ANGPTL6 expression is coupled with mitochondrial OXPHOS function to regulate adipose FGF21

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Abstract

Recent studies revealed that the inhibition of mitochondrial oxidative phosphorylation (OXPHOS) is coupled with the mitochondrial unfolded protein response, thereby stimulating the secretion of non-cell autonomous factors, which may control systemic energy metabolism and longevity. However, the nature and roles of non-cell autonomous factors induced in adipose tissue in response to reduced OXPHOS function remain to be clarified in mammals. CR6-interacting factor 1 (CRIF1) is an essential mitoribosomal protein for the intramitochondrial production of mtDNA-encoded OXPHOS subunits. Deficiency of CRIF1 impairs the proper formation of the OXPHOS complex, resulting in reduced function. To determine which secretory factors are induced in response to reduced mitochondrial OXPHOS function, we analyzed gene expression datasets in *Crif1*-depleted mouse embryonic fibroblasts. *Crif1* deficiency preferentially increased the expression of angiopoietin-like 6 (*Angptl6*) and did not affect other members of the ANGPTL family. Moreover, treatment with mitochondrial OXPHOS inhibitors increased the expression of *Angptl6* in cultured adipocytes. To confirm *Angptl6* induction *in vivo*, we generated a murine model of reduced mitochondrial OXPHOS function using adipose tissue-specific *Crif1*-deficient mice and verified the upregulation of *Angptl6* and fibroblast growth factor 21 (*Fgf21*) in white adipose tissue. Treatment with recombinant ANGPTL6 protein increased oxygen consumption and *Pparα* expression through the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway in cultured adipocytes. Furthermore, the ANGPTL6-mediated increase in *Pparα* expression resulted in increased FGF21 expression, thereby promoting β -oxidation. In conclusion, mitochondrial OXPHOS function governs the expression of ANGPTL6, which is an essential factor for FGF21 production in adipose tissue and cultured adipocytes.

Key Words

- ▶ *Angptl6*
- ▶ *Crif1*
- ▶ mitochondria
- ▶ oxidative phosphorylation

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Introduction

Mitochondrial oxidative phosphorylation (OXPHOS) function plays an essential role in energy metabolism in adipose tissue. The expression of OXPHOS genes in adipose tissue was significantly decreased in diet-induced obese and *db/db* mice compared to that in control mice (Wilson-Fritch *et al.* 2004, Rong *et al.* 2007). These findings suggest that OXPHOS function in adipose tissue plays an important role in the regulation of systemic and local homeostasis of energy metabolism, defects in which are implicated as causes of hepatic steatosis, insulin resistance and type 2 diabetes. The mitochondrial DNA copy number is decreased by aging and increasing body mass index and is also positively associated with insulin-stimulated lipogenesis in human white adipose tissue (Kaaman *et al.* 2007). The depletion of mitochondrial transcription factor A in the adipose tissue, which is necessary for mtDNA stability and transcription, paradoxically increases mitochondrial oxidation, thereby protecting mice against obesity and insulin resistance (Vernochet *et al.* 2012). A recent series of studies revealed that impaired OXPHOS function can increase lifespan in *Drosophila melanogaster*, *Caenorhabditis elegans* and mice (Dillin *et al.* 2002, Liu *et al.* 2005, Copeland *et al.* 2009). Furthermore, increased longevity resulting from reduced mitochondrial function in key tissues was mediated by a non-cell-autonomous secreted signaling factor, which was suggested to be a mitokine in *C. elegans* (Durieux *et al.* 2011). However, the induction of secreted factors as a result of reduced mitochondrial OXPHOS function has not been fully established in mammals.

Fibroblast growth factor 21 (FGF21) has long been studied as a critical metabolic regulator controlling glucose homeostasis and insulin sensitivity in metabolic tissues, including skeletal muscle, adipose tissue and hypothalamus (Hotta *et al.* 2009). Physiological secretion of FGF21 is mainly regulated by peroxisome proliferator-activated receptor (PPAR) α -driven expression, which is augmented in the fasting phase in liver (Inagaki *et al.* 2007). Recent studies revealed that FGF21 secretion by skeletal muscle is associated with mitochondrial OXPHOS impairment and stress, which activates the activating transcription factor 4 (ATF4)-mediated integrated stress response (Kim *et al.* 2013). However, the secreted factors whose expression is coupled with mitochondrial OXPHOS function in adipose tissue remain to be identified.

CR6-interacting factor 1 (CRIF1), also known as CR6/GADD45-interacting protein, is a mitochondrial protein

that is important for the insertion of intramitochondrial nascent OXPHOS polypeptides into the inner mitochondrial membrane (Kim *et al.* 2012). CRIF1 deficiency impairs proper formation of the OXPHOS complex because of aberrant synthesis of mitochondrial DNA-encoded OXPHOS polypeptides, resulting in severe OXPHOS dysfunction *in vitro* and *in vivo* (Kim *et al.* 2012). Therefore, conditional tissue-specific knockout of *Crif1* may be a useful model to determine the mouse phenotypes generated by reduced mitochondrial OXPHOS function (Kim *et al.* 2012, 2015, Ryu *et al.* 2013, Byun *et al.* 2015).

In this study, we investigated the non-cell autonomous factors induced in association with reduced OXPHOS function, using *Crif1*-deficient cells and adipose tissue-specific *Crif1*-knockout (AdKO) mice and the effects of adipo-mitokines on the expression of adipose FGF21. Based on these observations, we propose that adipose mitokines may mediate the specific adipose adaptive stress response or play broader systemic roles, alone or with other mitokines or hormones.

Materials and methods

Mice and ethical considerations

Floxed *Crif1* (*Crif1^{flox/flox}*) mice were generated as described previously (Kwon *et al.* 2008). *Adiponectin-Cre* transgenic mice were provided by Dr Evan Rosen. *Crif1^{flox/flox}* mice were bred with *Adiponectin-Cre* transgenic mice to generate adipocyte-specific *Crif1*-knockout mice. All mice were on a C57BL/6 background. All mice were maintained in a controlled environment (12-h light/12-h darkness cycle; humidity, 50–60%; ambient temperature, 22 $\pm$ 2°C) and fed a normal chow diet in a specific pathogen-free animal facility at the Chungnam National University Hospital Preclinical Research Center. All animals received humane care according to institutional guidelines, and all experimental procedures were approved by the Institutional Review Board of Chungnam National University School of Medicine (Daejeon, South Korea).

Generation of *Crif1*-deficient mouse embryonic fibroblasts (MEFs) and cell culture

Mouse embryonic fibroblasts (MEFs) were isolated from trypsin/EDTA-digested E13.5 wild-type (WT) and

Crif1-deficient embryos. SV40-large T antigen was transfected into MEFs for immortalization. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Gibco) in a humidified incubator with 5% CO₂. 3T3-L1 adipocytes (passage 5–6) obtained from ATCC were cultured with 10% newborn calf serum (Invitrogen) in DMEM. The cells were differentiated using 3-isobutyl-1-methylxanthine (0.5 mM), dexamethasone (1 μM), insulin (10 μg/mL) and 10% fetal bovine serum. 3T3-L1 adipocytes, primary hepatocytes, C2C12 myoblasts and mature immortalized brown adipocytes were treated with recombinant angiopoietin-like 6 (ANGPTL6) protein (rANGPTL6, 200 ng/mL) or the OXPHOS complex inhibitors oligomycin (2 μg/mL), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 5 μM) and rotenone (1 μM) for 12 h.

Isolation of adipocytes and stromal vascular cells (SVCs)

Epididymal and inguinal adipose tissues were dissected in mice and minced into fine pieces using sterile scissors. Minced samples were placed in HEPES-buffered PBS supplemented with 10 mg/mL BSA (Sigma-Aldrich) and type 1 collagenase (1 mg/mL) at 37°C on an orbital shaker for 30 min and then centrifuged at 1000g for 5 min at 4°C to pellet erythrocytes and other blood cells. Once digestion was complete, samples were passed through a sterile 70 μm nylon mesh (BD Falcon; Franklin Lakes, NJ, USA). The suspension was centrifuged at 1000g for 5 min. The pelleted cells were collected as the stromal vascular cells (SVCs), and the floating cells were collected as the adipocyte-enriched fraction. SVCs were resuspended in red blood cell (RBC) lysis buffer and incubated at room temperature for 5 min. The RBC-depleted SVCs were centrifuged at 500g for 5 min, and the pellet was frozen in liquid nitrogen.

Isolation of primary hepatocytes

As previously described (Yi *et al.* 2014), primary murine hepatocytes were isolated from mice using collagenase perfusion, followed by differential centrifugation on a Percoll (GE Healthcare) density gradient. Isolated cells were cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) at a density of 2×10^5 cells/well on 6-well plates.

Western blot analysis

3T3-L1 adipocytes, adipose tissue, liver and muscle from mice were lysed in RIPA buffer (30 mmol/L Tris, pH 7.5, 150 mmol/L sodium chloride, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 1% Nonidet P-40, 10% glycerol, containing phosphatase and protease inhibitors). Western blot analyses were performed with 30–50 μg protein from the tissue or cell homogenates using commercially available antibodies. Primary antibodies against OXPHOS complex subunits (NDUFA9, UQCRC2, and ATP5A1) were purchased from Invitrogen. Anti-COX4 (#4844), anti-extracellular signal-regulated kinase (ERK) (#3179) and anti-mitogen-activated protein kinase (MAPK) (#9212) antibodies were purchased from Cell Signaling. Anti-CRIF1 (sc-134882) antibody was obtained from Santa Cruz Biotechnology. Anti-β-actin (A2228) and anti-α-tubulin (T5168) antibodies were obtained from Sigma-Aldrich. Anti-ANGPTL6 (MAB7527) antibody was purchased from R&D Systems. Anti-FGF21 antibody was obtained from Novus Biologicals (Littleton, CO, USA). Immunoreactive bands were visualized on polyvinylidene difluoride membranes using alkaline phosphate-linked anti-rabbit or anti-mouse antibody and an enhanced chemiluminescence detection system (LI-COR Biosciences; Lincoln, NE, USA).

Enzyme-linked immunosorbent assay (ELISA)

Blood was collected from the hearts of mice under general anesthesia. Samples were centrifuged at 18,500g for 3 min, and the supernatants were collected. The measurement of ANGPTL6 (Cusabio Biotech, Wuhan, Hubei, China) or FGF21 (R&D Systems) was performed in serum or protein lysates from adipose or liver tissues according to the manufacturer's instruction.

Microarray analysis

Total RNA was prepared from WT and *Crif1*-deficient MEFs. RNA amplification and labeling were performed using the Low RNA Input Linear Amplification kit PLUS (Agilent Technologies). Array hybridization and scanning were performed using a DNA microarray chip and scanner (Agilent Technologies). Array data were analyzed using Feature Extraction and GeneSpring Software (Agilent Technologies).

Real-time polymerase chain reaction (PCR)

Total RNA was extracted from cells or tissues using TRIzol Reagent (Invitrogen) in accordance with the manufacturer's instructions. The same quantity of total RNA was reverse-transcribed into complementary DNA (cDNA) using M-MLV Reverse Transcriptase and oligo-dT primers (Invitrogen). Real-time polymerase chain reaction (PCR) was performed using cDNA, QuantiTect SYBR Green PCR Master Mix (QIAGEN) and the specific primers listed in Table 1. The comparative Ct method was used to quantify transcripts, which were normalized to β -actin expression. Values were expressed as fold changes vs control groups.

Measurement of oxygen consumption rate (OCR)

The mitochondrial oxygen consumption rate (OCR) was measured using a Seahorse XF-24 extracellular flux analyzer (Seahorse Bioscience Inc., North Billerica,

MA, USA) in 24-well plates. On the day before OCR measurement, the sensor cartridge was placed into the calibration buffer supplied by Seahorse Bioscience and incubated at 37°C in a non-CO₂-containing incubator. 3T3-L1 adipocytes and primary hepatocytes were cultured on Seahorse XF-24 plates at a density of 20,000 cells per well. The cells were washed and incubated with DMEM lacking sodium bicarbonate at 37°C in a non-CO₂-containing incubator for 1 h. The medium and mitochondrial OXPHOS inhibitors were adjusted to pH 7.4 on the day of the OCR assay. The basal OCR was measured three times, and three readings were taken after the addition of each mitochondrial OXPHOS inhibitor, before the injection of subsequent inhibitors. Oligomycin (2 µg/mL) and rotenone (1 µM) were used as the mitochondrial OXPHOS inhibitors. The basal and post-oligomycin OCRs were calculated by averaging the last three measurements after achieving a steady state. Coupled respiration was expressed as the percentage decrease from basal respiration. In addition, CCCP (5 µM) was used to measure maximal mitochondrial respiration. OCR was automatically calculated and recorded by the sensor cartridge and Seahorse XF-24 software.

Table 1 Primers used in real-time PCR (mouse).

Genes	Forward (5'-3')	Reverse (5'-3')
<i>Angptl1</i>	GCTGGGAGGTAAC-GAGATTCAG	TGGGAGAAGTTGCCAGAT-CAG
<i>Angptl2</i>	GATCCAGAGTGAC-CAGAATC	TCTCAGGCTTCACCAGGTAG
<i>Angptl3</i>	AGCACCAAGAAC-TACTCCCC	ATAAACGGCAGAGCAGTCGG
<i>Angptl4</i>	TCAAAGACTCCGAG-GATAGA	AAAGCCCTTTTCGTAGTTTT
<i>Angptl6</i>	ACTACGA-CAGCTTCTCCTTG	AGTGCTGAAAGGTTTGTCAT
<i>Angptl7</i>	TGCTGTGAGGAGAT-GAGGGA	TCCATCACCTGCATGACCAC
<i>Fgf21</i>	AGATCAGGGAG-GATGGAACA	TCAAAGTGAGGCGATCCATA
<i>Pparα</i>	AGAAGTTGCAG-GAGGGGATT	TTGAAGGAGCTTTGGGAAGA
<i>Pparγ</i>	ATCTTAAGTCCG-GATCCAC	TGGTGATTTGTCCGTTGTCT
<i>Acyl-CoA oxidase</i>	ATGACCCCACTTCCT-GACAC	GAAGGTCAGCCACCATGATT
<i>HSL</i>	AGACACCAGCCAAC-GGATAC	GCGGTTAGAAGCCACATAGC
<i>CD36</i>	TGCTGGAGCTGT-TATTGGTG	TGGGTTTTGCACATCAAAGA
<i>UCP2</i>	GATTGAAGGTCCCC-GTTTCT	ACATCTGTGGCCTTGAAACC
<i>UCP3</i>	TGCTGCTGACAC-GAGAACT	ATTTCCCATCAGGTCAAGTC
<i>PGC1α</i>	TCTCAGTA-AGGGGCTGGTTG	AGCAGCACACTCTATGT-CACTC
<i>18s</i>	GTAACCCGTT-GAACCCATT	CCATCCAATCGGTAGTAGCG

Luciferase reporter assay

To assess the PPAR α activity induced by ANGPTL6, 3T3-L1 adipocytes were transiently transfected with pGL3b-PPAR α -Luc provided by Dr Kook Hwan Kim (Yonsei University College of Medicine, Korea) using Lipofectamine plus (Invitrogen) and OptiMEM (Invitrogen) for 24 h. pGL3B-*Fgf21*(-2078/+129) and pGL3B-*Fgf21*(-1370/+129) vectors have been described (Kim *et al.* 2013). Cells were treated with rANGPTL6 protein (200 or 500 ng/mL, ABNOVA, Taipei, Taiwan), fenofibrate (5 µM, PPAR α agonist; Sigma-Aldrich) and GW6471 (1 µM, PPAR α antagonist; Sigma-Aldrich) for 24 h. Luciferase levels were determined using a luciferase assay kit (Promega). For each sample, luciferase activity was normalized to the protein concentration of whole cell lysates.

Statistical analysis

Data are presented as means $\pm$ standard deviation (s.d.). To compare values obtained from two or more groups, Student's *t*-test or one-way analysis of variance was performed. A *P* value less than 0.05 was considered statistically significant.

Results

Crif1 deficiency or impaired OXPHOS function increases ANGPTL6 expression

We previously reported that CRIF1 is essential for the translation and expression of respiratory chain subunits (Kim *et al.* 2012). Loss of *Crif1* function resulted in

profound impairment of OXPHOS function and was accompanied by reduced expression of mtDNA-encoded respiratory subunits (Kim *et al.* 2012, 2015, Ryu *et al.* 2013). To identify the signaling factors whose expression may be coupled with reduced mitochondrial OXPHOS function, we compared gene expression datasets in *Crif1*-deficient and WT MEFs. The genes related to inflammatory response,

Figure 1

Reduced oxidative phosphorylation (OXPHOS) function induces angiopoietin-related growth factor (ANGPTL6) expression. (A) Comparison of gene sets encoding signaling proteins between heterozygous and homozygous *Crif1*-deficient mouse embryonic fibroblasts. (B) Normalized fold expression of angiopoietin-like proteins in microarray analysis. (C) Western blotting for ANGPTL6 and CRIF1 in heterozygous and homozygous *Crif1*-deficient mouse embryonic fibroblasts. (D) Real-time PCR analysis of 3T3-L1 adipocytes, mature immortalized brown adipocytes, C2C12 myoblasts and primary hepatocytes treated with vehicle or mitochondrial inhibitors. (E) Western blotting for ANGPTL6 expression in 3T3-L1 adipocytes, mature immortalized brown adipocytes, C2C12 myoblasts and primary hepatocytes treated with vehicle or mitochondrial inhibitors. All data are representative of three independent experiments. Data are expressed as mean $\pm$ s.d. * P < 0.05, ** P < 0.01 vs the corresponding controls.

Table 2 Genes enriched in homozygous *Crif1*^{-/-} MEFs.

	<i>Crif1</i> ^{+/-}	<i>Crif1</i> ^{-/-}	Fold change
<i>Slamf6</i>	8.03	416.84	51.89
<i>Nlrp4f</i>	16.48	772.06	46.84
<i>S100a9</i>	8.03	187.17	23.30
<i>Hdgfl1</i>	8.03	178.33	22.20
<i>Tff3</i>	24.65	470.75	19.10
<i>Tenm1</i>	13.17	221.48	16.82
<i>Gstt4</i>	55.78	831.12	14.90
<i>Notch1</i>	159.83	2340.52	14.64
<i>Fgf21</i>	29.62	400.90	13.53
<i>Nrg2</i>	174.46	1459.84	8.37
<i>Gm5849</i>	8.03	66.55	8.28
<i>Dab1</i>	9.43	76.46	8.11
<i>Ly9</i>	9.01	62.40	6.93
<i>Siglecg</i>	57.95	366.19	6.32
<i>Sema7a</i>	339.99	1978.86	5.82
<i>Prl4a1</i>	8.03	44.82	5.58
<i>ADM2</i>	1074.60	5413.84	5.04
<i>Lta</i>	44.83	219.50	4.90
<i>Angptl6</i>	6089.92	29012.53	4.76
<i>Dab2ip</i>	116.27	546.43	4.70
<i>Fli1</i>	75.85	325.84	4.30
<i>Sema4d</i>	518.69	2190.16	4.22
<i>Tmprss9</i>	27.93	114.32	4.09

positive regulation of multicellular organismal processes and system development were enhanced in the *Crif1*^{-/-} MEFs compared to WT MEFs (Supplementary Fig. 1A, see section on supplementary data given at the end of this article). Interestingly, *Angptl6* and *Fgf21* were highly induced in homozygous *Crif1*-deficient MEFs (Fig. 1A and Table 2). By contrast, expression of other members of the ANGPTL family was not increased in *Crif1*-deficient MEFs (Fig. 1B). Moreover, the protein expression of ANGPTL6 was also enhanced in *Crif1*-deficient MEFs compared to that of control MEFs (Fig. 1C).

To extend our observation to adipocytes, we measured the expression of ANGPTL6 in adipocytes and non-adipocytes after inhibition of mitochondrial OXPHOS complex activity using oligomycin, CCCP and rotenone. As shown in Fig. 1D, E and Supplementary Fig. 1B, ANGPTL6 expression was significantly increased in 3T3-L1 adipocytes and mature immortalized brown adipocytes treated with mitochondrial OXPHOS inhibitors, according to real-time PCR, Western blot analyses and ELISA. However, there was no correlation between mRNA and protein levels in the adipocytes treated with mitochondrial OXPHOS inhibitors. Treatment with oligomycin or rotenone increased the mRNA levels, but protein levels were actually reduced in C2C12 myoblasts (Fig. 1D and E). Discrepancies between proteomic and transcriptomic data were also seen in several previous investigations (Wang 2008, Maier *et al.* 2009). Moreover, in the previous

report, these frequent discrepancies between variation in transcript and protein levels indicate that reanalysis of metabolic pathways using more comprehensive proteomic coverage may uncover unknown biological actions (Williams *et al.* 2016). In addition, differences between the associations at transcript and protein levels may indicate that post-transcriptional mechanisms might be important in the control of the protein expression. As no significant change in the expression of ANGPTL6 in primary hepatocytes was observed, this suggests that *Angptl6* induction by treatment with mitochondrial inhibitors might be a cell type-specific phenomenon (Fig. 1D and E). We also investigated the expression of *Angptl3* and *Angptl4* in cultured adipocytes treated with vehicle, oligomycin, CCCP or rotenone. These chemicals increased the expression of *Angptl6*, but did not induce any changes in *Angptl3* or *Angptl4* expression in cultured adipocytes (Supplementary Fig. 1C). Furthermore, inhibitors such as rotenone and CCCP are known to induce ER stress. Therefore, we showed *Angptl6* expression after treatment with doxycycline, which is more specific to mitochondria as a UPR^{mt} inducer. As expected, the expression of *Angptl6* was enhanced in the cultured adipocytes treated with doxycycline (Supplementary Fig. 2A). However, we did not observe a significant increase in *Angptl6* expression in cultured adipocytes treated with tunicamycin, which is an ER stress inducer (Supplementary Fig. 2B). Taken together, deficiency of CRIF1 or impaired OXPHOS function increased the expression of ANGPTL6 in cultured adipocytes.

Development of adipose tissue-specific *Crif1*-deficient mice

To confirm the *in vivo* effects of adipose OXPHOS reduction on ANGPTL6 expression, we generated adipocyte-specific *Crif1*-deficient mice (AdKO) by crossing *Crif1*^{flax/flax} mice with *Adiponectin-Cre* transgenic mice (Ryu *et al.* 2013) (Fig. 2A). AdKO mice were born viable and did not show gross abnormalities until 8 weeks of development, but had larger brown adipose tissue depots (Fig. 2B). At 10 weeks of age, AdKO mice showed lower body weight gain than WT mice on a normal chow diet, but there was no significant difference in fasting blood glucose between the two groups (Fig. 2C). Epididymal white adipose tissue (eWAT) mass was reduced, whereas brown adipose tissue mass was increased in AdKO mice (Fig. 2D). As mitochondrial OXPHOS function is essential for maintaining thermogenesis in brown adipose tissue, the increased mass and discoloration may be a result of

Figure 2

Development of adipose tissue-specific *Crif1*-deficient (AdKO) mice. (A) Strategy for generation of AdKO mice and genotype analysis using PCR. (B) Gross appearance of control and AdKO mice (left) and images of white adipose tissue (right upper), muscle and brown adipose tissue (right lower) from control and AdKO male mice. (C) Measurement of body mass, fasting glucose, fasting insulin and HOMA-IR in WT and AdKO mice. (D) Comparison of tissue masses between WT and AdKO mice. (E) Western blotting for mitochondrial oxidative phosphorylation complex subunits in adipose tissue of control and AdKO mice at 8 weeks of age. The same blot was stripped and reprobed with the various antibodies. All data are representative of three independent experiments ($n=5$ per group). Data are expressed as mean $\pm$ s.d. * $P<0.05$, ** $P<0.01$ vs the corresponding controls.

decreased OXPHOS function. Consistent with this finding, a previous study also showed that lipolysis is important for the maintenance of a BAT phenotype (Ahmadian *et al.* 2011). On the other hand, we also observed a decrease in the size of WAT from AdKO mice compared to that in WT mice. We thought that this finding might be due to increased levels of FGF21, which is an inducer of lipolysis in WAT. Adipose tissue from AdKO mice displayed significantly reduced levels of OXPHOS subunits, including NDUFA9, UQCRC2 and COX IV, compared to that from the control group (Fig. 2E). Moreover, the expression of COX1, which is an mtDNA-encoded OXPHOS subunit, was also reduced in the WAT and brown adipose tissue, but not in the liver (Supplementary Fig. 3). These data suggest that *Crif1* deficiency resulted in the decrease of OXPHOS subunits in adipose tissue and reduction in the mass of eWAT in mice.

ANGPTL6 expression is increased in AdKO mice

Emerging evidence suggests that reduced OXPHOS function in one organ can stimulate the activation of adaptive mitochondrial stress responses in other organs through non-cell-autonomous factors that are thought to be mitokines (Durieux *et al.* 2011, Houtkooper *et al.* 2013, Taylor *et al.* 2014). Although it is known that mitochondrial dysfunction induced by a deficiency in autophagy increases FGF21 expression (Kim *et al.* 2013), the identity of other secreted mediators and their interactions remain to be clarified. As shown in Fig. 1, ANGPTL6 expression was increased by the reduction in mitochondrial OXPHOS function in cultured adipocytes and MEFs. We also found that *Angptl6* and *Fgf21* expression was significantly increased in the white adipose tissue of AdKO mice (Fig. 3A). In Western blot

Figure 3

Angiopoietin-related growth factor (ANGPTL6) and fibroblast growth factor 21 (FGF21) expression in adipose tissue-specific *Crif1*-deficient (AdKO) mice. (A) Real-time PCR analysis of white adipose, liver and muscle RNA from control and male AdKO mice. (B) Immunoblotting for CRIF1, ANGPTL6 and FGF21 in white adipose tissue and liver of control and male AdKO mice. Relative quantification of each protein was undertaken using densitometry. The same blot was stripped and reprobed with the various antibodies. (C) Real-time PCR analysis of the adipocyte-enriched population and stromal vascular fraction from adipose tissue of WT and AdKO mice. All data are representative of three independent experiments ($n=5$ per group). Data are expressed as mean $\pm$ s.d. * $P<0.05$, ** $P<0.01$ vs the corresponding controls.

analysis, protein expression of ANGPTL6 and FGF21 was also higher in the white adipose tissue and brown adipose tissue of AdKO mice, but did not show any difference in the liver (Fig. 3B and Supplementary Fig. 4). Serum levels of FGF21 and Angptl6 were also increased in AdKO mice (Supplementary Fig. 5A and B). In addition, the adipose tissue concentration of FGF21 was also increased in AdKO mice compared to that in WT mice (Supplementary Fig. 5C). Moreover, epididymal and inguinal adipose (iWAT) depots were collected, digested and centrifuged to yield a buoyant adipocyte-enriched cell population and a pellet of SVCs. In real-time PCR analysis, *Angptl6* and *Fgf21* expression was significantly higher in the adipocyte-enriched cell population from the eWAT and iWAT of AdKO mice, whereas there was no significant difference from WT in expression in SVCs (Fig. 3C). Taken together, reduced mitochondrial OXPHOS function in

adipose tissue increased the expression of ANGPTL6 and FGF21 in mice.

ANGPTL6 increases *Ppara* expression by the regulation of ERK–MAPK pathways

To determine the paracrine role of ANGPTL6, we observed the effects of ANGPTL6 on cellular mitochondrial function. The OCR was measured in 3T3-L1 adipocytes and primary hepatocytes treated with vehicle or recombinant murine ANGPTL6 protein (rANGPTL6). Treatment with rANGPTL6 increased the basal OCR and enhanced maximal OXPHOS capacity in 3T3-L1 adipocytes, but did not affect the OCR of primary hepatocytes (Fig. 4A and B). In addition, treatment with rANGPTL6 significantly increased the mRNA expression of *Ppara*, *hormone-sensitive lipase (Hsl)*, *uncoupling protein 2 (Ucp2)* and *PPAR γ*

Figure 4

Angiopoietin-related growth factor (ANGPTL6) increases peroxisome proliferator-activated receptor- α (PPAR α) expression by regulating the extracellular signal-regulated kinase (ERK)-mitogen-activated protein kinase (MAPK) pathway. (A and B) Oxygen consumption rates measured in 3T3-L1 adipocytes and primary cultured hepatocytes, respectively, $\pm$ treatment with recombinant ANGPTL6 protein. (C and D) Real-time PCR analysis of RNA from 3T3-L1 adipocytes, mature immortalized brown adipocytes and primary hepatocytes treated with vehicle or recombinant ANGPTL6 protein. (E) Immunoblotting for ERK-MAPK signaling pathway components (phospho-ERK1/2, ERK1/2, phospho-p38 MAPK and p38 MAPK (Thr180/Tyr182)) in 3T3-L1 adipocytes treated with recombinant ANGPTL6 protein. All data are representative of three independent experiments ($n=5$ per group). Data are expressed as mean $\pm$ s.d. * $P<0.05$, ** $P<0.01$ vs the corresponding controls.

coactivator 1 α (PGC-1 α) in 3T3-L1 adipocytes and mature immortalized brown adipocytes (Fig. 4C). β -oxidation-related genes were also upregulated in 3T3-L1 adipocytes and mature immortalized brown adipocytes treated with rANGPTL6, but this treatment did not affect their expression in primary hepatocytes (Fig. 4D).

A previous study demonstrated that p38 MAPK phosphorylates and activates the transcription factor PPAR α in cardiac myocytes (Barger *et al.* 2001). To reveal the mechanism of the increased PPAR α expression in rANGPTL6-treated 3T3-L1 adipocytes, we investigated the effect of ANGPTL6 on the ERK-MAPK pathway. Interestingly, treatment with rANGPTL6 dramatically increased the levels of phosphorylated ERK and p38 MAPK (Thr180/Tyr182) in 3T3-L1 adipocytes (Fig. 4E). Taken together, these data indicate that ANGPTL6 increased

the expression of Ppara through activation of the ERK-MAPK pathway.

ANGPTL6 potentiates Ppara-mediated transcription of Fgf21

The increased Ppara expression observed after treatment with rANGPTL6 suggested the possibility that FGF21 is regulated by ANGPTL6 in adipocytes (Badman *et al.* 2007, Inagaki *et al.* 2007). Thus, we investigated the effect of ANGPTL6 on FGF21 expression in cultured adipocytes. Real-time PCR analysis established that treatment with rANGPTL6 enhanced Fgf21 expression in 3T3-L1 adipocytes and mature immortalized brown adipocytes (Fig. 5A). Moreover, rANGPTL6 enhanced luciferase activity driven by the Ppara promoter in 3T3-L1

Figure 5

The angiopoietin-related growth factor (ANGPTL6)-induced increase in peroxisome proliferator-activated receptor- α (PPAR α) results in increased fibroblast growth factor 21 (FGF21) expression. (A) Real-time PCR analysis of 3T3-L1 adipocytes and mature immortalized brown adipocytes treated with vehicle or recombinant ANGPTL6 protein. (B) Luciferase activity driven by the *Ppar α* promoter in 3T3-L1 adipocytes transfected with pGL3b-PPAR α -Luc. (C) Western blotting for FGF21 in 3T3-L1 adipocytes treated with vehicle or recombinant ANGPTL6 protein. (D) Real-time PCR analysis of *Fgf21* expression in 3T3-L1 adipocytes treated with recombinant ANGPTL6 protein, a PPAR α agonist, or a PPAR α antagonist. (E) Real-time PCR analysis of *Fgf21* expression in WT and *Ppar α* -deficient differentiated adipocytes treated with vehicle or recombinant ANGPTL6 protein. All data are representative of three independent experiments. Data are mean $\pm$ s.d. * P < 0.05 vs the corresponding controls.

adipocytes transfected with pGL3b-PPAR α -Luc (Fig. 5B). FGF21 expression was also increased in 3T3-L1 adipocytes and brown adipocytes treated with rANGPTL6 (Fig. 5C). In contrast to brown adipocytes, FGF21 levels were increased at 12, but not 24 h in 3T3-L1 adipocytes treated with rANGPTL6 (Fig. 5C). This discrepancy may be due to the normalized expression of recombinant ANGPTL6-induced PPAR α in cultured adipocytes at the 24-h time point (Supplementary Fig. 6). To define the effect of PPAR α on FGF21 expression in 3T3-L1 adipocytes, we also investigated ANGPTL6-mediated *Fgf21* expression using GW6471 as an inhibitor of PPAR α and fenofibrate as a PPAR α agonist. Interestingly, treatment with GW6471 significantly decreased ANGPTL6-mediated *Fgf21* expression in 3T3-L1 adipocytes (Fig. 5D). Furthermore, treatment with rANGPTL6 increased *Fgf21* expression in WT, but not *Ppar α* -deficient, differentiated adipocytes (Fig. 5E). These observations demonstrated that PPAR α is essential for ANGPTL6-mediated FGF21 expression in cultured adipocytes. Taken together, these data demonstrate that expression of ANGPTL6 is coupled with reduced mitochondrial OXPHOS function, and

its paracrine effects are characterized by enhanced mitochondrial oxidative function and stimulation of FGF21 expression in adipocytes.

Discussion

Mitochondria are crucial for cellular and systemic energy homeostasis in humans and rodents. The mitochondrial OXPHOS system, which is composed of five multimeric complexes embedded in the mitochondrial inner membrane, plays an important role in energy production and the generation of free radicals in cells. Recent studies in *D. melanogaster* and *C. elegans* revealed that mutation or reduced function of electron transport chain components increased longevity via activation of cell-autonomous and non-cell-autonomous factors (Durieux *et al.* 2011, Houtkooper *et al.* 2013). These studies suggested that reduced mitochondrial function induces a number of secreted factors. CRIF1 is known to interact with several nuclear receptors, including the nerve growth factor IB, androgen receptor, signal transducer and activator

of transcription 3, E74-like factor 3 and NF-E2-related factor 2, thereby regulating the function of these proteins and has been suggested to be a regulator of cell cycle progression and cell growth (Park *et al.* 2005, Kwon *et al.* 2008, 2009, Suh *et al.* 2008, Kang *et al.* 2010). CRIF1 is also important in mitochondria for the maintenance of OXPHOS function *in vitro* and *in vivo* (Kim *et al.* 2012). Therefore, we generated cellular and mouse models of reduced mitochondrial function (*Crif1*-deficient MEFs and AdKO mice, respectively) to clarify the effects of reduced OXPHOS function on the expression of secreted factors (Figs 1 and 2). We identified increased expression of ANGPTL6 in *Crif1*-deficient MEFs, as well as in white and brown adipose tissues from AdKO mice.

More recently, it was demonstrated that activation of mitochondrial stress responses is induced by specific conditions, including respiratory chain gene mutations (Baker *et al.* 2012, Nargund *et al.* 2012), imbalances between mtDNA-encoded and nuclear-encoded OXPHOS subunits (Houtkooper *et al.* 2013), inhibition of mitochondrial proteases, chaperones, respiratory chain complex assembly factors (Pulliam *et al.* 2014) and treatment with respiratory chain inhibitors (Nargund *et al.* 2012, Liu *et al.* 2014). In previous investigations, CRIF1 was identified as a mitoribosomal factor required for intramitochondrial production and insertion of OXPHOS polypeptides into the inner membrane (Kim *et al.* 2012, Greber *et al.* 2014). Therefore, the absence of *Crif1* results in profound impairment of cellular respiration. In the current study, we clarified the effects of impaired mitochondrial OXPHOS function using *Crif1*-deficient MEFs and AdKO mice. Moreover, we suggest that ANGPTL6 is a non-cell-autonomous factor induced by the reduction in OXPHOS function in 3T3-L1 adipocytes and adipose tissue from AdKO mice. Based on our observations, mitochondrial function in white adipose tissue may be regulated not only through cell-autonomous mechanisms but also through non-cell-autonomous effects, thereby regulating local and systemic energy homeostasis by paracrine and endocrine mechanisms.

ANGPTL6, also known as AGE, was identified as an orphan peptide containing an N-terminal coiled-coil domain and a C-terminal fibrinogen-like domain, which does not bind to the angiopoietin receptor (Oike *et al.* 2003). ANGPTL6 was initially described as a secreted angiogenic factor involved in promoting angiogenesis and cutaneous wound healing (Oike *et al.* 2003, 2004). However, recent emerging evidence suggests that ANGPTL6 has biological functions in non-vascular cells. For example, disruption of *Angptl6* in mice leads to marked

obesity and lipid accumulation in skeletal muscle and liver, thereby producing insulin resistance, accompanied by decreased energy expenditure (Oike *et al.* 2005). However, *Angptl6*-transgenic mice were resistant to obesity, insulin resistance and non-adipose tissue steatosis (Oike *et al.* 2005). Furthermore, serum ANGPTL6 was significantly increased in patients with type 2 diabetes compared with that in non-diabetic controls and positively correlated with fasting blood glucose, independent of age, sex, body mass index and glomerular filtration rate (Ebert *et al.* 2009, Namkung *et al.* 2011). However, little is known about the mechanism of action or the physiological relevance of ANGPTL6 in adipose tissue because its cognate receptor has not been identified.

ANGPTL6 was previously reported to be an angiogenic and liver-derived secreted factor. In the present study, we observed ANGPTL6 expression at both the RNA and protein levels in adipose tissue and 3T3-L1 adipocytes, suggesting that ANGPTL6 may also be secreted from adipose tissue. Moreover, this study showed that reduced OXPHOS function in adipose tissue results in increased production of ANGPTL6, which promotes the gene expression of β -oxidation enzymes in cultured adipocytes. ANGPTL6 also suppresses glucose production through AKT/forkhead box class O1-dependent signaling in hepatocytes (Kitazawa *et al.* 2007). Treatment with rANGPTL6 increased adenosine monophosphate-activated protein kinase (AMPK) activity, thereby improving insulin signaling in the skeletal muscle of diet-induced obese mice (Hato *et al.* 2008). Furthermore, ANGPTL6 enhances the ligand-binding activities of PPAR α and PPAR δ in C2C12 myoblasts and activates p38 MAPK in skeletal muscles (Oike *et al.* 2005). Taken together, these findings demonstrate that ANGPTL6 is closely associated with enhanced systemic energy expenditure.

Notably, *Angptl6* expression was markedly increased in *Crif1*-deficient MEFs and 3T3-L1 adipocytes treated with inhibitors of the mitochondrial OXPHOS complex. In parallel, AdKO mice displayed significant increases in ANGPTL6 expression in both visceral and subcutaneous adipose tissue. Moreover, ANGPTL6 modulated PPAR α expression and β -oxidation via the ERK–MAPK pathway in 3T3-L1 adipocytes. Collectively, these findings provide evidence for the non-cell-autonomous factor ANGPTL6 as a regulator of FGF21 production in cultured adipocytes and AdKO mice.

Emerging evidence shows that FGF21 is involved in mitochondrial dysfunction and diseases in human patients. Serum FGF21 increased in patients with mitochondrial translation defects or mitochondrial DNA

deletions (Lehtonen *et al.* 2016). FGF21 secretion by muscle cells with mitochondrial alterations may be an adaptive physiological mechanism to regulate systemic energy metabolism (Ribas *et al.* 2014). In the current study, we also observed increased levels of FGF21 in adipose tissue and the serum of mice with adipose tissue-specific mitochondrial OXPHOS dysfunction. This finding suggests that serum levels of FGF21 can be increased by mitochondrial dysfunction in adipose tissue as well as in skeletal muscle.

PPAR α is a critical transcription factor that is regulated as part of metabolic stress responses under both physiologic and pathologic conditions. In the current study, we showed that ANGPTL6 increased the expression of *Ppara* via activation of the ERK–MAPK signaling pathway in 3T3-L1 adipocytes (Fig. 3). In line with our findings, p38 MAPK also phosphorylates and activates PPAR α , leading to an increase in the ligand-mediated transactivation of targets and enhanced cooperation with the transcriptional coactivator PGC-1 in cardiac myocytes (Barger *et al.* 2001). Moreover, the p38 MAPK signaling pathway is involved in the adiponectin-mediated activation and transcriptional activity of PPAR α in C2C12 myoblasts (Yoon *et al.* 2006), whereas insulin-induced activation of PPAR α is mediated via the ERK–MAPK pathway in hepatocytes (Shalev *et al.* 1996). By contrast, ERK–MAPK signaling is known to reduce PPAR α activity and induce gene expression in hypertrophied cardiac myocytes (Barger *et al.* 2000). The detailed molecular mechanism underlying this discrepancy has not been established, but the context-specific nature of nuclear receptor phosphorylation is likely of importance. Thus, further studies are needed to determine the effects of MAPK activation on PPAR α activity and expression under diverse conditions.

In summary, inhibition of mitochondrial OXPHOS function-mediated ANGPTL6 production increases the expression of *Ppara* via the activation of the ERK–MAPK signaling pathway in 3T3-L1 adipocytes and the adipose tissue of AdKO mice. Therefore, this study suggests that mitochondrial OXPHOS function influences the expression of non-cell-autonomous factors in adipose tissue, and therefore represents a novel regulator of fat metabolism.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-16-0549>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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