Original Paper

Browning of Pig White Preadipocytes by Co-Overexpressing Pig PGC-1 α and Mice UCP1

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Key Words

Ucp1 • Mitochondria • AMPK-SIRT1 pathway • PGC-1 α • Uncoupled respiration

Abstract

Background/Aims: Brown adipose tissue (BAT) is critical for mammals' survival in the cold environment. BAT-dependent non-shivering thermogenesis is attributed to uncoupling protein 1 (UCP1)'s disengagement of oxidative phosphorylation from ATP synthesis and dissipates energy as heat. Thus individuals with a substantial amount of BAT are better equipped during cold stress and less likely to become obese. Recently, our laboratory has shown pig adipocytes have no UCP1 protein. The inability of newborn piglets to generate heat contributed to its high death rate. Repairing the genetic defect of UCP1 in pig adipocytes has implications in defending against cold for piglets and developing an alternative treatment for human obesity.

Methods: Q-PCR, western blotting (WB) and oxygen consumption measurement were used to enable functional UCP1 protein in preadipocytes. Immunoprecipitation (IP), chromatin immunoprecipitation (CHIP), and dual-luciferase reporter assay system were used to clarify the thermogenesis mechanism of functional UCP1. **Results:** Only co-overexpressing mice UCP1 and pig PGC-1 α increased not only the mitochondrial number but also the uncoupled respiration rate in the transfected pig adipocytes. The functional mice UCP1 increased the pig PGC-1 α activity through the AMPK-SIRT1 pathway. The active form PGC-1 α interacted with transcription factors Lhx8, Zic1, ERR α , and PPAR α to regulate the expression of mitochondrial energy metabolism and adipocytes browning-related genes. **Conclusion:** Our data suggest a model in which pig PGC-1 α and mice UCP1 work collaboratively to restore uncoupling respiration in pig preadipocytes. These results have great implications for piglet survival and developing an alternative treatment for human obesity in the future.

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Introduction

Obesity has become a worldwide epidemic, and more research is focused on the physiology and biology of the adipose tissues. Most mammals have two different types of adipose tissues: white and brown [1]. The white adipose tissue (WAT) is mainly for energy storage while the brown adipose tissue (BAT) is for generating heat [2, 3]. In addition to the classic white and brown adipocytes, brown-like fat cells or beige adipocytes have been identified in recent years [4, 5]. The non-shivering thermogenesis in the BAT disengages oxidative phosphorylation from ATP synthesis and dissipates energy as heat [6]. Individuals with a substantial amount of BAT are better equipped during cold stress and less likely to become obese [7-10]. Recent studies show humans have BAT, raising the question of whether activation of BAT and recruitment of the beige adipocytes can be a tool to combat the current obesity pandemic [11].

The uncoupling respiration mediated by UCP1 is the source of the non-shivering thermogenesis in brown and beige adipocytes [12-15]. *UCP1* knockout mice were used to study the process of activation of brown adipocytes and recruitment of beige adipocytes [16-18]. *UCP1* null mice are cold sensitive, and *UCP1* is responsible for the brown adipose tissue non-shivering thermogenesis function [19]. However, over one-half of adult humans have no functional UCP [1, 20]; therefore, there is still much work to do to establish the molecular mechanism of UCP1 thermogenesis.

Our laboratory has shown the *UCP1* gene is naturally truncated and is not translated in the pig adipose tissues [21]. Our findings indicate pigs have no brown adipose tissue, which explains the weak thermogenic ability of the piglets [21]. A recent study has shown UCP1 transgenic pigs could improve the piglet's weak thermogenic ability [20]. However, their *in vitro* study did not detect the uncoupling respiration in the adipocytes isolated from the transgenic pigs [20]. Additional research will be needed to establish the mechanisms associated with the browning of the pig adipocytes. Since pigs have no brown adipose tissue and lots of white adipose tissue, it is an excellent model for studying the browning of white adipose tissue.

The peroxisome proliferator-activated receptor coactivator-1 α (*PGC-1 α*) is a transcriptional co-activator that interacts with many transcription factors (NRFs, ERRs, and PPARs) to regulate mitochondrial biogenesis and adaptive thermogenesis [22-25]. Overexpressing *PGC-1 α* in human white adipocytes has been shown to induce human white adipocytes to acquire the brown adipocyte features [26]. However, there is no report on the roles of *PGC-1 α* in pig mitochondrial biogenesis and adaptive thermogenesis.

The objectives of this study are to repair the *UCP1* gene defect in pig adipocytes enabling the adipocytes to obtain the uncoupling respiration *in vitro* and to explore the underlying mechanisms involved. Our results demonstrated co-overexpressing mice *UCP1* and pig *PGC-1 α* enabled pig preadipocytes to acquire uncoupling respiration ability. We also found the functional mice UCP1 increased pig PGC-1 α activity in pig preadipocytes. In turn, PGC-1 α interacts with transcription factors Lhx8, Zic1, ERR α , and PPAR α to regulate mitochondrial biogenesis and adaptive thermogenesis in pig preadipocytes. These results have implications for neonatal pigs to defend themselves against the cold and for developing an alternative treatment for human obesity.

Materials and Methods

All animals used in this study were reared and euthanized with the approval of the College of Animal Science, South China Agricultural University. All experiments were performed following the guidelines and regulations of 'the instructive notions with respect to caring for laboratory animals' issued by the Ministry of Science and Technology of the People's Republic of China.

RNA extraction and PCR analysis

Methods used for the RNA extraction and PCR analysis have been described previously [21]. The relative expression of mRNAs and microRNA were normalized with β -actin levels using the $\Delta\Delta$ Ct method

[27]. $\Delta\Delta C_t$ is defined as the ratio of the relative mRNA level of the target gene between the experimental group and the control group. Primers were designed using Primer Premier 5 according to the pig genes sequence obtained from NCBI. The sequences of the primers used in this study are listed in Table 1.

Cell experiment

The pig preadipocytes in primary culture were isolated from the subcutaneous back fat pad of 1-day old male Landrace pigs according to previously published protocols [21] with following modifications. Briefly, subcutaneous adipose tissue was dissected and finely minced after removing all visible connective tissues. Then the minced tissue was digested for 40 min at 37°C in isolation buffer (125 mM NaCl, 5 mM KCl, 1.3 mM CaCl₂, 5 mM glucose, 100 mM HEPES, 4% BSA, 1.5 mg/mL Collagenase B). Digested tissue was filtered through a 70 μ m cell strainer to remove large pieces, and the flow-through was centrifuged for 10 min at 500 $\times$ g to collect stromal-vascular fraction (SVF) cells in the pellet. The SVF cells were suspended in the growth medium, which is DMEM/F12 (GIBCO, Grand Island, NY, USA) containing 15% fetal bovine serum (FBS, GIBCO), 100, 000 units/L of penicillin sodium, and 100 mg/L of streptomycin sulfate (GIBCO). The SVF cells were then plated in 75 cm² cell culture flasks. For plasmid transfection, primary adipocytes with 60% confluency were incubated with plasmids for six hours in the growth medium. Then the medium was replaced with new growth medium and cells were maintained in growth medium for an additional 24 h. All other chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

Mitochondrial DNA content quantification by q-PCR

Genomic DNA was isolated from different cells. The cells were digested with Proteinase K overnight in a lysing buffer for DNA extraction by the conventional phenol-chloroform method. The results were calculated from the difference in the threshold cycle values for mitochondrial DNA and nuclear-specific

Table 1. PCR primers used in this study

Gene name	Forward primer sequence (5'-3')	Reversed primer sequence (5'-3')
β -actin	GCGGGACATCAAGGAGAAGC	TGTCGGCGATGCCTGGGTA
Pig UCP1	GGTCACCGCCAAAGTCCG	CAGCCCTCTGTAGTGCTTCATT
Mice UCP1	AGAGGTCGTGAAGGTCAGAATG	GGCTTGGTACGCTTGGGT
PGC-1 α	TGACAGCGAAGATGAAAGTGA	GATTTGGGTGGTGATACGG
AP2	AAAACCTACGGAGATTGCCTTCA	GCTACATTCCACCACCAACTTAT
Leptin	CAAGCAGTGCCTATCCAGAAAG	GGTGAAGCCCAGGAATGAAGT
AdipoQ	ACCTTTGTGGGAACCTGGAA	CCTGTCTGTGCGGTGATT
CD24	GGTAGACCACTCGCATTCCTTT	CCCACAGACTCGGCACTCA
HMGA2	CGCACACCGCCAAAGTC	ATGCCAGTCACCAGAAGGAA
Lhx8	GAGGCACATCCATTCTACCG	AGGAGGGCACCTTCAACAC
Zic1	TCTACCTTCCTTCTGGTGACTG	TGCCGTGACTCCATCAACTCT
Cidea	ATGACAGTTCTCTGAAGTGCCC	GCGGTGTCCAAAGTGCAA
Hspb7	GGGCTGGCAACATCAA	CTTCTCAGCTCGCACCTCAAT
Dio2	CGGGACATCAAGGAGAAGC	GCACTGTGTTGGCGTAGAG
PDK4	AGATTTCAGACAGAGGAGGTGGT	TATGCTGGCAAAGAGTAGAGGTT
CD40	CCTGCCCAGTTGGCTTCT	CTGCTCCTTGGTCACCTTTCT
CD137	CCAGGACTGTATGTGGAGCG	AGCCAAGGGTTCGGGACT
TBX1	ATCTTCTCATTGTTATTGGGTCT	TTGTGTTCTGTGGAGGCTTTT
Tmem26	TGGTCCCTTCTCTGGTTGTG	GCCTGCTGGCTTACTTCTCA
SLC27A1	GGATGGGAAGGTTGGTGC	AGAGGGTCTGCTGGTTGAT
TFAM	GACCTCTGTGCGGTTTGTG	ACCTGCCAGTCTGCCCTAT
FABP1	AAGGGGAAGGACATCAAGG	CAGTCAGGGTCTCCATCTCAC
MCAD	GTGCCCCCTTATTATTGGTGG	TTCTGCTTTGGTCTTTATCCC
CPT-1 β	GCACGGCAACTGCTACAACA	CAGGGCGATCTGCACGAA
ACOX I	GCAACCGAGGAGTTCATTCT	CTTCACCTGGGCATACTTCAT
ATP synthase	CTCTAATAACCGCACATCTCG	TGTAATGTTGGCTGTCTAGTCGT
Cyt C	CGACTCGTCCAAACCTCCA	CTTCCCTTTCTCCCTTCTTCTTA
Lhx8	ATGGCCTCGGGCTCCGTCTGC	TTAGGTATGACTTATTGGCAGT
Zic1	ATGCTCCTGGACGCCGCC	TTAAACGTACCATTCTGTTAAA
AMPK	GTATGCTGGTCCAGAGG	AAAGGCTAATCACAGAAGG
SIRT1	AGTACAGACTTCTAGGAGCAT	TACCATCAAGCCGTTTA
COX III	ATGGCATCTGGAGGTGGTG	GCTGGGTCTCGCCTTTGT

gene by q-PCR. The data are expressed as mitochondrial DNA-specific 16S ribosomal RNA normalized to nuclear-specific gene hexokinase, which was described by Lagouge et al [28, 29].

Western blot analysis

The method used for the Western blot analysis has been described previously [21]. The sources and names of antibodies used in this study are listed in Table 2. The protein quantification was performed using Image J (Bethesda, USA).

Determination of Cellular Respiration

We used Oxygen Consumption/Mito Membrane Potential Dual Assay Kit (Cayman, Michigan, USA) to measure the oxygen consumption rate of the pig adipocytes. This kit utilizes the phosphorescent oxygen probe MitoXpress-Xtra to measure oxygen consumption rate in living cells. The MitoXpress-Xtra probe lifetime signal has a negative correlation with oxygen concentration. In short, pig preadipocytes transfected with different recombinant vectors were collected 24 h later by 0.05% trypsin digestion. Cells (30, 000 cells per well) were seeded in 96-well cell culture plate, and 120 μ L culture medium, 10 μ L MitoXpress-Xtra Solution and 10 μ L different treatments (oligomycin, FCCP or Antimycin A) were added to each well. Then, 100 μ L HS Mineral Oil was gently added to each well. Each plate was read once every five min for 90 min. Uncoupled and maximal oxygen consumption rates were determined using oligomycin (14 mM) and FCCP (10 mM). Antimycin A (4 mM) was used to inhibit Complex III-dependent respiration.

The ATP concentration of the pig adipocytes was determined using an ATP Assay Kit according to the manufacturer's instructions (Beyotime, Shanghai, China).

Transmission Electron Microscopy

The mitochondrial number in pig adipocytes was examined by transmission electron microscopy (TEM). Pig adipocytes were collected 36 h after transfecting with different vectors using 0.05% trypsin. Cells were fixed in 2.5% (w/v) glutaraldehyde in PBS (pH 7.4) at 4 °C for 24 h and then fixed in 2% (w/v) osmium tetroxide at 4 °C for 1 h. Dehydration was done in an ascending concentration series of ethanol at 25°C. Samples were embedded in Quetol 812 and sectioned to 90 nm by Leica EM UC6 Ultramicrotome (Leica Company). Then the section was viewed with a Tecnai 12 transmission electron microscope (FEI Company).

Statistical analysis

All data are expressed as the mean $\pm$ standard error of the mean (S.E.M.), and at least three independent individuals or replicates were used per group. The assumptions of normality of data and homogeneity of variances between the groups were analyzed by SPSS. Significant differences between treatment groups were determined by one-way ANOVA (SPSS 18.0, Chicago, IL, USA). Significance was achieved when $p < 0.05$. * is $p < 0.05$ and ** is $p < 0.01$.

Results

Overexpression of truncated pig UCP1 protein failed to induce uncoupled respiration in pig preadipocytes

We have shown pig has truncated UCP1 gene with only three exons, and two of them are transcribed, but not translated in the adipose tissues. We wanted to know what happens if we overexpress the cloned truncated pig UCP1 gene (two exons) in pig preadipocytes. So we expressed the truncated pig UCP1 protein in pig preadipocytes using the pcDNA3.1 (+)-pig UCP1 recombinant vector. Through q-PCR (Fig. 1A) and WB (Fig. 1B), we found the

Table 2. The details of antibodies used in this study

Primary antibody	Clone	Company	Catalog No.	Dilution
PGC-1 α	Monoclonal	Abcam	ab54481	1:2000
TBX1	Polyclonal	Abcam	ab109313	1:2000
Zic1	Monoclonal	Abcam	ab134951	1:2000
Lhx8	Monoclonal	Abcam	ab137036	1:2000
UCP1	Polyclonal	Bioss	bs-1925R	1:700
FABP1	Polyclonal	Bioss	bs-0824R	1:500
MCAD	Polyclonal	Bioss	bs-4047R	1:500
ATP synthase	Polyclonal	Bioss	bs-8600R	1:500
β -Actin	Monoclonal	Bioss	bsm-33036M	1:1000
Secondary antibody	Conjugate Used	Company	Catalog No.	Dilution
Goat Anti-rabbit IgG	HRP	Bioss	bs-0295G	1:3000

recombinant vector expressed pig *UCP1* mRNA and pig *UCP1* protein highly in the pig preadipocytes. Then we examined whether the truncated pig *UCP1* protein has the thermogenic ability by measuring the uncoupled respiration rate in the preadipocytes. The truncated pig *UCP1* did not increase pig preadipocytes oxygen consumption in the presence of oligomycin (an ATP synthase inhibitor) (Fig. 1C). These results indicate the truncated pig *UCP1* protein is not functional.

Overexpression of mice *UCP1* protein failed to induce uncoupled respiration in pig preadipocytes

Since pig *UCP1* is a truncated and non-functional gene, we want to know whether by introducing the mice *UCP1* gene we can restore the uncoupled respiration in pig preadipocytes. We transfected pig preadipocytes with pcDNA3.1 (+) - mice *UCP1* recombinant vector or pcDNA3.1 (+). Using q-PCR (Fig. 2A) and WB (Fig. 2B), we found the recombinant vector expressed highly the mice *UCP1* mRNA and mice *UCP1* protein in pig preadipocytes. However, mice *UCP1* did not increase pig preadipocytes oxygen consumption in the presence of oligomycin (Fig. 2C) when we measured the uncoupled respiration rate in these preadipocytes. These results indicate overexpression of mice *UCP1* protein does not affect uncoupled respiration of pig preadipocytes.

Overexpression of pig *PGC-1 α* and mice *UCP1* enabled pig preadipocytes to acquire uncoupled respiration

PGC-1 α is recognized as a master regulator of mitochondrial biogenesis and has a close positive relationship with adaptive thermogenesis. Since overexpression of mice *UCP1* alone did not restore the uncoupled respiration in the pig preadipocytes, we explored whether co-overexpressing *PGC-1 α* and *UCP1* allows pigs to acquire uncoupled respiration. Results from the q-PCR and WB indicate the construction of overexpression models were successful (Fig. 3A, 3B). Moreover, the Western blot (WB) result shows co-overexpressing pig *PGC-1 α*

Fig. 1. Overexpression of truncated pig *UCP1* protein failed to induce uncoupled respiration in pig preadipocytes. (A) *UCP1* mRNA level was increased in pig preadipocytes after transfected with pcDNA3.1 (+)-pig *UCP1* recombinant vector. (B) *UCP1* protein was expressed in pig preadipocytes after transfected with pcDNA3.1 (+)-pig *UCP1* recombinant vector. (C) Overexpression of the truncated pig *UCP1* gene did not affect uncoupled respiration of pig preadipocytes. We used Oxygen Consumption/Mito Membrane Potential Dual Assay Kit to measure continuous oxygen consumption in transfected pig preadipocytes. The MitoXpress-Xtra probe phosphorescent lifetime signal increased as the oxygen concentration decrease. The results are presented as Mean $\pm$ S.E.M. of three replicates for each group. *** P<0.001.

Fig. 2. Overexpression of mice *UCP1* protein failed to induce uncoupled respiration in pig preadipocytes. (A) Mice *UCP1* mRNA level was increased in pig preadipocytes after transfected with pcDNA3.1 (+)-mice *UCP1* recombinant vector. (B) Mice *UCP1* protein was expressed in pig preadipocytes after transfected with pcDNA3.1 (+)-mice *UCP1* recombinant vector. (C) Overexpressing the mice *UCP1* gene did not affect uncoupled respiration of pig preadipocytes. We used Oxygen Consumption/Mito Membrane Potential Dual Assay Kit to measure continuous oxygen consumption in transfected pig preadipocytes. The results are presented as Mean $\pm$ S.E.M. of three replicates for each group. *** P<0.001.

and mice *UCP1* increased mice *UCP1* protein compared with overexpressing mice *UCP1* alone. However, co-overexpressing *PGC-1 α* and pig *UCP1* did not increase pig *UCP1* protein compared with overexpressing pig *UCP1* alone. By measuring the uncoupled respiration rate in preadipocytes, we found only co-overexpressing pig *PGC-1 α* and mice *UCP1* in pig preadipocytes increased oxygen consumption in the presence of oligomycin (Fig. 3C). These results indicate co-overexpressing pig *PGC-1 α* and mice *UCP1* induced pig preadipocytes to acquire the ability for uncoupled respiration.

Overexpression of PGC-1 α increased the mitochondrial biogenesis in pig preadipocytes

Since *PGC-1 α* is a master regulator of mitochondrial biogenesis, we examined the number and morphology of mitochondria in these pig preadipocytes by transmission electron microscopy. We found there are more mitochondria (indicated by the red arrowheads) in *PGC-1 α* overexpression groups (Fig. 4A, 4B). Consistent with the TEM observation results, we found *PGC-1 α* overexpression increased pig preadipocytes mitochondrial DNA copy number (Fig. 4C).

Co-overexpression of pig PGC-1 α and mice UCP1 increased the expression of genes associated with mitochondrial energy metabolism and oxygen consumption in pig preadipocytes

Since mitochondrial H⁺ is the substrate for UCP1 to exert its uncoupling respiration function, we measured the expressions of mitochondrial oxidative phosphorylation-related genes and fatty acid oxidation-related genes, and the maximal oxygen consumption of the preadipocytes. Fig. 5A shows overexpressing pig *PGC-1 α* increased ($P < 0.05$) mRNA levels of the mitochondrial oxidative phosphorylation-related genes such as ATP synthase, Cytochrome C (*Cyt C*), and Cytochrome C oxidase subunit III (*COX3*); and mRNA levels of the fatty acid oxidation-related genes, such as fatty acid binding protein 1 (*FABP1*), carnitine palmitoyltransferase 1B (*CPT-1 β*), acyl-CoA oxidase 1 (*ACOX1*), and medium chain acyl-Coenzyme A dehydrogenase (*MCAD*) in pig preadipocytes. Fig. 5B shows overexpressing pig *PGC-1 α* increased the protein levels of the ATP synthase, COX3, FABP1, and MCAD in pig preadipocytes. However, compared with overexpressing *PGC-1 α* , co-overexpressing pig *PGC-1 α* and mice *UCP1* increased *CPT-1 β* and ATP synthase mRNA levels in pig preadipocytes (Fig. 5A). As a robust uncoupled agent, FCCP transports H⁺ through the mitochondrial inner membrane to provide energy for the oxidative phosphorylation. In the presence of FCCP, cells showed the maximal oxygen consumption. This oxygen consumption reflects mitochondrial H⁺ production rate. As shown in Fig. 5C, after co-overexpressing pig *PGC-1 α* and mice *UCP1*, pig preadipocytes acquired the highest oxygen consumption among all the groups in the presence of FCCP. These results indicate pig preadipocytes co-transfected with pig *PGC-1 α* and mice *UCP1* have elevated mitochondrial energy metabolism. Most likely, increased

Fig. 3. Overexpression of pig *PGC-1 α* and mice *UCP1* enabled pig preadipocytes to acquire uncoupled respiration. (A) *PGC-1 α* and *UCP1* mRNA levels were increased in pig preadipocytes after transfected with both recombinant vectors. (B) Overexpressing *PGC-1 α* increased mice *UCP1* protein level in pig preadipocytes after transfected with both recombinant vectors. (C) Co-overexpressing pig *PGC-1 α* and mice *UCP1* gene induced uncoupled respiration in pig preadipocytes. We used Oxygen Consumption/Mito Membrane Potential Dual Assay Kit to measure continuous oxygen consumption in transfected pig preadipocytes. The results are presented as Mean $\pm$ S.E.M. of three replicates for each group. ** $P < 0.001$.

Fig. 4. Overexpression of PGC-1 α increased the mitochondrial biogenesis in pig preadipocytes. (A) The morphology and number of mitochondria were observed by transmission electron microscopy. The arrows show some representative mitochondria. Fig. a-f represent pig preadipocytes transfected with pcDNA3.1 (+), pcDNA3.1 (+)-pig UCP1, pcDNA3.1 (+)-mice UCP1, pcDNA3.1 (+)-PGC-1 α , pcDNA3.1 (+)-PGC-1 α + pig UCP1, pcDNA3.1 (+)-PGC-1 α + mice UCP1, respectively. (B) The quantitative data of mitochondrial number in Fig. A. (C) Mitochondrial DNA copy number was increased in pig preadipocytes after overexpressing PGC-1 α . The results are presented as Mean $\pm$ S.E.M. of three replicates for each group. ** $P < 0.001$.

Fig. 5. Co-overexpression of mice UCP1 and pig PGC-1 α increased the expression of genes associated with mitochondrial energy metabolism and oxygen consumption in the pig preadipocytes. (A) Co-overexpressing mice UCP1 and pig PGC-1 α elevated mRNA levels of the mitochondrial oxidative phosphorylation-related and fatty acid oxidation-related genes in pig preadipocytes. (B) Co-overexpressing mice UCP1 and pig PGC-1 α elevated protein levels of mitochondrial biogenesis, fatty acid oxidation, and oxidative phosphorylation-related genes in pig preadipocytes. (C) Co-overexpressing mice UCP1 and pig PGC-1 α increased mitochondrial oxygen consumption in the pig preadipocytes. We used Oxygen Consumption/Mito Membrane Potential Dual Assay Kit to measure continuous oxygen consumption in transfected pig preadipocytes. The results are presented as Mean $\pm$ S.E.M. of three replicates for each group. * indicates a difference between treatments (transfected with different recombinant vectors) and control (pcDNA3.1), and # indicates a difference between pcDNA3.1-PGC-1 α transfection group and PGC-1 α + Mice UCP1 co-transfection group. N = 3, **, $P < 0.01$; #, $P < 0.05$; ##, $P < 0.01$.

mitochondrial H $^{+}$ production provided sufficient substrate allowing the UCP1 to exert its uncoupling respiration.

Co-overexpression of pig PGC-1 α and mice UCP1 increased the expression of brown/beige adipocyte marker genes in pig preadipocytes

Since co-overexpressing pig PGC-1 α and mice UCP1 induced pig preadipocytes to acquire the ability for uncoupled respiration, we also measured the expression of brown and beige adipocyte marker genes in preadipocytes transfected with pig PGC-1 α and mice UCP1. Compared with control, co-overexpressing pig PGC-1 α and mice UCP1 in pig preadipocytes increased mRNA levels of brown and beige adipose tissue markers *Lhx8*, *Zic1*, *Cidea*, *PDK4*, *CD137*, and *SLC27a1* (Fig. 6). However, compared with overexpressing pig PGC-1 α alone, co-overexpressing pig PGC-1 α and mice UCP1 in preadipocytes decreased ($P < 0.05$) *Lhx8* mRNA level; and increased ($P < 0.05$) mRNA levels of *Cidea*, *CD137*, and *SLC27a1* (Fig. 6). These results indicate co-overexpressing pig PGC-1 α and mice UCP1 induced browning of pig preadipocytes.

Functional UCP1 increased PGC-1 α activity through activating the AMPK-SIRT1 signal pathway

Since the function of UCP1 is to disperse the energy used to generate ATP in the form of heat, we measured the ATP level in the pig preadipocytes transfected with pig PGC-1 α and mice UCP1. As shown in Fig. 7A, compared with overexpressing pig PGC-1 α alone, co-overexpressing pig PGC-1 α and mice UCP1 decreased the cellular ATP level. The reduction of cellular ATP led to elevated mRNA levels of AMPK and SIRT1 (Fig. 7B). Reduction of cellular ATP level also increased the protein levels of pAMPK, the active form of AMPK, and SIRT1. The active form of PGC-1 α is either de-acetylated or phosphorylated, and SIRT1 activation reduced PGC-1 α acetylation (Fig. 7C). To further investigate the phosphorylation of PGC-1 in this process, we mutated the acetylation site of the PGC-1 gene (PGC- α -M) and transfected it into pig preadipocytes. By measuring the uncoupled respiration rate in these transfected pig preadipocytes, we found there was still uncoupled respiration in the pig preadipocytes after transfected with pig PGC- α -M and mice UCP1. These results indicate functional UCP1 increased PGC-1 α activity through activating the AMPK-SIRT1 signal pathway.

PGC-1 α regulated the expression of mitochondrial energy metabolism and adipocytes browning-related genes

To further explore the mechanism of co-overexpressing pig PGC-1 α and mice UCP1 induced preadipocytes browning mediated by the active PGC-1 α , we investigated the

Fig. 6. Co-overexpression of pig PGC-1 α and mice UCP1 increased the expression of brown/beige adipocyte marker genes in pig preadipocytes. The expressions of brown and beige adipocyte marker genes were measured in preadipocytes transfected with pig PGC-1 α and mice UCP1. The results are presented as Mean $\pm$ S.E.M. of three replicates for each group. * indicates a difference between treatments (transfected with different recombinant vectors) and control (pcDNA3.1), and # indicates differences between PGC-1 α transfection group and PGC-1 α + Mice UCP1 co-transfection group. N = 3, *, $P < 0.05$, **, $P < 0.01$; #, $P < 0.05$; ##, $P < 0.01$.

interactions of browning-related transcription factors with PGC-1 α through IP. As shown in Fig. 8A, after pig preadipocytes transfected with pig *PGC-1 α* and mice *UCP1* interacted with transcription factors Lhx8, Zic1, ERR α , and PPAR α . Through CHIP, we found a binding site in the promoter region of *CPT-1 β* for Lhx8, a binding site in the promoter region of *TFAM* for Zic1, a binding site in the promoter region of *PDK4* for ERR α , and a binding site in the promoter region of *SLC27a1* for PPAR α (Fig. 8B). Finally, we confirmed the regulatory relationship between the genes and transcription factors through the dual luciferase reporter system. As shown in Fig. 8C, after the recombinant vector transfected in HEK-293 cell, Lhx8 decreased the relative luciferase activity of pGL3-basic-CPT-1 β promoter recombinant vector, and even more pronounced reduction in the presence of PGC-1 α (Fig. 8C). Zic1 increased the relative luciferase activity of pGL3-basic-TFAM promoter recombinant vector, and the trend was further increased in the presence of PGC-1 α (Fig. 8C). Similar results were also observed for the ERR α with PDK5 and PPAR α with SLC27a1 (Fig. 8C). These results indicate PGC-1 α , as a transcriptional co-activator, regulated mitochondrial energy metabolism and the expression of adipocytes browning-related genes. Thus, our data suggest a model in which pig PGC-1 α and mice UCP1 work collaboratively to restore uncoupling

Fig. 7. Functional UCP1 increased PGC-1 α activity through activating the AMPK-SIRT1 signal pathway. (A) Functional UCP1 decreased the ATP level in preadipocytes. (B) Functional UCP1 increased AMPK and SIRT1 mRNA levels in pig preadipocytes. (C) Functional UCP1 increased pAMPK and SIRT1 protein levels and decreased the acetylation level of PGC-1 α in pig preadipocytes. (D) The gray-scale scan of the WB results. (E) Pig preadipocytes acquired uncoupling respiration after co-transfected with pig PGC-1 α -M and mice UCP1. The results are presented as Mean $\pm$ S.E.M. of three replicates for each group. * $P < 0.05$, ** $P < 0.01$.

Fig. 8. PGC-1 α regulated the expression of mitochondrial energy metabolism and adipocytes browning related genes. (A) IP assays showed transcriptional co-activator PGC-1 α interacted with transcription factors Lhx8, Zic1, ERR α , and PPAR α . (B) ChIP assays showed Lhx8 binds directly to the CPT-1 β promoter region, Zic1 binds directly to the TFAM promoter region, ERR α binds directly to the PDK4 promoter region, and PPAR α binds directly to the SLC27a1 promoter region. (C) Dual-Luciferase Report Gene System results confirmed the regulatory relationship between the transcription factors and the genes. The results are presented as Mean $\pm$ S.E.M. of three replicates for each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

respiration in pig preadipocytes (Fig. 9).

Discussion

BAT is essential for most mammals to maintain body temperature homeostasis in cold environment. BAT-dependent non-shivering thermogenesis requires the UCP1 [30]. Increasing non-shivering thermogenesis through activating BAT or obtaining BAT-like characteristics in WAT might be an alternative strategy for piglets surviving the cold environment or treating obesity in humans [31, 32]. Since the UCP1 defect leads to poor thermogenic ability in piglets, we attempted to restore the ability of uncoupling respiration in the pig preadipocytes and analyze the mechanism of the pig *PGC-1 α* and mice *UCP1* work collaboratively to restore uncoupling respiration in pig preadipocytes.

To perform the uncoupling respiration function, UCP1 needs to be transported and assembled to the mitochondrial inner membrane, and the H⁺ must be available as the substrate [33]. Consistent with this, brown or beige adipocytes contain a large number of mitochondria and UCP1 that correspond to the high level of PGC-1 α , whereas PGC-1 α expression is much lower in white adipocytes [34]. PGC-1 α has been shown to have a close relationship with adaptive thermogenesis and is a master regulator of mitochondrial biogenesis [35]. These mechanisms may be the causes for overexpressing either pig or mice UCP1 alone, failed to induce uncoupled respiration of pig preadipocytes. Therefore, we introduced pig *PGC-1 α* and *UCP1* to repair the *UCP1* defect. Our results demonstrate co-overexpressing pig *PGC-1 α* and mice *UCP1* enabled pig preadipocytes to acquire the ability for uncoupled respiration. These results indicate overexpressing pig *PGC-1 α* promoted mitochondrial biogenesis in pig preadipocytes, and assembled mice UCP1 into the mitochondrial inner membrane to exert its function. In addition, our result shows the PGC-1 α was barely detectable in pig adipose tissue (Fig. 3B). The lower expressed PGC-1 α level may explain why UCP1 knock-in pigs did not obtain the uncoupling respiration ability in the adipose tissue [20]. We did not observe any noticeable color change in pig preadipocytes transfected with pig *PGC-1 α* and mice *UCP1*. The single-layer cells grown in the cell culture plates may have contributed to this lack of color change. However, we did observe increased mitochondria number.

TBX1 is a specific beige adipocyte marker for mice, but not for humans because there is no difference in *TBX1* expression between human brown and beige adipocytes [36]. While *Eva1* is thought to be a specific marker for mice brown adipocytes, but not for human infants, because *Eva1* is highly expressed in the perirenal adipose tissue (a WAT site), not the interscapular adipose tissue (a BAT site) [20]. These studies indicate that the brown and beige adipocyte markers are only species-specific. At the same time, we found that

Fig. 9. Model of how pig PGC-1 α and mice UCP1 interact at the genetic and functional levels to restore uncoupling respiration in pig preadipocytes. Overexpression of pig PGC-1 α promotes mitochondrial biogenesis in pig preadipocytes. In this process, mice UCP1 assembled into the mitochondrial inner membrane to exert its function. The uncoupled respiration of functional UCP1 increased pAMPK and SIRT1 protein levels. The de-acetylation of SIRT1 and the phosphorylation of pAMPK increased the PGC-1 α activity. The active transcriptional co-activator PGC-1 α interacted with transcription factors Lhx8, Zic1, ERR α , and PPAR α regulated mitochondrial energy metabolism and the expression of preadipocytes browning-related genes. Thus, co-overexpressing mice UCP1 and pig PGC-1 α restored uncoupling respiration in pig preadipocytes.

most brown and beige adipocyte markers are related to the cellular energy metabolism. Since the objective of this study was to establish whether we could restore the uncoupled respiration in the pig preadipocytes, we do not distinguish the markers between brown or beige adipocytes.

In our study, compared with overexpressing pig *PGC-1 α* alone, co-overexpressing pig *PGC-1 α* and mice *UCP1* in pig preadipocytes elevated the mRNA levels of *CPT-1 β* , *ATP synthase*, *Cidea*, *CD137*, and *SLC27a1*; and lowered the *Lhx8* mRNA level. These changes may result from the functional UCP1 increased the activity of the PGC-1 α . Since UCP1 disengages ATP synthesis from the oxidative phosphorylation and dissipates energy as heat, this process leads to faster substrate oxidation and a lower rate of ATP production. AMPK is the cellular energy sensor; low ATP level activates AMPK [37]. Then the AMPK activates PGC-1 α via phosphorylation and de-acetylation through the AMPK-SIRT1 pathway. In our study, we confirmed the functional mice UCP1 increased pig PGC-1 α activity through activating the AMPK-SIRT1 signal pathway in the pig preadipocytes.

PGC-1 α as a transcriptional co-activator interacts with other transcription factors, which regulate the expressions of fatty acid oxidation, oxidative phosphorylation, and mitochondrial biogenesis related genes [38-40]. Through the use of IP assay, we found ERR α , PPAR α , Lhx8, and Zic1 interact with PGC-1 α in pig preadipocytes transfected with pig *PGC-1 α* and mice *UCP1*. Of all these four transcription factors, only ERR and PPAR have been reported to exist in the mice and human brown adipocytes [41]. Lhx8 and Zic1 are used only as the brown/beige adipocyte marker genes in the previous studies [42, 43]. We are not aware of any report on the function of Lhx8 and Zic1 in adipocytes. The function of Lhx8 is mostly related to the WNT signaling pathway and regulation of Maxillary Arch Cells proliferation [44]. Most studies about Zic1 are focused on the differentiation of neurons [45, 46]. However, Zic1 is known to regulate the expression of BMPs for neural fate determination [47]. Meanwhile, BMP4 increases the browning of white adipose tissue [48], and BMP8b increases the non-shivering thermogenesis in the brown adipose tissue [49]. Future studies should include whether Zic1 regulates brown adipose tissue non-shivering thermogenesis through BMPs.

In conclusion, our results show co-overexpressing pig *PGC-1 α* and mice *UCP1* induced pig preadipocytes to acquire the ability for uncoupled respiration. The functional UCP1 increased the PGC-1 α activity through the AMPK-SIRT1 pathway. Finally, the active transcription co-activator PGC-1 α interacted with transcription factors Lhx8, Zic1, ERR α , and PPAR α to regulate mitochondrial energy metabolism and the expression of preadipocytes browning-related genes. Additional work *in vivo* is needed to ascertain whether the same can be achieved *in vivo*. These results have implications for newborn piglet survival and developing an alternative treatment for human obesity.

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Disclosure Statement

The author(s) declare no competing financial interests.

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