressed in brown but not white fat, increased 3.4-fold ( $P < 0.01$ ) and 1.8-fold ( $P < 0.05$ ), respectively, in resveratrol group. Moreover, resveratrol increased the mRNA expression of PGC1 $\alpha$  (3.3-fold,  $P < 0.01$ ), the master regulator of mitochondrial biogenesis and oxidative phosphorylation. Finally, resveratrol promoted the mRNA expression of beige adipocytes selective markers such as CD137 (1.8-fold,  $P < 0.05$ ), Tbx1 (1.9-fold,  $P < 0.01$ ), and TMEM26 (2.1-fold,  $P < 0.01$ ).

Immunostaining results showed that the expression of UCP1 in the resveratrol-treated group was higher than that of control group (Fig. 2B) and this was confirmed by western blotting as the UCP1 protein level in resveratrol treated cells was 1.5-fold higher than that of control cells ( $P < 0.05$ ). Consistent with mRNA expression, the protein level of PRDM16 was also markedly increased in the resveratrol treatment group (1.2-fold versus control,  $P < 0.05$ ). Moreover, the protein levels of cytochrome C (Cyto C) (1.4-fold versus control,  $P < 0.05$ ) and pyruvate dehydrogenase (PDH) (1.2-fold versus control,  $P < 0.05$ ), which represent the mitochondrial content, were also elevated by resveratrol (Fig. 2C, D). The mRNA and protein expression data together provided evidence that resveratrol promoted the formation of brown-like adipocytes of iWAT SVC.

Browning of WAT is expected to similarly increase cellular respiration. To investigate whether resveratrol elevated cellular respiration, O<sub>2</sub> consumption of differentiated iWAT SVC was measured after 7 days of treatment. Consistent with increased browning, the basal oxygen consumption in resveratrol group was 1.6-fold higher than that of control cells ( $P < 0.001$ ) (Fig 2E).

### Resveratrol stimulates the phosphorylation of AMPK $\alpha$ in SVC

In order to determine whether AMPK $\alpha$  was involved in the resveratrol-mediated browning effects, we examined the effects of resveratrol on the phosphorylation of AMPK $\alpha$  (p-AMPK $\alpha$ ). As shown in Fig. 3, resveratrol increased the phosphorylation of AMPK $\alpha$  in differentiated wild-type iWAT SVC cells (1.3-fold versus control,  $P < 0.05$ ), with no effect on total AMPK $\alpha$  (t-AMPK $\alpha$ ). In addition, the ratio pAMPK $\alpha$ / t-AMPK $\alpha$  was elevated (1.2-fold versus control,  $P < 0.01$ ) in resveratrol treated group. Furthermore, the protein level of Sirt1 was also higher (1.5-fold versus control,  $P < 0.05$ ) due to resveratrol treatment. When the confluent iWAT SVC was treated with 4-OHT to knockout AMPK $\alpha$ 1 acutely before brown adipogenic differentiation, the expressions of p-AMPK $\alpha$ , t-AMPK $\alpha$  and Sirt1 in differentiated iWAT SVC were much lower than seen in wild type cells. Moreover, we

found that resveratrol had no effect on the protein levels of p-AMPK $\alpha$ , t-AMPK $\alpha$  or Sirt1 in AMPK $\alpha$ 1 knockout SVC cells.

### **AMPK inhibition or AMPK $\alpha$ 1 deletion eliminate the browning effects of resveratrol on mouse iWAT SVC**

AMPK inhibitor Compound C was used to examine the effects of AMPK inhibition on the resveratrol mediated browning effects on mouse iWAT SVC. We found that Compound C (1 $\mu$ M) did inhibit the activation of AMPK (p-AMPK/t-AMPK) (Fig. 4A, B). Although Compound C had no effects on the expression of UCP1, PRDM16, Cyto C and PDH. However, Compound C inhibited the promotional effects of resveratrol on the expression of these genes (Fig. 4A, B).

We also tested whether acute Ampk $\alpha$ 1 deletion affected the browning effects of resveratrol on iWAT SVC. To this end, iWAT SVC isolated from weaning Rosa<sup>cre</sup>/Ampk $\alpha$ 1<sup>flox/flox</sup> mice that ubiquitously express a tamoxifen-inducible Cre recombinase were treated with 4-OHT to induce AMPK $\alpha$ 1 knockout acutely. In the absence of AMPK $\alpha$ 1, resveratrol had no effects on the mRNA expression of PRDM16, UCP1, Cidea, Elovl5 and PGC1 $\alpha$  (Fig. 4C). Consistently, after deletion of AMPK $\alpha$ 1, the protein levels of UCP1, PRDM16, Cyto C and PDH in the resveratrol treatment group did not differ from those in the control group (Figure 4E, F). Furthermore, after knocking out AMPK $\alpha$ 1, the basal oxygen consumption of differentiated iWAT SVC was not affected by resveratrol treatment (Fig. 4D). These results suggested that AMPK $\alpha$ 1 has a major role in mediating the browning effect of resveratrol on iWAT SVC.

### **Resveratrol reduces body weight, iWAT index and stimulates browning of iWAT**

To further analyze browning effects of resveratrol on iWAT *in vivo*, 5-month-old CD1 mice were treated HFD or HFD containing 0.1% resveratrol for 4 weeks. While there was no apparent difference in food intake (Fig. 5A), the body weight gain in resveratrol supplemented group was lower than that of control group (2.99  $\pm$  0.91 g versus 5.91  $\pm$  0.90 g of control,  $P < 0.05$ ) (Fig. 5B). In addition, resveratrol markedly reduced the body iWAT index (iWAT mass/body weight) (24.15  $\pm$  2.56 versus 34.57  $\pm$  0.76 of control,  $P < 0.05$ ) (Fig. 5C), with no effect on blood glucose level. H&E staining results revealed that the average adipocyte diameter in resveratrol supplemented group was much smaller than that of control group (33.29  $\pm$  1.90  $\mu$ m versus 81.07  $\pm$  1.94  $\mu$ m of control,  $P < 0.001$ ) (Fig. 5D). Inspection of the distribution of cell sizes indicated that diameter of most adipocytes (about 70%) in the control group was in the range of 61-100  $\mu$ m. In contrast, diameter of most adipocytes (about 90%) in the resveratrol supplemented group was smaller than 60  $\mu$ m (Fig. 5E). Moreover, iWAT from resveratrol treated mice showed the appearance of multiocular adipocytes within white fat, a characteristic of brown adipocytes (Fig. 5D), suggesting that resveratrol induced brown-like remodeling (browning) of iWAT. Subsequently, IHC staining of UCP1 indicated an enhanced UCP1 staining in resveratrol treated mice (Fig. 5D). In agreement, the UCP1 protein content in the resveratrol group was 1.5-fold higher than that of the control group ( $P < 0.05$ ). Furthermore, resveratrol supplement resulted in increased protein contents of PRDM16 (1.8-fold versus control,  $P < 0.01$ ) and Cyto C (1.3-fold versus control,  $P < 0.05$ ), which was accompanied by the elevated expression of p-

AMPK $\alpha$  (1.5-fold versus control,  $P < 0.01$ ) (Fig. 5F, G), suggesting that AMPK $\alpha$  was involved in the resveratrol-induced browning of iWAT. We also found that resveratrol supplement had no effect on the protein expression levels of PPAR $\gamma$  and aP2.

We also analyzed the serum profiles (Table 2). While there was no difference in non-fasting glucose, the insulin level was lower in resveratrol treated mice ( $P < 0.05$ ). In addition, the triglyceride concentration was also reduced in resveratrol supplemented compared to control mice ( $P < 0.05$ ).

### Resveratrol promotes lipid oxidation in iWAT

To explore why resveratrol feeding reduced the body weight gain and iWAT mass under HFD, we measured the oxygen consumption of mice. Resveratrol treatment significantly increased the oxygen consumption ( $\text{VO}_2$ ) of mice ( $2,359 \pm 43$  ml/kg/h versus  $2,103 \pm 61$  ml/kg/h of control,  $P < 0.01$ ) (Fig. 6A). There was no difference in  $\text{CO}_2$  production ( $\text{VCO}_2$ ) between control and resveratrol group before and after treatment (Fig. 6B). As a result, resveratrol decreased the respiratory exchange rate (RER,  $\text{VCO}_2/\text{VO}_2$ ) ( $0.731 \pm 0.017$  versus  $0.791 \pm 0.038$  of control,  $P < 0.01$ ) (Fig. 6C), suggesting that there was a shift to primarily utilize fatty acids for oxidation in the resveratrol group. This was consistent with the reduced serum triglyceride concentration in resveratrol group (Table 2). Furthermore, resveratrol exerted the tendency to increase average heat production ( $0.727 \pm 0.024$  versus  $0.671 \pm 0.010$  kCal/h of control,  $P = 0.065$ ) (Fig. 6D). Moreover, resveratrol increased the basal oxygen consumption (2.1-fold versus control,  $P < 0.01$ ) (Fig. 6E) of iWAT *in vitro*.

### Discussion

In this paper, we investigated the effects of resveratrol on the formation of brown-like adipocytes and the mechanism underlying this process. Our results demonstrated that resveratrol induces the browning of mouse iWAT by promoting the expression of brown adipocyte selective genes through the activation of AMPK $\alpha$ 1. It has been reported that resveratrol reduces adiposity<sup>19</sup>, via inhibiting white adipogenesis<sup>24</sup> and stimulating the lipolysis<sup>27, 28</sup>. To date, however, no study assessed resveratrol's effects on the brown adipogenesis or the formation of brown-like adipocytes. Furthermore, the concentrations used in previous *in vitro* studies of white adipogenesis<sup>24, 25</sup> are much higher than the plasma concentration<sup>20, 49</sup>. These concentrations can stimulate apoptosis<sup>25, 50</sup> and might be less relevant to the physiological effects of resveratrol. In the present study, we found that high concentrations (20 or 40  $\mu\text{M}$ ) of resveratrol inhibited lipid accumulation during the brown adipogenic differentiation of iWAT SVC. And these results agreed with previous reports that high concentration (50  $\mu\text{M}$ ) of resveratrol inhibits the adipogenic differentiation of 3T3-L1<sup>24, 25</sup> and SGBS preadipocytes<sup>51</sup>. However, at the lower concentrations ( $\leq 10$   $\mu\text{M}$ ), which is closer to the plasma concentration<sup>20, 49</sup>, resveratrol did not affect lipid content in induced brown adipocytes. It has been reported that the plasma resveratrol concentration is  $1.56 \pm 0.28$   $\mu\text{M}$  in rat fed a high fat diet containing 4 g resveratrol per kg diet<sup>49</sup>. While in mice fed high fat diet containing 0.4% resveratrol, the highest plasma resveratrol concentration is about 0.5  $\mu\text{M}$ <sup>20</sup>. Thus, relative low resveratrol concentration (10

μM) was selected to investigate its role in the formation of brown-like adipocytes during the brown adipogenic differentiation of iWAT SVC.

Our results showed that resveratrol boosts UCP1 mRNA expression in differentiated iWAT SVC, which is consistent with the reports in maturing 3T3-L1 preadipocytes<sup>25</sup> and primary MEF-derived adipocytes<sup>30</sup>. In addition, the mRNA expression of other brown adipocytes selective genes such as PRDM16, Cidea, Elovl3, and PGC1α as well as the protein levels of UCP1, Cyto C and PDH were also markedly elevated by resveratrol treatment. Moreover, the expressions of beige adipocyte selective markers such as CD137, Tbx1 and TMEM26 in resveratrol treated group were much higher than those of control group. These data strongly support the notion that resveratrol promotes the formation of brown-like adipocytes in differentiated mouse iWAT SVC.

*In vivo* studies were conducted to further address the biological effects of resveratrol on the formation of brown-like adipocytes in WAT. We found that resveratrol significantly decreased the body weight gain compared with the control group when challenged with an obesogenic diet. The reduced body weight gain in resveratrol treated mice might be due to the lower body fat accumulation. Our findings confirmed the body fat lowering effects of resveratrol, which have been reported in both animals<sup>21, 29, 52</sup> and humans<sup>22</sup>. It has been reported that thermogenesis is involved in the body-fat lowering effects of resveratrol<sup>53, 54</sup>. However, in these previous studies, their primary focuses were on the BAT and/or skeletal muscle, but not WAT. In our study, we found that resveratrol resulted in decreased adipocyte size in WAT, which is in agreement with a recent report in humans<sup>23</sup>. More importantly, we observed brown-like adipocytes, with an appearance of multiocular lipid droplets, in iWAT, which has not been observed before. The presence of brown-like adipocytes was further confirmed by UCP1 IHC staining. Moreover, the UCP1 protein content was also elevated in the resveratrol group, accompanied with the elevated expression of PRDM16 and Cyto C, two markers of brown adipogenesis<sup>55, 56</sup>. These findings strongly suggested the browning effects of resveratrol on iWAT.

Increased browning of iWAT could lead to increased energy expenditure and oxygen consumption. It has been reported that resveratrol improves mitochondrial oxidation function in BAT and skeletal muscle<sup>20</sup>, but whether resveratrol elicits similar effects in iWAT has not been evaluated. Our findings indicate that resveratrol increased oxygen consumption (VO<sub>2</sub>) and decreased respiratory exchange ratio (RER) (CO<sub>2</sub> production/O<sub>2</sub> uptake) in mice, which is highly consistent with our *in vitro* data. RER is commonly used to determine the relative contribution of carbohydrate and lipids to overall energy expenditure. A high RER indicates that carbohydrates are being predominantly catabolized, whereas a low RER suggests lipid oxidation<sup>57</sup>. Thus, the decreased RER in resveratrol treated mice suggests that a higher ratio of lipids were being oxidized. We also found that resveratrol had the tendency to increase ( $P = 0.065$ ) the average heat production. It should be noted that the increased oxygen consumption (VO<sub>2</sub>), heat production and lipid oxidation might be partially due to the activation of BAT by resveratrol<sup>53</sup>. Meanwhile, we also found that oxygen consumption of tissue (iWAT) and cells (differentiated iWAT SVF) in resveratrol treated group was higher than that of control group. Thus, a lower iWAT adipocyte size in the HFD-fed mice supplemented with resveratrol might be due to the increase of lipolysis and

subsequent elevated fat oxidation and heat production with increased oxygen consumption. Moreover, the expression of genes related to mitochondrial fatty acid oxidation such as PGC1 $\alpha$ , PDH, Cyto C was elevated in the resveratrol group. These data were consistent with the enhanced fatty acid oxidation observed in 3T3-L1 and MEF-derived adipocytes following resveratrol treatment<sup>30</sup>. Together, our data suggested that the anti-obesity effects of resveratrol at least partially resulted from the enhanced fat oxidation in iWAT.

It has been reported that resveratrol may exert its effects on metabolic health in part through the activation AMPK<sup>49, 58</sup>. To investigate whether AMPK was involved in the resveratrol mediated browning of iWAT, we first analyzed the activation of AMPK $\alpha$  (the ratio phosphorylation level of AMPK $\alpha$  (p-AMPK $\alpha$ ) to total AMPK $\alpha$  (t-AMPK)) in the differentiated iWAT SVC and found that the ratio p-AMPK $\alpha$ /t-AMPK $\alpha$  was increased in the resveratrol treated group. Meanwhile, AMPK $\alpha$  inhibition by Compound C, which could inhibit the activation of AMPK $\alpha$ , led to the complete elimination of the stimulating effects of resveratrol on the expression of markers of beige adipocytes, including UCP1, PRDM16, Cyto C and PDH. Furthermore, our *in vivo* study also revealed the increased AMPK $\alpha$  phosphorylation and p-AMPK $\alpha$ /t-AMPK $\alpha$  ratio in iWAT of resveratrol treated mice, in agreement with the previous report<sup>21</sup>. These results suggested that AMPK $\alpha$  is involved in the browning effects of resveratrol on iWAT.

Because the predominant isoform of  $\alpha$  catalytic subunit expressed in adipose tissue is  $\alpha 1$ <sup>31, 32</sup>, we speculated that AMPK $\alpha 1$  but not AMPK $\alpha 2$  participated in resveratrol induced browning effects. To verify our hypothesis, we acutely delete AMPK $\alpha 1$  by treating the confluent iWAT SVC isolated from Rosa26<sup>Cre</sup>/Ampk $\alpha 1$ <sup>fllox/fllox</sup> mice with 4-OHT and, then, induced brown adipogenic differentiation. As expected, only trace amount of p-AMPK $\alpha$  and t-AMPK $\alpha$  was detected in SVC after the acute deletion of AMPK $\alpha 1$ , showing that  $\alpha 1$  isoform accounts for most of the total activity of this kinase in SVC<sup>32, 35</sup>. In the absence of AMPK $\alpha 1$ , the effects of resveratrol on the expression of brown adipocyte selective genes were abolished, suggesting that AMPK $\alpha 1$  is the key mediator linking resveratrol to the browning of iWAT. Our study is consistent with a previous study showing that AMPK $\alpha 1$  knockout abolished the effect of resveratrol on metabolic rate in mice<sup>21</sup>, though no brown adipogenesis or browning of white adipocytes were examined. Here, building on that study, for the first time, we demonstrate that resveratrol improves metabolism at least partially through enhancing brown-like, or beige adipogenesis in WAT, which is mediated by AMPK $\alpha 1$ .

In a recent study, resveratrol was shown to induce thermogenesis by increasing Sirt1 expression<sup>53</sup>. And Sirt1 is required for the mitochondrial biogenesis induced by resveratrol<sup>58</sup>. Consistently, in our study, the Sirt1 content in SV cells was also activated due to resveratrol treatment, which was absent in AMPK $\alpha 1$  KO cells; in addition, AMPK $\alpha 1$  deficiency dramatically reduced Sirt1 content. These data suggest that AMPK and Sirt1 likely reinforce each other to induce the browning of iWAT. Indeed, AMPK and Sirt1 coordinate to regulate mitochondriogenesis<sup>59</sup>.

In conclusion, we provide evidence that resveratrol induces the formation of brown-like adipocytes in mouse iWAT by increasing expression of genes specific to brown adipocytes

and stimulating fatty acid oxidation, which appeared to be primarily mediated by AMPK $\alpha$ 1. These data demonstrate, in addition to the inhibition of adipogenesis and stimulation of lipolysis, a novel browning role of resveratrol in WAT, which contributes to the beneficial effects of resveratrol in metabolism. Moreover, it extends our knowledge on dietary polyphenols and beige adipogenesis and provides new strategies for the prevention and treatment of obesity and related diseases.

## Acknowledgments

Special thanks to Joseph Maricelli for his help in the measurement of basal metabolic rate (BMR). This work was supported by grants from National Institutes of Health (R01HD067449), the National Natural Science Foundation of China (31372397), the Muscular Dystrophy Association (216602) and the National Science Foundation (1147275). This activity was also funded, in part, with an Emerging Research Issues Internal Competitive Grant from the Agricultural Research Center at Washington State University, College of Agricultural, Human, and Natural Resource Sciences.

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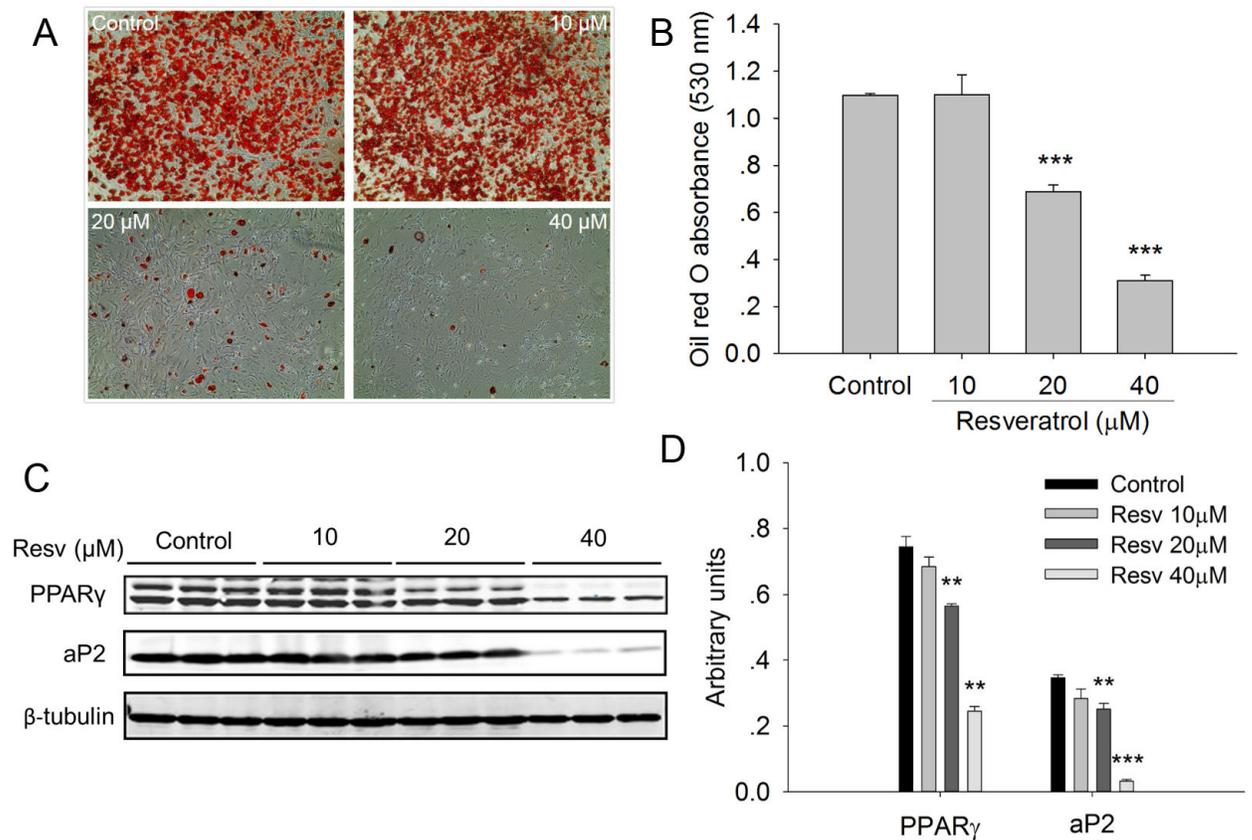
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## Abbreviations

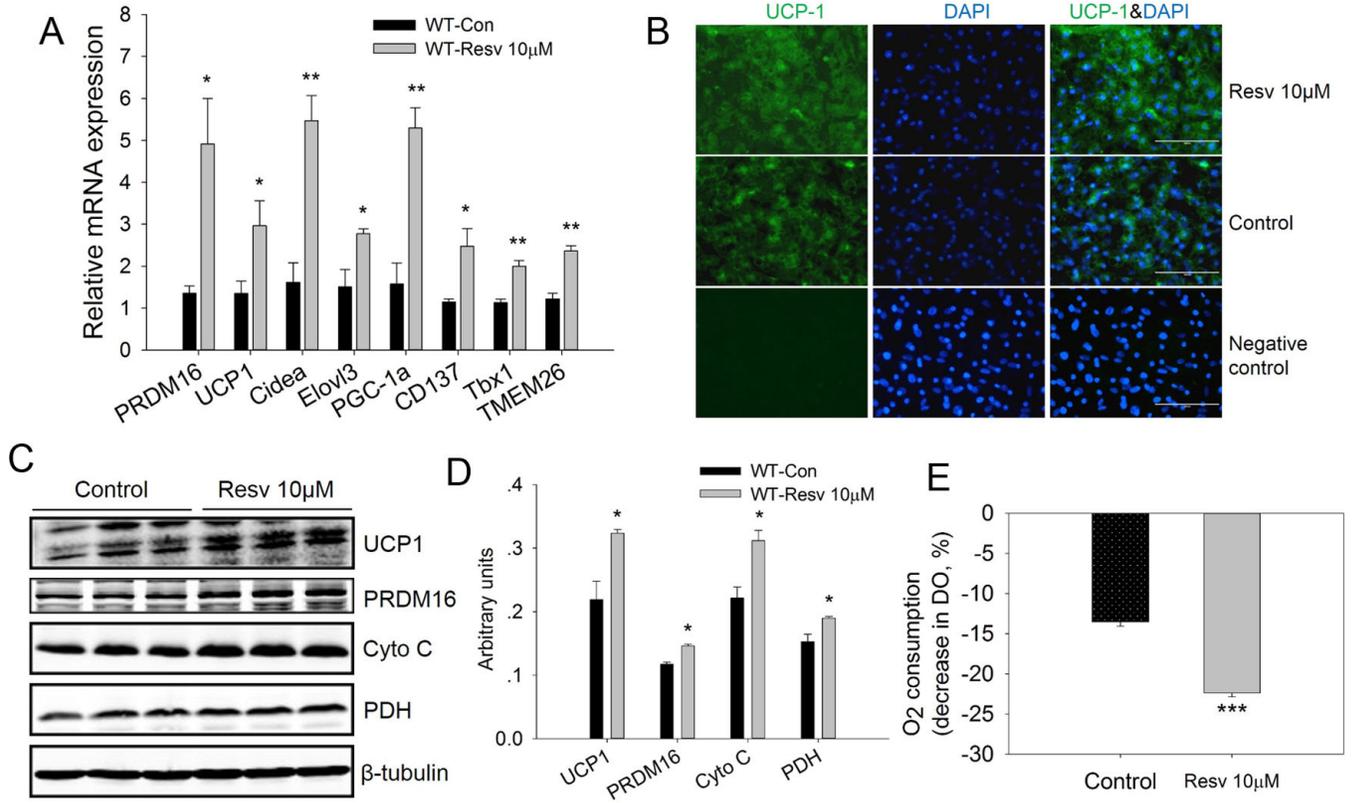
<b>4-OHT</b>	4-hydroxytamoxifen
<b>AMPK</b>	AMP-activated protein kinase
<b>aP2</b>	adipocyte protein 2
<b>BMR</b>	basal metabolic rate
<b>BMP7</b>	bone morphogenetic protein 7
<b>BAT</b>	brown adipose tissue
<b>CC</b>	Compound C
<b>Cidea</b>	Cell death-inducing DFFA-like effector A
<b>Cre</b>	cre recombinase
<b>Cyto C</b>	cytochrome C
<b>Elovl3</b>	elongation of very long chain fatty acids protein 3
<b>FGF21</b>	fibroblast growth factor 21
<b>H&amp;E</b>	hematoxylin and eosin
<b>HFD</b>	high fat diet
<b>IHC</b>	immunohistochemical
<b>iWAT</b>	inguinal white adipose tissue
<b>MEF</b>	mouse embryonic fibroblasts
<b>PDH</b>	pyruvate dehydrogenase
<b>PGC1<math>\alpha</math></b>	peroxisome proliferator-activated receptor- $\gamma$ coactivator 1 $\alpha$

<b>PPAR<math>\gamma</math></b>	peroxisome proliferator-activated receptor $\gamma$
<b>PRDM16</b>	PR domain-containing 16
<b>RER</b>	respiratory exchange ratio
<b>SVC</b>	stromal vascular cells
<b>UCP1</b>	uncoupling protein 1
<b>WAT</b>	white adipose tissue

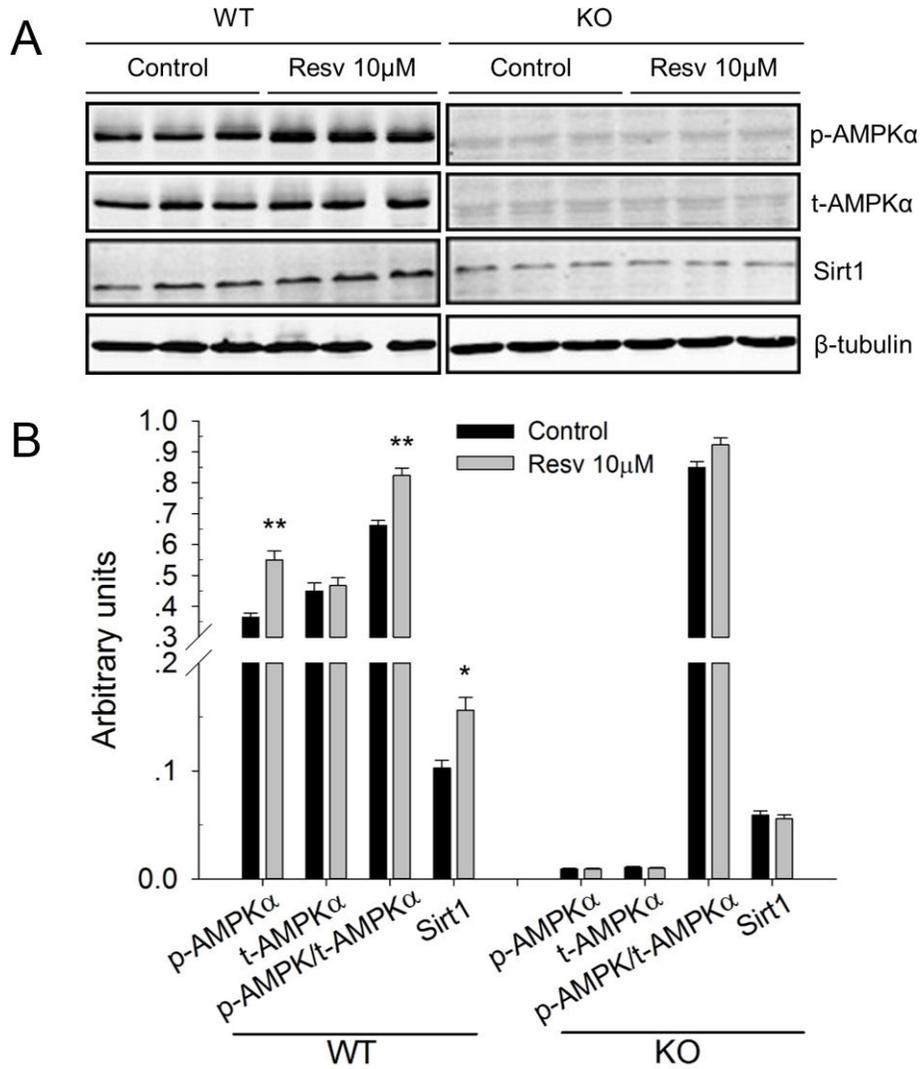


**Figure 1.**

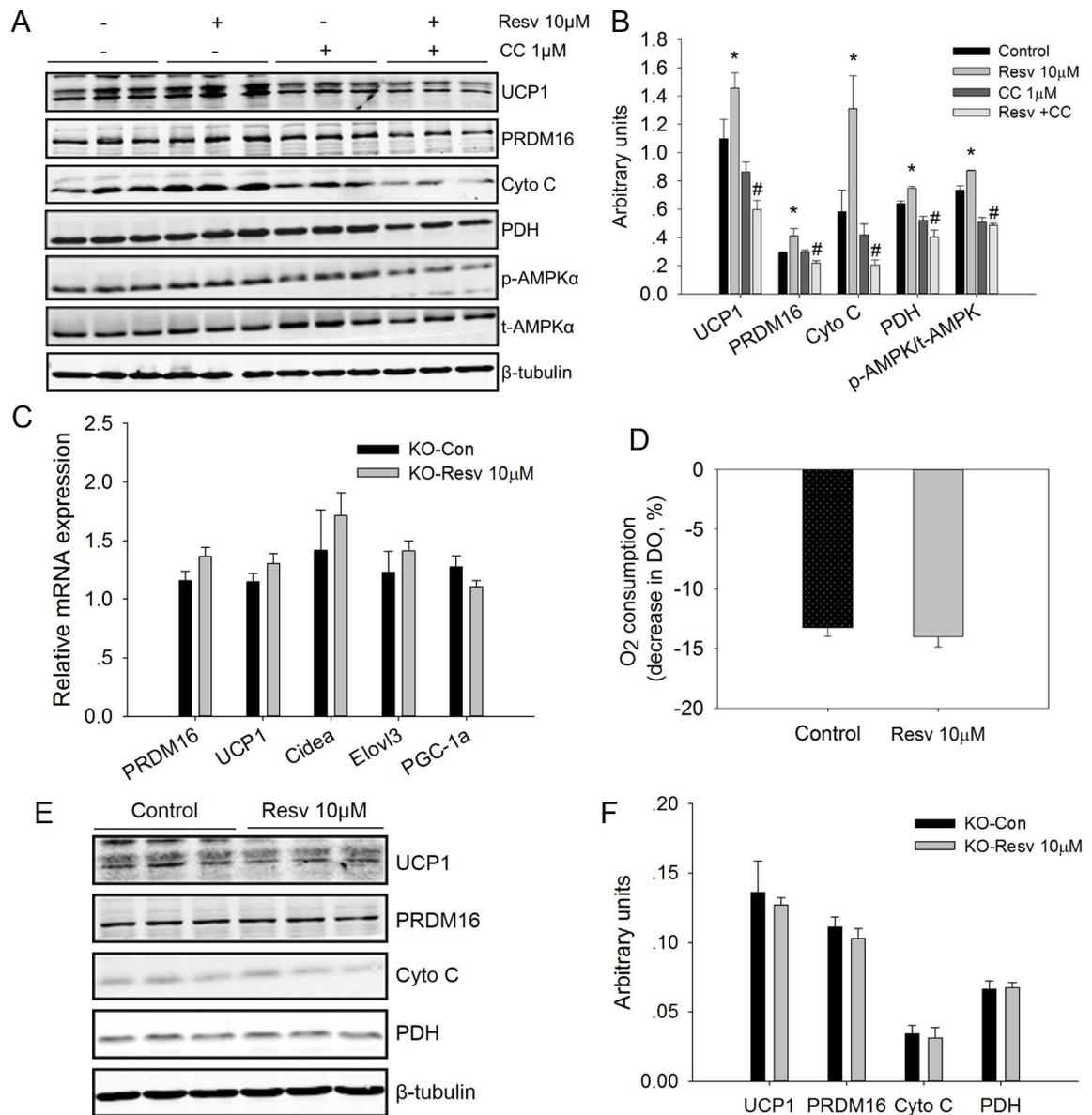
Effects of resveratrol on the lipid accumulation and the expression of adipogenic marker genes in differentiated inguinal WAT (iWAT) SVC. *A*) Oil-Red O staining was conducted in the differentiated iWAT SVC after 7-day brown adipogenic differentiation. Microscopic pictures were taken on day 7 with  $\times 100$  magnification. *B*) The stained Oil-Red O was extracted with isopropanol. The absorbance of the extracted Oil-Red O was spectrophotometrically determined at 530 nm to measure triglyceride (TG) accumulation. *C*, *D*) Western blot analysis of adipogenic marker genes (PPAR $\gamma$  and aP2) in the differentiated iWAT SVC after 7-day brown adipogenic differentiation, and  $\beta$ -tubulin was used as a loading control (*C*). Mean  $\pm$  SEM of immunoblotting bands of PPAR $\gamma$  and aP2 (*D*). The intensities of the bands were expressed as arbitrary units. \*\*  $P < 0.01$  and \*\*\*  $P < 0.001$  versus control.



**Figure 2.** Resveratrol promoted formation of brown-like adipocytes in the differentiated iWAT SVC cells from wild-type mice after 7-day adipogenic differentiation. *A*) Relative mRNA levels of brown adipocytes selective genes (PRDM16, UCP1, Cidea, Elovl3 and PGC-1a) and beige adipocytes selective genes (CD137, Tbx1 and TMEM26). *B*) UCP1 immunofluorescence staining for UCP1 in the differentiated iWAT SVC. Nuclei were stained with DAPI (scale bar, 100  $\mu$ m). *C*, *D*) Western blot analysis of brown adipocytes selective genes (UCP1, PRDM16, Cyto C, and PDH) in the differentiated iWAT SVC, and  $\beta$ -tubulin was used as loading control (*C*); Mean  $\pm$  SEM of immunoblotting bands of UCP1, PRDM16, Cyto C and PDH (*D*). The intensities of the bands were expressed as arbitrary units. *E*) Basal O<sub>2</sub> consumption of differentiated iWAT SVC from control and resveratrol treated groups. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  versus control.



**Figure 3.** Effects of resveratrol on the phosphorylation of AMPK $\alpha$  and Sirt1 in wild type and AMPK $\alpha$ 1 knockout iWAT SVC. *A*) Western blot analysis of phospho-AMPK $\alpha$  (p-AMPK $\alpha$ ), t-AMPK $\alpha$  (t-AMPK $\alpha$ ) and Sirt1 in the differentiated iWAT SVC of wild type (left part) and AMPK $\alpha$ 1 deletion (right part).  $\beta$ -Tubulin was used as loading control. *B*) Mean  $\pm$  SEM of immunoblotting bands of p-AMPK $\alpha$ , t-AMPK $\alpha$ , p-AMPK $\alpha$ /t-AMPK $\alpha$  and Sirt1 in wild type and AMPK $\alpha$ 1 knockout cells. The intensities of the bands were expressed as arbitrary units. \* $P < 0.05$  versus control.

**Figure 4.**

AMPK inhibition or AMPK $\alpha$ 1 deletion eliminated the browning effects of resveratrol on mouse differentiated iWAT SVC. **A**) Effects of AMPK inhibitor Compound C (CC) in the protein contents of UCP1, PRDM16, Cyto C, PDH, phospho-AMPK $\alpha$  (p-AMPK $\alpha$ ), and t-AMPK $\alpha$  (t-AMPK $\alpha$ ) in the differentiated iWAT SVC after 7-day brown adipogenic differentiation.  $\beta$ -Tubulin was used as loading control. **B**) Mean  $\pm$  SEM of immunoblotting bands of UCP1, PRDM16, Cyto C, PDH, p-AMPK $\alpha$ /t-AMPK $\alpha$ . The intensities of the bands were expressed as arbitrary units. \* $P$  < 0.05 versus control, # $P$  < 0.05 versus Resv 10  $\mu$ M. **C**) Relative mRNA levels of brown adipocyte selective genes (PRDM16, UCP1, Cidea, Elovl3, and PGC1 $\alpha$ ) in the differentiated SVC after 7-day differentiation with classical brown adipogenic induction cocktails. SVC cells from iWAT of weaning Rosa26Cre/AMPK $\alpha$ 1flox/flox mice were treated with 4-hydroxytamoxifen (4-OHT) to delete AMPK $\alpha$ 1

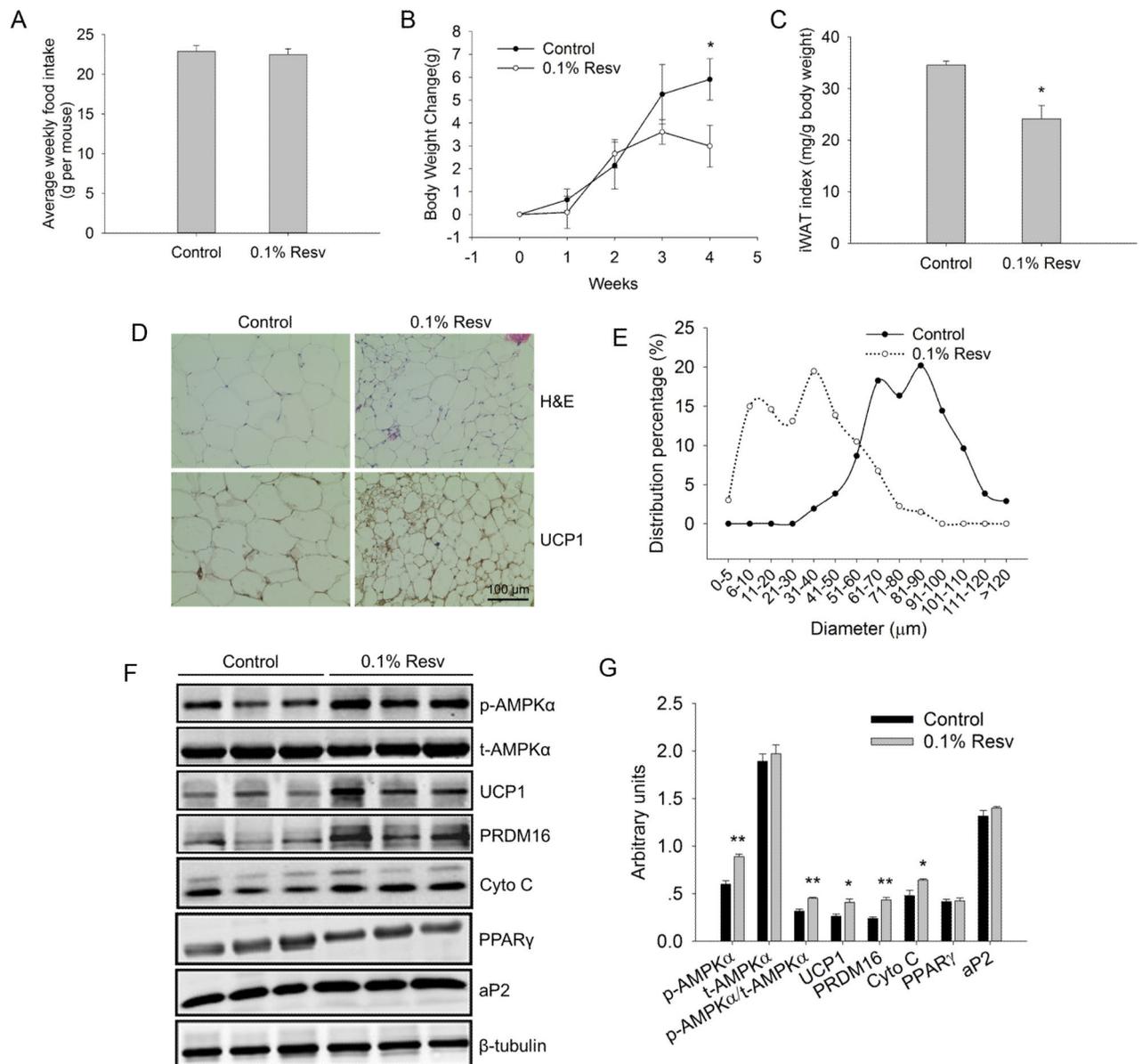
before being induced to undergo brown adipogenic differentiation. *D*) Basal O<sub>2</sub> consumption of differentiated AMPK $\alpha$ 1 knockout iWAT SVC from control and resveratrol treated groups. *E, F*) Western blot analysis of brown adipocyte selective genes (UCP1, PRDM16, Cyto C, and PDH) in the differentiated SVC after 7-day brown adipogenic differentiation, and  $\beta$ -tubulin was used as loading control (*E*). Mean  $\pm$  SEM of immunoblotting bands of UCP1, PRDM16, Cyto C and PDH (*F*). The intensities of the bands were expressed as arbitrary units.

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**Figure 5.** Resveratrol induced brown-like adipocytes in iWAT. *A*) Weekly food intake were measured in control (n=6) and 0.1% Resv (n=6) groups. *B*) Body weight changes were compared between control and 0.1% Resv groups during 4 weeks. *C*) iWAT index was compared between control and 0.1% Resv groups. *D*) Representative images of H&E and UCP1 IHC staining in sections of iWAT of control and 0.1% Resv treated mice. All images were obtained at  $\times 400$  magnification. *E*) Distribution percentage of adipocyte diameters from control and 0.1% Resv treated mice. Data analysis from the H&E staining sections. *F*, *G*) Western blot analyses of p-AMPK $\alpha$ , t-AMPK $\alpha$ , UCP-1, PRDM 16, Cyto C and adipogenic marker genes (PPAR $\gamma$  and aP2) were performed in iWAT of control and resveratrol treated mice, and  $\beta$ -tubulin was used as the loading control (*F*). Mean  $\pm$  SEM of immunoblotting bands of p-AMPK $\alpha$ , t-AMPK $\alpha$ , p-AMPK $\alpha$ /t-AMPK $\alpha$ , UCP-1, PRDM16, Cyto C, PPAR $\gamma$

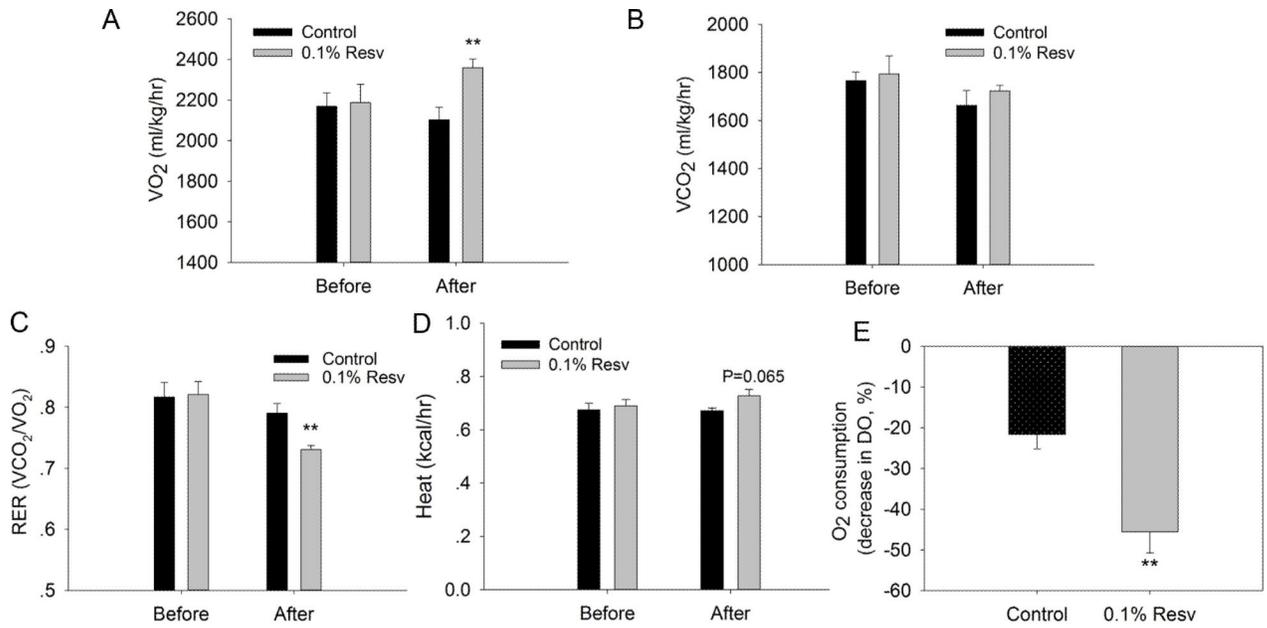
and aP2 (*G*). The intensities of the bands were expressed as arbitrary units.\**P* < 0.05, \*\**P* < 0.01 versus control.

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**Figure 6.**

Resveratrol promoted the lipid oxidation of iWAT. *A*) O<sub>2</sub> consumption of control and resveratrol treated mice were recorded during a 3-h period. *B*) CO<sub>2</sub> production of control and resveratrol treated mice was recorded during a 3-h period. *C*) Respiratory exchange ratio (RER) of control and resveratrol treated mice was recorded during a 3-h period. *D*) Average heat production of control and resveratrol treated mice during a 3-h period. *F*) O<sub>2</sub> consumption of iWAT of control and resveratrol treated mice was measured as the decrease in dissolved oxygen (DO). \*\**P* < 0.01 versus control.

**Table 1**  
**The primer sequences used for real-time quantitative PCR**

Gene	Forward (5'-3')	Reverse (3'-5')	Amplicon size (bp)	Gene access number
18s	GTAACCCGTTGAACCCATT	CCATCCAATCGGTAGTAGCG	151	<a href="#">NR_046233.2</a>
Cidea	ATCACAACCTGGCCTGGTTACG	TACTACCCGGTGTCCATTCT	136	<a href="#">NM_007702.2</a>
CD137	GTCGACCCTGGACGAACTGCTCT	CCTCTGGAGTCACAGAAATGGTGTA	132	<a href="#">NM_001077509.1</a>
Elovl3	GATGGTTCTGGGCACCATCTT	CGTTGTTGTGTGGCATCCTT	73	<a href="#">XM_006526624.1</a>
PGC1 $\alpha$	CCCTGCCATTGTTAAGACC	TGCTGCTGTTCTGTTTTTC	161	<a href="#">XM_006503779.1</a>
PRDM16	CAGCACGGTGAAGCCATTC	GCGTGCATCCGCTTGTG	87	<a href="#">NM_001291029.1</a>
Tbx1	TGAAGAAGAACCCGAAGGTGG	ACTTGAACGTGGGAACATT	133	<a href="#">XM_006536887.1</a>
TMEM26	GAAACCAGTATTGCAGCACCCAAT	AATATTAGCAGGAGTGTGGTGGA	205	<a href="#">NM_177794.3</a>
UCP1	ACTGCCACACCTCCAGTCATT	CTTGCCTCACTCAGGATTGG	123	<a href="#">NM_009463.3</a>

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**Table 2**  
**Serum profiles of Con and Resveratrol treated CD1 mice fed a high fat diet**

Treatments	Control (Con)	Resveratrol (Resv)	P-value
Insulin (ng/ml)	1.48 ± 0.279	0.85 ± 0.136	< 0.05
Triglyceride (mg/dl)	145.0 ± 28.2	87.4 ± 9.33	< 0.05
Glucose (mg/dl)	188 ± 16.7	196 ± 8.2	n.s.

Mice were fed a high energy diet with 45% energy from fat for 4 weeks, and with/without 0.1% resveratrol.

Mice were not fasted before collection of blood samples for analyses. n = 6.

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