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Journal of Cellular Biochemistry 116:1090–1100 (2015)

Journal of Cellular Biochemistry

Adiponectin Prevents Reduction of Lipid-Induced Mitochondrial Biogenesis via AMPK/ACC2 Pathway in Chicken Adipocyte

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ABSTRACT

Adiponectin (APN) stimulates mitochondrial biogenesis and reduces lipid content in human and animal adipocytes. However, the mechanism of adiponectin in regulating mitochondrial biogenesis in chicken adipocytes has never been reported. The objective of this study is to examine the mechanism that adiponectin plays in lipid-induced mitochondrial biogenesis and mitochondrial function in chicken adipocytes. We found that the overexpression of adiponectin reduced the membrane DAG content and elevated the membrane translocation of PKCθ. In contrast to control groups, the overexpression of adiponectin increased mitochondrial density and mitochondrial DNA contents and peroxisome proliferator-activated receptor αcoactivator 1α (PGC1-α) expression. Mitochondrial membrane potential and cytochrome C (Cyt C) content were detected by JC-1 fluorescent staining and immunofluorescence which indicated that overexpression of adiponectin enhanced mitochondrial ATP synthesis. Moreover, AMPK/ACC2 signaling pathway was activated along with the elevation of PGC1-α and TFAM by the overexpression of adiponectin, meanwhile the lipid transcription marker genes were down-regulated. This effect was alleviated by reducing adiponectin and a specific inhibitor of AMPK pathway. We concluded that adiponectin could prevent reduction of lipid-induced mitochondrial biogenesis via AMPK/ACC2 pathway in chicken adipocytes. J. Cell. Biochem. 116: 1090–1100, 2015.

KEY WORDS: ADIPOCYTOKINE; AMPK; FAT CELLS; MITOCHONDRIA; POULTRY

Adiponectin (APN) is a 30 kDa adipokine, a hormone secreted from adipose tissues. APN consists of an N-terminal collagen domain and a C-terminal globular domain as the receptor binding effector (Scherer et al., 1995; Maedak et al., 1996). It has many positive effects on metabolism, including enhancing insulin action and reducing atherosclerotic processes. Circulating adiponectin concentrations were reduced in patients with insulin resistance, type 2 diabetes, obesity, or cardiovascular disease (Arita et al., 1999; Lindsay et al., 2002; Lindsay et al., 2003). A negatively association between adiponectin and belly fat deposition in chicken has been reported (Hendricks et al., 2009). Bauche et al. (2007) demonstrated overexpression of adiponectin in white adipose tissue of mice corresponded to the reduction in adiposity due to increased energy expenditure. Adiponectin has also been shown to increase mitochondrial biogenesis and palmitate oxidation, enhance citrate synthase activity, and reduce reactive oxygen species production in human myotubes (Civitarese et al., 2006; Liu and Sweeney, 2013). Moreover, mitochondrial biogenesis is positively related with plasma adiponectin concentration in obesity mice (Koh et al., 2007). However, most works were conducted in human skeletal muscles and adipocytes, and there has been no report on chicken adipocytes. Thus, we devoted to find the role of adiponectin on regulating mitochondrial biogenesis in chicken adipocyte.

Mitochondrion is a main organelle for fatty acid oxidation in animal cells, where oxidative phosphorylation happens and ATP is generated (Kadenbach et al., 2012). Mitochondrion is also essential to other vital metabolic processes, such as glucose homeostasis, energy metabolism, cell signal transduction, and apoptosis (Diaz and Moraes, 2008). Mitochondrial dysfunction contributes to a variety of human diseases, such as diabetes, obesity, cancer, aging, cardiomyopathy, and neural degenerative diseases (Wallace, 2005). Mitochondrial biogenesis involves several processes, including mitochondrial maintenance, number of mitochondria, and mitochondrial activity. These processes are regulated dynamically during cell differentiation.

Lu Gan and Jun Yan contributed equally to this paper.

Grant sponsor: National Nature Science Foundation of China; Grant number: 31172185; Grant sponsor: National Major Scientific Project; Grant number: 2015CB943102.

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Manuscript Received: 8 May 2014; Manuscript Accepted: 18 December 2014

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 23 December 2014

DOI 10.1002/jcb.25064 • © 2014 Wiley Periodicals, Inc.

PGC1- α, a transcriptional coactivator, is a key inducer of mitochondrial biogenesis in cells (Austin and St-Pierre, 2012). Although mitochondria morphology in fatty liver did not vary much from normal liver, its function was impaired (Bruguera et al., 1977; Yang et al., 2000; Gonçalves et al., 2013). Adiponectin has been shown to promote mitochondrial biogenesis and enhance oxidative capacity in the skeletal muscles using adiponectin gene knocked out and transgenic mouse models (Civitarese et al., 2006; Iwabu et al., 2010; Qiao et al., 2012). Adiponectin could protect mitochondrial biogenesis from lipid-induced impairment. And thereby, contribute to improvement of mitochondrial functions. However, this hypothesis has not been tested in chicken adipocytes.

The molecular mechanisms leading to mitochondrial dysfunction in obesity have been debated intensively over the past decades. Some studies showed AMP-activated protein kinase (AMPK) plays a vital role on regulating mitochondrial function (Civitarese et al., 2006). Zong et al. (2002) demonstrated a mutation of the AMPK-α2 gene disturbed mitochondrial biogenesis, and no active signaling pathway was involved in the process of disturbance. Bergeron et al. (2001) also showed that activation of AMPK increased both mitochondrial biogenesis and oxidative capacity in skeletal muscles of rodents. In animal models of obesity, both adiponectin and globular adiponection (gAD) treatments activated AMPK and increased fat oxidation and insulin sensitivity in hepatocytes and skeletal muscles (Yamauchi et al., 2002; Steinberg and Beck, 2007). Collectively, these data suggest that AMPK can increase both the number and oxidative capacity of mitochondria, hence establishing a direct link between adiponectin signaling and mitochondrial biogenesis. These effects are attributed to the activation of AMPK by adiponectin binding to the adiponectin receptor 1 (AdipoR1), resulting in increased phosphorylation of acetyl-coenzyme A carboxylase (ACCβ), reduced malonyl-coenzyme A production, and increased CPT-1 (Carnitinepalmitoyltransferase 1) activity and mitochondrial fatty acid oxidation (Yamauchi et al., 2007; Osler and Zierath, 2008). In summary, AMPK can increase both the number and oxidative capacity of mitochondria, and there is a linkage between adiponectin signaling and mitochondrial biogenesis. Based on those reports, we hypothesized that AMPK pathway and ACC are vital regulators between adiponectin and mitochondrial biogenesis in chicken adipocytes.

In this study, we used an over-expression vector of adiponectin gene and an interference vector of adiponectin gene in cultured chicken adipocytes to investigate the regulatory role of adiponectin on lipid- induced impairment of mitochondrial biogenesis. It was anticipated to develop a new approach to prevent and treat metabolic disorders associated with lipid metabolism in poultry. We demonstrated that AMPK/ACC2 signaling pathway was activated in the process that adiponectin alleviated lipid- induced reduction of mitochondrial biogenesis.

MATERIALS AND METHODS

CHICKEN PRE- ADIPOCYTES CULTURE

The use of the animals and the experimental procedures were approved by the Animal Ethics Committee of the Northwest A&F University. Nine male Cobb broilers were used as a source of adipose tissue. Chicks were maintained on a 24 h constant- light schedule and allowed ad libitum access to water and a commercial diet. The diet was formulated to meet or exceed the nutrient requirements for broilers (NRC, 1994).

Broiler chicks aged of 7 to 12 days was anesthetized using an intraperitoneal injection of 50 mg/kg barbiturate, followed immediately by sterile dissection. The abdominal adipose tissues were removed and collected. Visible fibers and blood vessels in the tissues were removed. The adipose tissue was washed three times with PBS buffer containing 200 U/mL penicillin (Sigma, St. Louis) and 200 U/mL streptomycin (Sigma). Then the adipose tissue was chopped to fine sections ($1mm^3$) with scissors and incubated in 10 mL of digestion buffer at 37°C for 50 min in water bath. The digestion buffer contained Dulbecco′s modified Eagle medium (DMEM)/F-12 (Gibco, Life Technology, CA), 100 mM HEPES (Sigma), 1.5% bovine serum albumin (Sigma), 2 mg/mL type I collagenase (Sigma). After the incubation, a growth medium DMEM/F-12 (50:50), 10% fetal bovine serum (Sigma), 100 U/mL penicillin and 100 U/mL streptomycin were added into the digestion flask. The solution was then filtered through nylon screens with 250 μm and 20 μm mesh to remove undigested tissues and large cell aggregates. The filtered cells were centrifuged at 1, $300 \times g$ for 7 min at room temperature to separate floating adipocytes from cell pellets. Cell pellets were incubated in 5 mL red blood cell lysate containing 0.154 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA (all the chemicals were obtained from Sigma) for 10 min, and then centrifuged at 1, $300 \times g$ for 7 min at 25°C. Cells were then seeded in 60-mm culture dish at a density of 5×10^4 cells per cm^2 and cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C until confluence. The medium was freshed every other day.

ADENOVIRAL VECTORS AND PLASMIDS CONSTRUCTION

Total RNA was extracted from abdominal fat of chicks. Recombinant adenovirus vector encoding adiponectin (Ad-APN) and empty adenovirus vector containing green fluorescent protein (Ad-GFP) were constructed using the method as described by Luo (Luo et al., 2007). Adiponectin (GeneBank, NM_206991) gene had been cloned previously in our laboratory.

Plasmids pcDNA 3.1-ADPN (pC-APN) was created by subcloning the cDNA into pcDNA 3.1 vector. Small-interfering RNA against adiponectin was designed based on the principle of shRNA construction and was synthesized by Invitrogen Company (Shanghai, China) using pGPU6/GFP/Neo shRNA expression vector (Genepharma). The sequences of shRNAs are as followed: pGPU6/GFP/Neo-ADPN-952 (bases 952–972): (forward 5′- CAC CGG GTC TAT GCT GAC AAC ATC ATT CAA GAG ATG ATG TTG TCA GCA TAG ACC CTT TTT TG -3′, reverse 5′- GAT CCA AAA AAG GGT CTA TGC TGA CAA CAT CAT CTC TTG AAT GAT GTT GTC AGC ATA GAC CC-3′). pGPU6/GFP/Neo-shRNA-GAPDH (glyceraldehyde 3- phosphate dehydrogenase) was used as a positive control (forward 5′- CAC CGT ATG ACA ACA GCC TCA AGT TCA AGA GA C TTG AGG CTG TTG TCA TAC TTT TTT G-3′, reverse 5′-GAT CCA AAA AA G TAT GAC AAC AGC CTC AAG TCT CTT GAA CTT GAG GCT GTT GTC ATA C-3′). shRNA plasmids were transfected into chicken pre- adipocytes by using Lipofectamine reagent (Invitrogen, CA).

CELL LIPID MEASUREMENT

The adipose cells were washed three times in PBS buffer and then fixed in 10% (v/v) formaldehyde for 30 min. The fixed cells were then washed three times with PBS buffer, and stained with a working solution of Oil Red O for 30 min at room temperature. The cells were then washed with deionized water, and the images of the cells were observed with an inverted microscope (Nikon Instruments Europe BV, England). Then Oil Red O was extracted by 100% avantin for colorimetric analysis of TG at 510 nm. The Bodipy 493/503 staining (Life Technology, CA) was used to visualize lipid droplet frequency in the adipocytes transfected with indicated plasmids. After 6 days treatment, cells were washed in PBS buffer, and 4% formaldehyde diluted in PBS buffer was added to fix the cells for 30 min. Bodipy dye was diluted in PBS buffer to the final concentration of 1 mg/mL, and applied to cells for 15 min. Then DAPI (4′,6-diamidino-2-phenylindole) solution (10 μg/mL) was added in, and incubated for 30 min. Lipid droplet frequency was quantitated by counting the number of BODIPY-positive cells in 10 random microscopic fields (×40), as described previously (Alkhouri et al., 2010). Digital images were obtained with a Nikon TE2000-U Fluorescence Microscope (Tokyo, Japan). The area of the stained cells with Bodipy and the droplet diameter frequency distributions was measured by Image-Pro Plus analyzer (Media Cybernetics, MD).

The cytosol and membrane DAG content of the adipocytes were determined using the procedure described by Choi et al. (2007). Cells were homogenized in buffer (20 mM Tris-HCl, pH 7.4; 1 mM EDAT; 250 mM sucrose; 2 mM phenylmethanesulfonyl fluoride; protease inhibitor cocktail (Roche)). Cells were then centrifuged at 12,000 × g at 4°C for 1 h. The supernatant contained the cytosolic fraction. Homogenized the pellets one more time in the above buffer and then sonicated thoroughly. This was considered the membrane fraction. The total DAG content in both the cytosolic and membrane fractions were determined using ELISA kit (Jiangcheng, China), and the total DAG content was expressed as the sum of individual DAG species.

For glucose uptake measurement, the culture medium containing 0.2% BSA and high glucose medium (25 mM glucose) was used. After 24 h incubation, the media were collected. The glucose content in the medium was measured using an enzymatic colorimetric glucoseoxidase/peroxidase assay. The absorbance was recorded in a microplate reader at a wavelength of 505 nm (Multiskan MK3, Thermo). Medium glucose content, before and after the cultivation, was used to calculate the glucose uptake by the cells over the culture period (Accorsi et al., 2005). Free cell culture medium was removed at day 8, and used to determine fatty acid (FA) content using FA Assay kit (Jiangcheng, China) at wave length 570 nm. The triglyceride concentration in this medium was determined using Infinity Triglyceride kit (Sigma).

MITOCHONDRIAL CONTENT ASSAY BY TRANSMISSION ELECTRON MICROSCOPY

The medium was removed at day 4 after transfection with Ad-APN, shAPN and Ad-GFP. Cells were dissociated with 0.25% trypsin, and transferred into 10 mL tube, centrifuged at 1,200 × *g* at 4°C for 8 min. The supernatant was discarded, and the pellet was washed in pre-cooled (4°C) PBS buffer and then centrifuged again. After discarding the supernatant, the cell pellet was fixed with pre- cooled (4°C) 2.5% glutaraldehyde for 4 h. After washing three times with PBS buffer, the cells were fixed in 1% osmium tetroxide for 2 h, dehydrated in a graded series of ethanol dilutions (from 50% up to 100%), and embedded in epoxy resin 812. Then semi-thin section and ultra-thin section were processed and samples were double stained with uranylacetate and lead citrate (all the chemicals were purchased from Sigma). The section slides were examined and photographed under JEM- 1230 transmission electron microscopy (JEOL, Japan). Five images from three random sections were examined for each of the samples, and the averaged volume density of those images was calculated as the indicator of the mitochondrial content.

The mtDNA copy number was determined using QPCR method. A pair of primers for the Cox2 mtDNA region were: F: TGA CAG TCC ACC TAC TTA CAA T; R: CTC CAC CAA TGA CCT GAT AT. The QPCR system performed as described in the real-time quantitative PCR analysis manual. After treatment with Ad-APN and shAPN, ATP concentrations were determined using the luciferase-based ATP-assay from Roche (Mannheim, Germany). Mitochondria biogenesis markers PGC1- α, CPT1 and MDH1 were analyzed by the western blot method.

DETERMINATION OF MITOCHONDRIAL MEMBRANE POTENTIAL (ΔΨM) AND CYT C

The ΔΨm was determined by using fluorescent probe JC-1 (Beyotime, China). Briefly cells were incubated at 37°C for 10 min with 5 μg/mL JC-1, then washed twice with PBS buffer and placed in fresh medium without serum. Images were scanned by a Fluorescence Microscope (Nikon TE2000-U, Japan). In addition, cells were gently harvested with trypsin, and transferred on ice to the flow cytometer. JC-1 was excited at 488 nm and the monomer signal (green) was analyzed at 525 nm (JC-1 monomer) on a flow cytometer using a minimum of 10,000 cells per sample. Simultaneously, the aggregate signal (red) was recorded at 590 nm (JC-1 aggregates). The ratio of red/green fluorescent intensity was calculated.

Immunofluorescence analysis was performed by method as described by Lu et al. (2010). Four day after treatment, adipocytes were washed three times with PBS buffer, and fixed with 10% neutral formalin for 30 min and washed with PBS buffer again, then incubated with the rabbit against rat CytC antibody (Boster Biological Technology Co., China), diluted 1:100 in PBS buffer, at 4°C for 12 h. After the incubation, cells were washed twice with PBS buffer for 3 min, and then incubated for 1 h at room temperature with fluorescein isothiocyanate- conjugated goat against rabbit IgG antibody (Boster) diluted 1:100 in PBS buffer, and then washed again in PBS buffer. Finally the cells were illuminated with the appropriate laser line and photographed with a TE2000 Nikon fluorescence microscopy (excitation filter BP 450–490, a beam splitter FT510 and an emission filter LP520, Tokyo, Japan).

PKC TRANSLOCATION AND AMPK SIGNALING ASSAY

PKCθ membrane translocation was assessed using the protein extract from cells by western blot. 80μg crude membrane and cytosol protein extracts were separated by electrophoresis on 8% SDS-PAGE gels using slab gel apparatus. After transfer, the membranes were blocked in blocking buffer for 2 h at room temperature. Membranes were then incubated with primary antibodies against anti-PKCθ

(Epitomics, UK) diluted 1:800 in rinsing solution at 4°C overnight and incubated with the appropriate HRP- conjugated secondary antibody (Bioworld) for 2 h at room temperature. Membrane translocation of PKCθ was expressed as the ratio of membrane bans to cytosol bands (Choi et al., 2007).

Phosphorylated-AMPK, phosphorylated- Akt2 and phosphorylated ACC2, and mitochondrial biogenesis marker genes and lipid metabolism- related key factors were assessed in the protein extract from the whole cell by using western blot. Relative values from band intensities were normalized against β-actin. The primary anti-bodies used in this procedure were as follows: anti-AMPK/phosphor-AMPK^{T172}, anti-ACC2/phosphor-ACC2^{S212}, anti-Akt2/ phosphor-Akt2^{S474} (Santa Cruz). TFAM, CPT1, FAS, perilipin, ATGL and HSL were all from Epitomics (Cambridge, UK). AMPK inhibitor Compound C was purchased from Sigma (USA).

REAL-TIME QUANTITATIVE PCR ANALYSIS

The RNA in adipocytes was extracted with TRIpure Reagent kit (Takara, Dalian, China), and then 400 ng of total RNA was reverse transcribed using the M-MLV reverse transcriptase kit (Takara, China). Primers for *fatty acid synthase* (*FAS*), *adipose triglyceride lipase* (*ATGL*), *mitochondrial transcription factor A* (*TFAM*), *peroxisome proliferator activated receptor gamma coactivator 1 alpha* (*PGC1-α*), *mitochondrial superoxide dismutase 2* (*SOD2*), *catalase* (*CAT*), *malate dehydrogenases 1*(*MDH1*), *facilitated glucose transporter member 4* (*SLC2A4*), *carnitinepalmitoyltransferase 1* (*CPT-1*), *uncoupling protein 2* (*UCP2*), *Cyt C* were all synthesized by Shanghai Sangon Ltd (Shanghai, China). Primer sequences for the genes are shown in Table I. *GAPDH* and *β-actin* were used as internal controls in PCR amplification. Quantitative PCR was performed in a volume of 25 μL that contained 12.5 μL SYBR Premix EX Taq (Takara, Dalian, China), 0.5 μL each of upstream and downstream primers, 1.0 μL cDNA, and 10.5 μL deionized water. Reaction mixtures were incubated with the initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and then 60°C for 1 min. A melting curve analysis was incorporated at the end of each run to control for amplification specificity. The levels of mRNAs were normalized to β-*actin* and *GAPDH*. The expression of the genes were analyzed by method of $2^{-\triangle\triangle Ct}$ (Sun et al., 2011).

STATISTICAL ANALYSIS

The one-way ANOVA procedure of SAS v8.0 (SAS Institute, Cary, NC) was used to examine the effects of the treatments. Comparisons among the individual means were made by Fisher's least significant difference (LSD) post hoc test after ANOVA. Data are presented as mean ± SD. $P < 0.05$ was considered to be significant.

RESULTS

EFFICIENCY DETECTION OF ADIPONECTIN RECOMBINANT VECTORS AND EFFECTS ON ADIPONECTIN RECEPTORS

As shown in Figure 1A, 24 h after treating with Ad-GFP, Ad-APN, shGH and shAPN, GFP was observed by fluorescence microscopy. Compared with control groups, the expression of adiponectin increased by 80% in Ad- APN infection group, whereas decreased by 55% in shAPN transfection group ($P < 0.01$) (Fig. 1B). Figure 1C indicated the APN protein levels from each treatment group, APN protein increased in Ad-APN infection group, while decreased in the shAPN transfection group ($P < 0.01$). Neither overexpression nor inhibition of APN had any significant effect on AdipoR2 ($P > 0.05$), while the AdipoR1 protein level was elevated ($P < 0.01$) in Ad-APN group and reduced in shAPN group ($P < 0.05$) (Fig. 1D).

ADIPONECTIN REDUCED MEMBRANE DAG CONTENT AND PKCΘ TRANSLOCATION TO PLASMA MEMBRANE ACTIVITY IN CHICKEN ADIPOCYTE

The morphologic changes of chicken adipocytes were recorded in Figure 2A. Oil Red O staining showed that over-expression of APN inhibited lipid droplets formation at d 8, while reduced expression of APN increased the lipid droplets formation. Adiponectin reduced the TG concentration in adipocytes at d 8 ($P < 0.01$). Six days after transfection with an eukaryotic over-expression vector (pC-APN), the over-expression of adiponectin reduced lipid content in the

TABLE I. Primers Used for PCR

Gene	Primer sequences (5′-3′)	Product size (bp)	Accession number
β-actin	F[a]: GGCTGTGCTGTCCCTGTATGC R[b]: CTCTCGGCTGTGGTGGTGAAG	207	NM_205518
GAPDH	F: GGTGGTGCTAAGCGTGTTA R: CCCTCCACAATGCCAA	179	NM_204305
Adiponectin	F: AAGGAGAGCCAGGTCTACAAGGTG R: GTGCTGCTGTCGTAGTGGTTCTG	238	NM_206991
TFAM	F: AGCAGGTTTACGAGGAAGCA R: TTGAAGCCACTTCGAGGTCT	202	NM_204100.1
PGC1-a	F: CATCTCCAGCCAGTACAGCA R: GCGTCATGTTCATTGGTCAC	177	NM_001006457.1
SOD2	F: CCTTCGCAAACTTCAAGGAG R: ATTCCCAGCAATGGAATGAG	166	NM_204211.1
CAT	F: CCACGTGGACCTCTTCTTGT R: AAACACTTTCGCCTTGCAGT	165	NM_001031215.1
SLC2A4	F: GAGACACCAGCCATGTCCTT R: GAAGTGGGTGCTGTCAGGTT	189	XM_003642462.2
MDH1	F: AAGGCATGGAGAGGAAGGAT R: TGTGATCCAAGCGAGTCAAG	201	NM_001006395.1
CPT-1	F: TGAACACGGCAAACTTTCTG R: CATAAGTGGCCGGACTGATT	171	AY675193.1
UCP2	F: GTGGATGCCTACAGGACCAT R: GAAGTGACAGGGGACGTTGT	178	AF287144.1
Cyt C	F: AAAAGGAGGCAAGCACAAGA R: CTTGATACCCGCAAAAATCA	199	NM_001079478

GAPDH = gly ceraldehyde 3-phosphate de-hydrogenase, TFAM = mitochondrial transcription factor A, PGC1- α = peroxisome proliferator activated receptor gamma coactivator 1 alpha, SOD2 = mitochondrial superoxide dismutase 2, CAT = catalase, SLC2A4 = acilita ted glucose transporter member 4, MDH1 = malate dehydrogenases 1, CPT- 1 = carnitinepalmitoyltransfe rase 1, UCP2 = uncoupling protein 2, Cyt C = cytochrome C.
F[a] and R[b] indicate forward and reverse primers respectively.

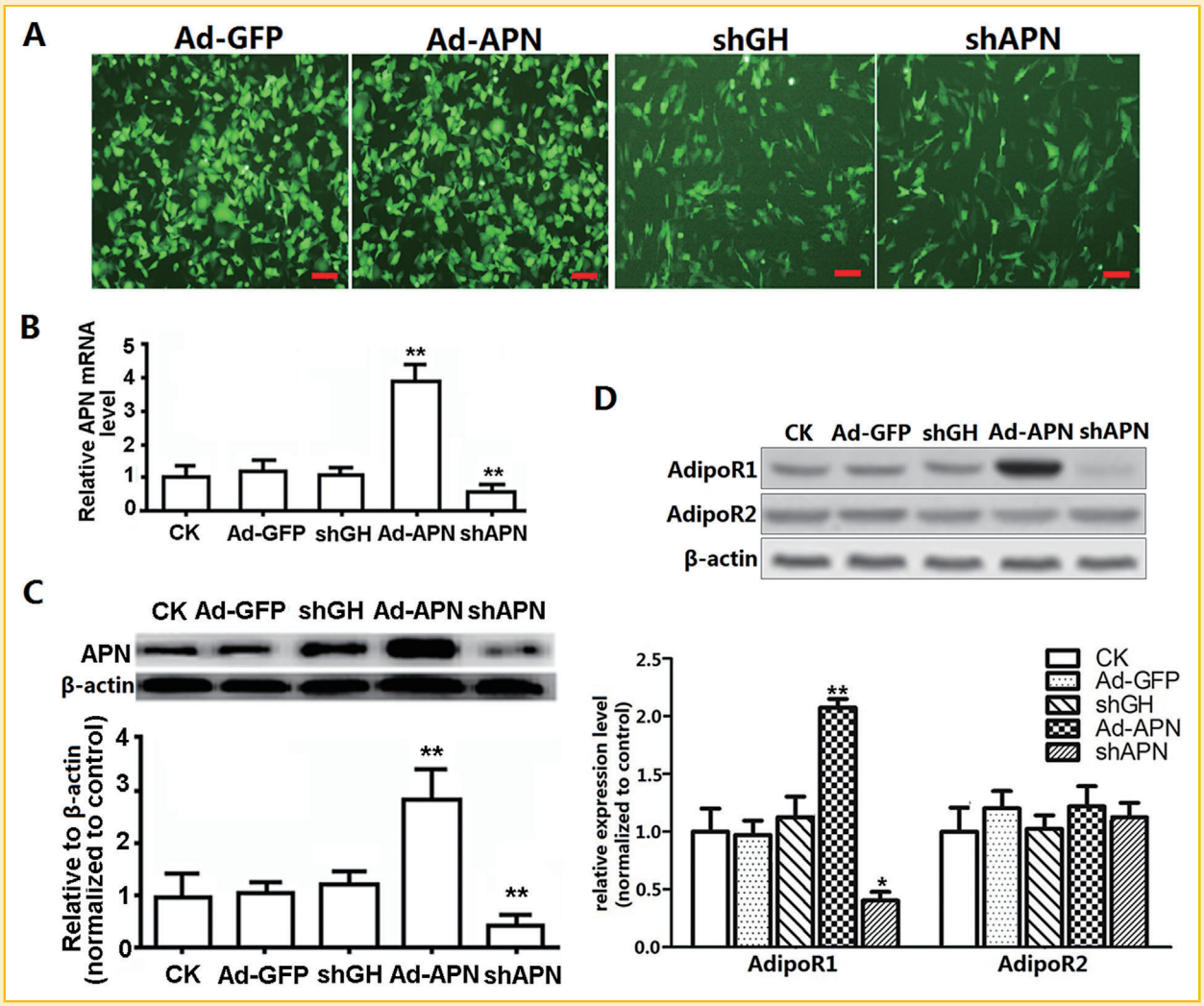

Fig. 1. Efficiency detection of adiponectin recombines vectors and effect on adiponectin receptors. A: Green fluorescent protein (GFP) observed by fluorescence microscopy with recombinant adenovirus vector encoding adiponectin (Ad-APN) infection and small interfering RNA of APN (shAPN) transfection and control vectors Ad-GFP, interference RNA of GAPDH (shGH). Scale bar, 100 μm. B: The expression of APN gene after 24 h treatment with Ad-APN, Ad-GFP, shAPN, or shGH ($n = 3$). C: The expression of adiponectin protein after 72 h treatment with Ad-APN, Ad-GFP, shAPN, or shGH ($n = 3$). D: The expression of AdipoR1 and AdipoR2 protein after 72 h treatment with Ad-APN, Ad-GFP, shAPN, or shGH ($n = 3$). CK: control group; Ad-APN: recombinant adenovirus vector encoding adiponectin; Ad-GFP: empty adenovirus vector containing green fluorescent protein; shGH: pGPU6/GFP/Neo-GAPDH; shAPN: pGPU6/GFP/Neo-APN-952. Values are means ± SD (error bar). ** $P < 0.01$, * $P < 0.05$, compared with control group.

adipocytes, as shown by BODIPY staining (Fig. 2B). Compared with control group, the over-expression of adiponectin increased glucose uptake of the adipocytes (Fig. 2C) and release of FA (Fig. 2D) during 24 h, whereas shAPN inhibited glucose uptake ($P < 0.05$) and FA release ($P < 0.01$). The TG content data (Fig. 2E) further reinforced the result of FA release ($P < 0.01$). To further examine the potential effects of adiponectin on lipid content in chicken adipocyte, DAG content was chosen as the marker. Membrane DAG content (Fig. 2F) was decreased by 30% in Ad-APN group ($P < 0.05$), which was associated with decreased PKCθ activation, as reflected by 40% reduction of PKCθ translocation to the plasma membrane ($P < 0.05$) (Fig. 3G) and by a 30% increase ($P < 0.01$) in activation of Akt2 (Fig. 3H). In contrast, shAPN increased membrane DAG content, PKCθ activation and similar inactivation of Akt2 compared with Ad-APN group. Thus, the over-expression of adiponectin decreased membrane DAG content and prevented intracellular lipid content increase.

ADIPONECTIN PROTECTED MITOCHONDRIA FROM LIPID-INDUCED REDUCTION IN MITOCHONDRIA BIOGENESIS

The copy number of intracellular mitochondria in the adipocytes was increased by 60% ($P < 0.01$) in Ad-APN group compared with that in shAPN group (Fig. 3A). Mitochondrial DNA and ATP synthesis were also significantly higher in Ad-APN group (Fig. 3B and 3 C). To further investigate the potential effect of APN on mitochondrial biogenesis, the expressions of the genes encoding *PGC1*-α, *TFAM*, *SOD2*, *CAT*, *SLC2A4*, *MDH1*, *CPT-1* and *UCP2* were determined on mRNA level (Fig. 3D). Compared with control group, the over-expression of adiponectin promoted the expressions of all the above genes ($P < 0.01$). The PGC1- α, MDH1 and CPT-1 protein levels were

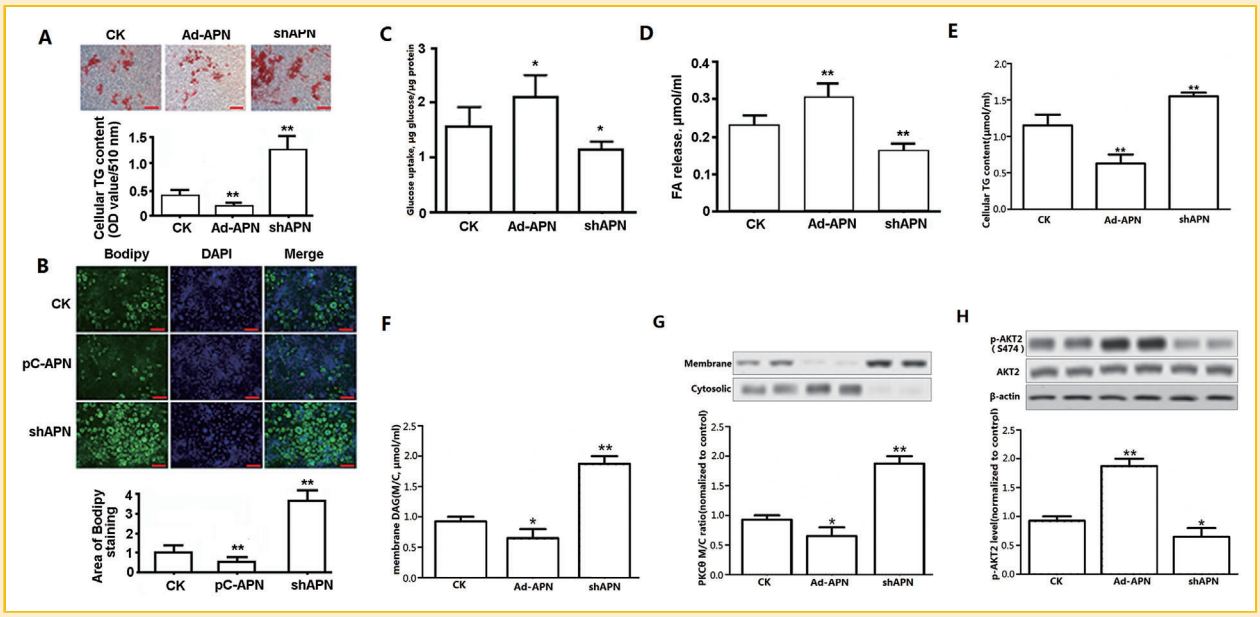

Fig. 2. Adiponectin reduced membrane DAG content and PKCθ translocation to membrane activity in chicken adipocytes. A: Representative images of Oil Red O stained sections of three groups at d 8. Scale bar, 100 μm. Lipid content was assessed by the quantification of A510 in destained Oil Red O with isopropyl alcohol (n = 3). B: Representative images of Bodipy-stained sections of three groups at d 8 after treatments with pcDNA 3.1-ADPN (pC-APN) and shAPN (n = 3). Scale bar, 100 μm. The area of stained with Bodipy was assessed by Image Pro-Plos (n = 3). C: Glucose uptake during 24 h. D: FFA release in medium during 24 h. E: cellular TG concentration. F: Membrane DAG concentration (n = 3). G: Membrane translocation of PKCθ (n = 3). M: membrane; C: cytosol; H: Representative immunoblots and densitometric quantification of phosphorylated Akt2 (n = 3); CK: control group; Ad-APN: recombinant adenovirus vector encoding adiponectin; shAPN: pGPU6/GFP/Neo-APN-952. Values are means ± SD. ** $P < 0.01$,* $P < 0.05$, compared with control group.

also higher ($P < 0.01$) in Ad-APN group compared with those in shAPN group (Fig. 3E).

ADIPONECTIN PREVENTED MITOCHONDRIA FROM LIPID-INDUCED REDUCTION IN MITOCHONDRIAL FUNCTION

To examine the effect of adiponectin on mitochondrial function, Cyt C content and mitochondrial membrane potential were studied by immunofluorescent staining and JC-1 staining. The Cyt C content, as reflected by the fluorescence intensity, was higher ($P < 0.01$) in Ad-APN group than that in control group, while the shAPN treatment decreased ($P < 0.01$) the fluorescence intensity of Cyt C (Fig. 4A). Real-time and western blot analysis also verified that the over-expression of adiponectin increased ($P < 0.01$) the expression of Cyt C gene, whereas the shAPN treatment inhibited ($P < 0.01$) the expression of Cyt C gene (Fig. 4B). Flow cytometry analysis showed that the over-expression of adiponectin increased ($P < 0.01$) the ratio of the red/green light by 50%, while shRNA treatment decreased ($P < 0.01$) the red/green ratio (Fig. 4C, 4D and 4 E).

AMPK/ACC2 PATHWAY WAS INVOLVED IN ADIPONECTIN REGULATION OF MITOCHONDRIA BIOGENESIS

To further characterize the underlying mechanisms for the regulation of adiponectin on mitochondrial biogenesis, we evaluated the changes of the AMPK pathway in response to the adiponectin treatments. The ratio of phosphorylated $AMPK^{T172}$ (p-$AMPK^{T172}$) to total AMPK protein level in Ad-APN group was increased ($P < 0.01$), and the change was accompanied by the increases in acetyl-CoA carboxylase (ACC2) phosphorylation and elevated expressions of PGC1- α, TFAM and CPT1 (Fig. 5A, 5B and 5 C). In contrast, suppression of AMPK pathway by the AMPK specific inhibitor Compound C markedly reduced ($P < 0.01$) the ACC2 phosphorylation level, and meanwhile reduced the PGC1-α, TFAM and CPT1 expression. The protein levels of lipid metabolism related genes, FAS, perilipin, ATGL and HSL were further determined, and the results are show in Figure 5C. As shown in Figure 5, AMPK up-regulated the ATGL and HSL proteins, but decreased the expression of FAS and perilipin. And Compound C reversed those proteins expression notably (Fig. 5C).

DISCUSSION

Adiponectin is an adipocyte-derived hormone that exhibits multiple positive effects in glucose and lipid metabolism (Yamauchi et al., 2002; Liu et al., 2012). Bauche et al. (2007) showed that over-lexpressing of adiponectin in white adipose tissue reduced adiposity through increased energy expenditure and decreased adipocyte number. Our previous studies have found that adiponectin impaired differentiation of chicken pre-adipocytes through the influence on the p38 MAPK/ATF-2 and TOR/p70 S6 kinase pathways and negatively regulated fat deposition in chicken (Yan et al., 2013, 2014). In this study we demonstrated a direct connection between mitochondrial biogenesis and membrane DAG. A study of mice showed mitochondrial damage could lead to the increase in

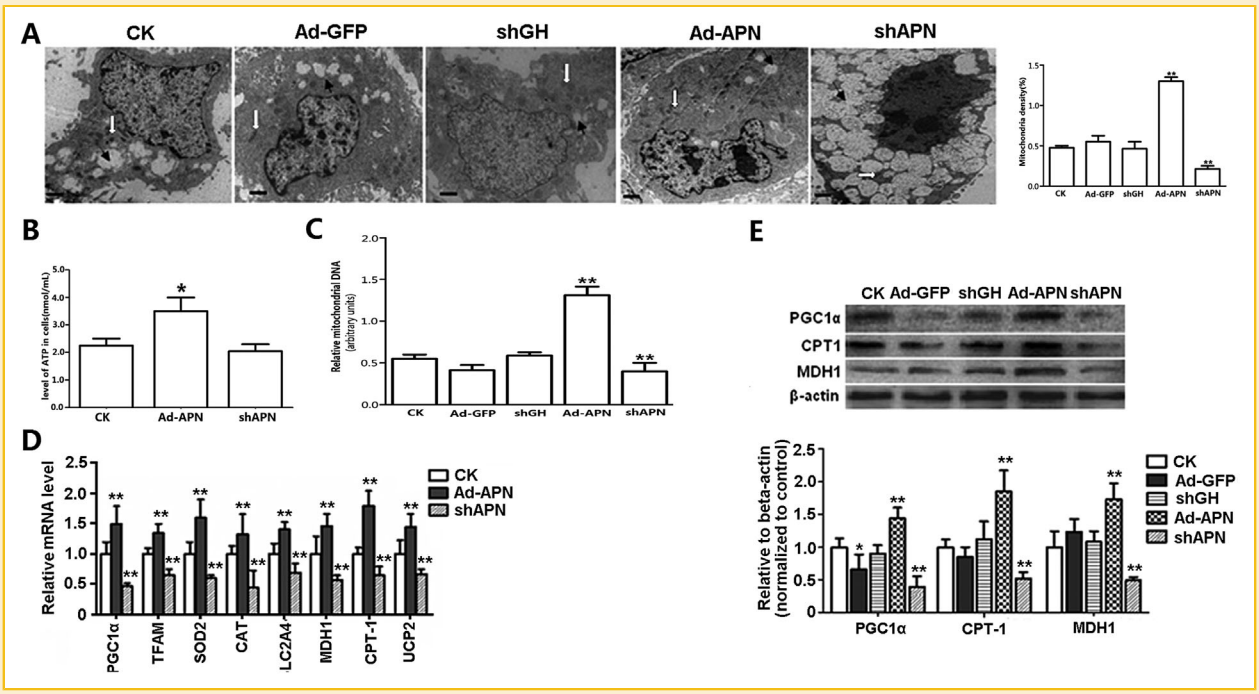

Fig. 3. Adiponectin protected mitochondria from lipid-induced reduction in mitochondria biogenesis in chicken adipocytes. A: Representative figures for the intracellular mitochondria. Scale bar: 1 μm. White arrows indicate mitochondria, and black arrows indicate lipid droplets. B: Level of ATP synthesis (n = 3). C: Mitochondrial DNA content (n = 3). D: Expression of mitochondrial maker genes after adiponectin treatment (n = 3). E: Protein levels of mitochondrial marker genes after adiponectin treatment (n = 3). CK: control group; Ad-APN: recombinant adenovirus vector encoding adiponectin: Ad-GFP: empty adenovirus vector containing green fluorescent protein; shGH: pGPU6/GFP/Neo-GAPDH; shAPN: pGPU6/GFP/Neo-APN-952; TFAM: Mitochondrial transcription factor A: PGC1-α: peroxisome proliferator activated receptor gamma coactivator 1 alpha: SOD2: mitochondrial superoxide dismutase 2; CAT: Catalase: MDH1: Malate dehydrogenases 1: CPT-1: carnitinepalmitoyltransferase 1: UCP2: uncoupling protein 2; SLC2A4: Solute carrier family 2, facilitated glucose transporter member 4. Values are means ± SD. ** $P < 0.01$, * $P < 0.05$, compared with control group.

intramyocellular DAG concentration and the translocation of PKCθ, and then reduced Akt2 activity on the phosphorylation level; in addition, individual obese mice showed dysfunction mitochondria (Lee et al., 2010). That the low level of adiponectin in obese chicken inspired us to hypothesize that adiponectin has an effect on lipid content, and likely the effect is via its influence on mitochondrial biogenesis. Our results that adiponectin decreased the membrane DAG content and reduced the PKCθ translocation to membrane support this hypothesis. In addition the decreases in the FA and TG further corroborated this hypothesis (Fig. 6).

Mitochondrial number and function are altered in response to external stimuli in eukaryotes (Wu et al., 1999). Both structural and functional impairments of mitochondria have been seen in the skeletal muscle of insulin-resistant diabetic animal models and humans (Bugger and Abel, 2010). A study on pre- adipocyte differentiation with electron microscopy revealed that there was a massive increase in the copy number of mitochondria in cells at the beginning and early stages of adipogenesis (Wilson-Fritch et al., 2003). It implied that an excess of lipid content might impair mitochondrial biogenesis. Another study presented that the over-expression of adiponectin in C2C12 cells increased the mitochondrial mass and mitochondrial DNA content (Qiao et al., 2012). In our study, we found the development of mitochondrial biogenesis was activated by over-expression of adiponectin, confirming the connection between mitochondrial function and adiponectin. TFAM, a 25 kD protein encoded by a nuclear DNA, starts the transcription process of mitochondrial DNA (mtDNA). The protein profiles in TFAM over-expression and knockout mice were found to be related to the copy number of mtDNA (Ekstrand et al., 2004). Our study also showed that the over-expression adiponectin stimulated the transcription of TFAM, further supports the function of adiponectin in promoting mtDNA synthesis. As oxidation may blunt lipid synthesis, we measured the expressions of oxidative respiration marker genes PGC1-α, SOD2, CAT, SLC2A4, MDH1, CPT-1 and UCP-2 in this study, and found over-expressed adiponectin up- regulated ($P < 0.01$) these genes.

The Cyt C is a carrier of electrons in mitochondrial respiratory chain along with an electron transformation from reductive state to oxidative state, occurring in the mitochondrial inner membrane (Aouadi et al., 2006). The change of Cyt C protein is exploited standing for the quantity of mitochondrial proteins. In this experiment the expression of Cyt C was found significantly elevated by the over-expression of adiponectin, which also supports the active role of adiponectin on mitochondria development in chicken adipocytes. The electron gradient across the mitochondrial inner membrane ($\Delta\Psi m$), established by electron transport chain (ETC) activity and permitted mitochondrial Ca^{2+} sequestration, is a typical indicator of mitochondrial permeability transition and mitochondrial dysfunction (Ly et al., 2003). Lower $\Delta\Psi m$ indicates an increased permeability of mitochondrial membrane, opening permeable transition hole, and impaired cell

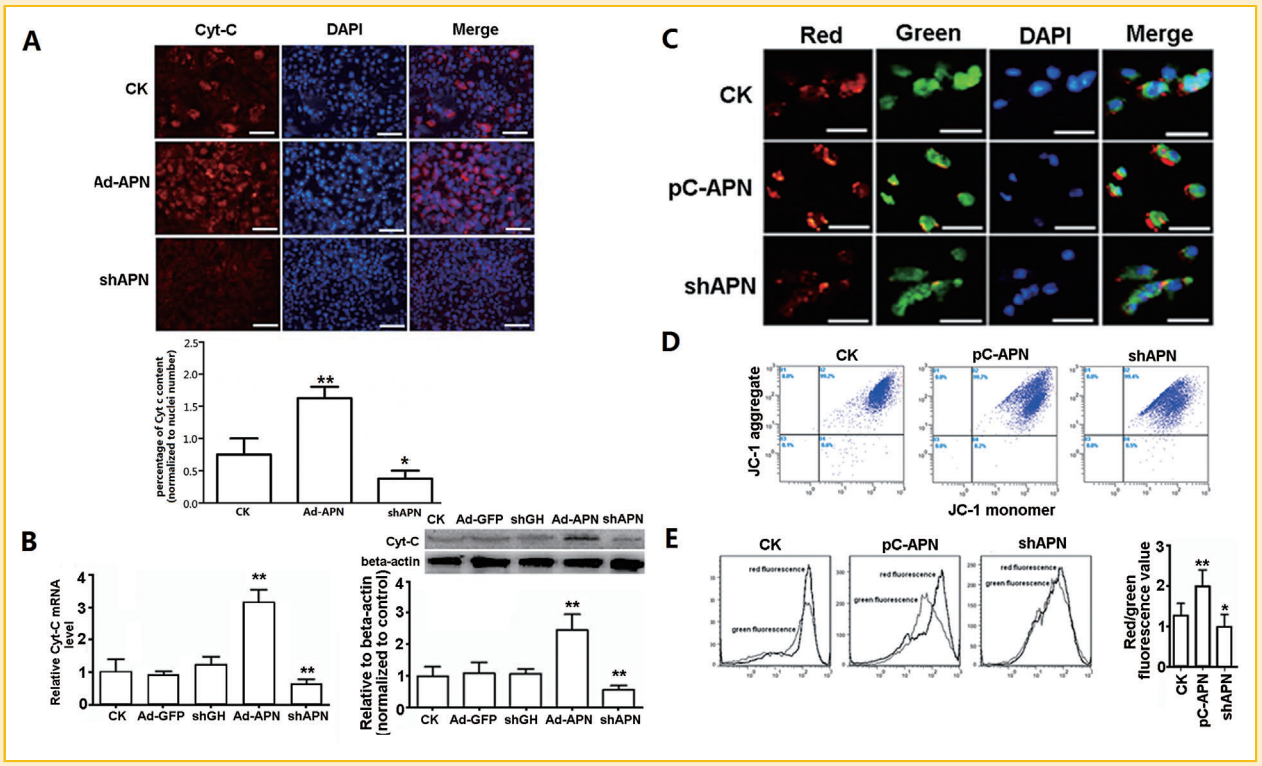

Fig. 4. Adiponectin protected mitochondria from lipid-induced reduction in mitochondrial function. A: Images of cytochrome C (Cyt C) immunofluorescent stains. Scale bar: 100 μm. The percentages of cells displaying Cyt C distribution was determined by counting fluorescent cells on 20 randomly selected fields under the microscope. 100% represents the total cells on the respective fluorescence per field (n = 3). B: mRNA and protein levels of Cyt C gene (n = 3). C: Images of mitochondrial membrane (Mb) integrity was observed under a fluorescence microscope after JC-1 dyeing (n = 3). Cells were analyzed by flow cytometry after staining with JC-1. D: Dot plots of cells in different treatment groups. E: The amounts of shift in green and red fluorescence intensities were calculated by subtracting the mean fluorescence intensity of treated cultures from the mean of the untreated control cultures. The ratio of red/green fluorescence intensity was then used to quantitate adiponectin (APN), and the level of mitochondrial membrane potential (n = 3). CK: control group; Ad-APN: recombinant adenovirus vector encoding adiponectin; Ad-GFP: empty adenovirus vector containing green fluorescent protein; shGH: pGPU6/GFP/Neo-GAPDH; shAPN: pGPU6/GFP/Neo-APN-952. Values are means ± SD. ** $P < 0.01$, * $P < 0.05$, compared with control group.

energy metabolism, uncoupling of oxidative respiration chain, disturbed calcium homeostasis and many other damages to cells. JC-1, a specific mitochondrial fluorescent dye, can be used to assess $\Delta\Psi$m, which is effective and more sensitive than the use of DiOC6 and Rho-123–1 (Reers et al., 1994; Salvioli et al., 1997). We used JC-1 to stain the membrane, followed with fluorescence microscope and flow cytometry examination to determine $\Delta\Psi$m, and our results suggested that the over-expressing adiponectin helped to maintain a higher potential of mitochondrial membrane compared with control group.

PGC1-α is a transcriptional co-activator and plays an important role in mitochondrial biogenesis (Iwabu et al., 2010; Qiao et al., 2012). Our results suggest that adiponectin enhanced mitochondrial biogenesis through the up-regulation of PGC1- α. Studies showed that adiponectin and adipoR1 may regulate the expression of PGC1-α and mitochondrial biogenesis in parallel with its activation of AMPK. Adiponectin is known to stimulate phosphorylation of acetyl coenzyme A carboxylase (ACC), fatty-acid oxidation, and glucose uptake and lactate production in myocytes (Wakil, 1989; Iwabu et al., 2010). By examining translocation of PKCθ, we found that the Akt2 activity was negatively correlated with PKCθ translocation, suggesting that it influenced the AMPK pathway. By using a phosphor-specific antibody of AMPK, we found that both the phosphorylation levels of AMPK Thr172 and PGC1- α Ser570 were markedly increased in adiponectin-treated chicken adipocytes (Fig. 5A and 5B). Our results showed acetyl-CoA carboxylase2 (ACC2) activity was responsible, in an inverting manner, to the adiponectin-increased phosphorylation of AMPK and PGC1-α activity. The ACC is important for adipogenesis and fatty acid oxidation, and AMPK phosphorylation inhibits ACC1 and ACC2 activities (Fullerton et al., 2013; Galic et al. 2013). ACC1 is located in cytoplasm of the cells, and is considered to be the primary ACC involved in de novo fatty acid synthesis. In contrast, ACC2 was reported to regulate fatty acid oxidation through malonyl-CoA mediated inhibition of CPT-1 (Castle et al. 2009). ACC2 is expressed in muscle, heart, liver, and mammary gland (Oh et al., 2005). Physiological studies have also established a link between ACC2 expression in muscle to physical exercise, corroborating a link between ACC2 and fatty acid oxidation (Phillips et al. 2010). Deletion of ACC2 gene had beneficially metabolic effects in white adipose tissue, although its expression in white adipose was low, moreover, ACC2$^{-/-}$ mice have not only increased fatty acid oxidation but also depleted body fat (Olson et al. 2010). Recent research

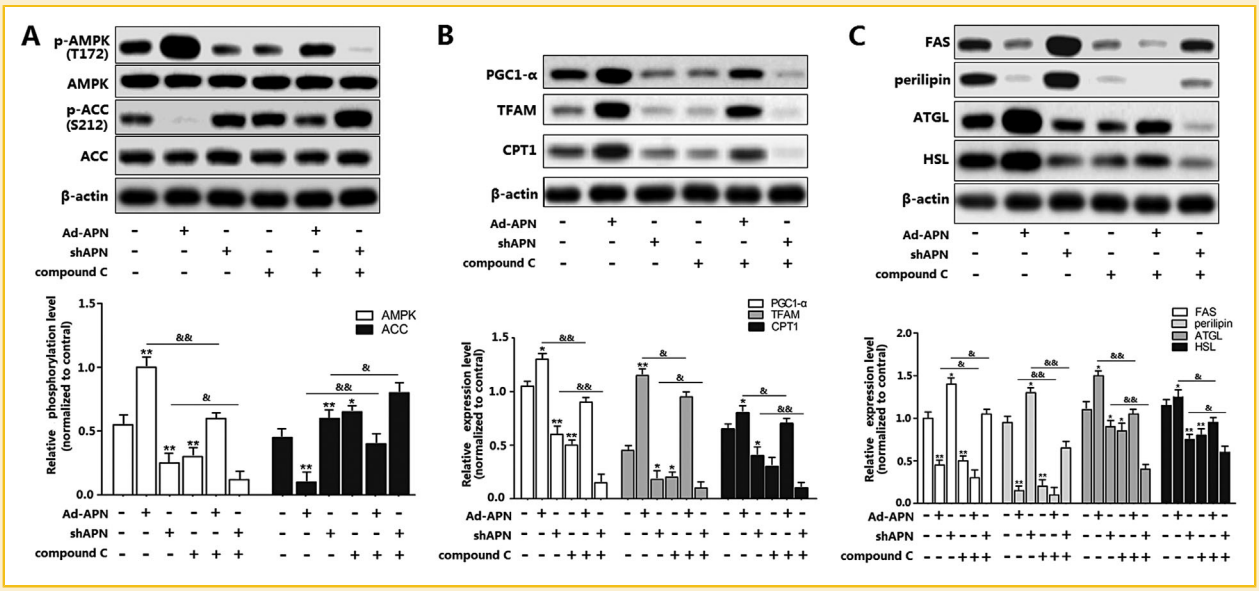

Fig. 5. AMPK/ACC2 pathway was involved in regulation of mitochondria biogenesis in chicken adipocytes by adiponectin. Chicken adipocytes were pretreated with Ad-APN or shAPN or compound. A: Representative immunoblots and densitometric quantification for p- AMPK^{T172}, p-ACC2^{S212}. B: Representative immunoblots and densitometric quantification for PGC1-α, TFAM and CPT1. C: Representative immunoblots and densitometric quantification for FAS, periliplin, ATGL and HSL. The level of total β-actin was determined as loading control. CK: control group; Ad-APN: recombinant adenovirus vector encoding adiponectin; shAPN: pGPU6/GFP/Neo-APN-952. Values are means ± SD. ** $P < 0.01$, * $P < 0.05$, $^{\&\&}$ $P < 0.01$, $^{\&}$ $P < 0.05$, compared with control group.

suggested that ACC2 is primarily localized in the mitochondrial membranes and regulated by AMP- activated protein kinase (AMPK) pathway (Cho et al., 2010). Our study showed the same kind of effects on lipid oxidation by ACC2 in chicken adipocytes. ACC2 also inhibits carnitinepalmitoyltransferase 1 (CPT1) and hence mitochondrial biogenesis. Pretreatment with Compound C, a specific inhibitor of AMPK pathway, blocked the adiponectin-induced mitochondrial biogenesis and prevented the increase of lipid oxidation in this study. Therefore, our study suggests that adiponectin regulated mitochondrial biogenesis through inhibiting lipid accumulation and activating AMPK/ACC2 signal pathway in chicken adipocytes.

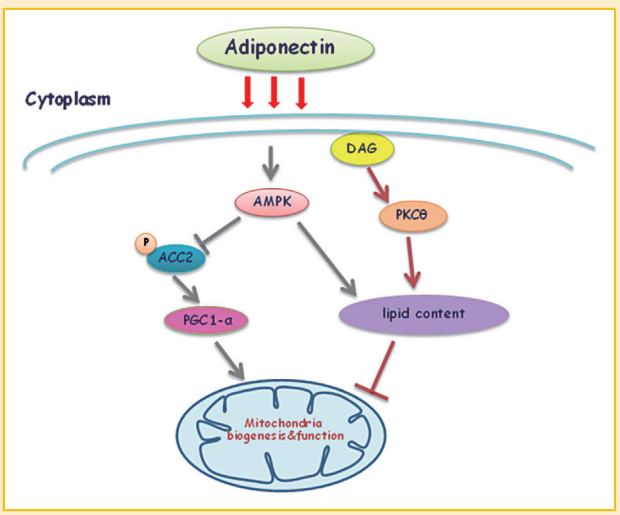

Fig. 6. A summary of the effects of adiponectin on lipid-induced reduction in mitochondrial biogenesis in chicken adipocytes through AMPK/ACC2 signal pathway. Data demonstrated that over-expression of adiponectin in chicken adipocytes prevented lipid-induced (1) reduction in mitochondrial function and mitochondrial biogenesis, (2) decreases in membrane DAG content, PKCθ activity, and increases Akt2 activity, and (3) increases in AMPK, ACC2 and PGC1-α activity.

In conclusion, our results demonstrate that adiponectin is a novel regulator that prevents lipid-induced reduction of mitochondrial biogenesis in chicken adipocyte. The regulatory mechanisms could include adiponectin (1) reduced membrane DAG content and the amount of intracellular TG, (2) enhanced the expression of PGC1-α, and (3) activated AMPK/ACC2 signaling pathway.

ACKNOWLEDGEMENTS

This work was supported by National Nature Science Foundation of China #31172185, and National Major Scientific Project #2015CB943102.

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