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Pig has no uncoupling protein 1

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Brown adipose tissue (BAT) is critical for mammal's survival in the cold environment. Uncoupling protein 1 (UCP1) is responsible for the non-shivering thermogenesis in the BAT. Pig is important economically as a meat-producing livestock. However, whether BAT or more precisely UCP1 protein exists in pig remains a controversy. The objective of this study was to ascertain whether pig has UCP1 protein. In this study, we used rapid amplification of cDNA ends (RACE) technique to obtain the *UCP1* mRNA 3′ end sequence, confirmed only exons 1 and 2 of the *UCP1* gene are transcribed in the pig. Then we cloned the pig *UCP1* gene exons 1 and 2, and expressed the UCP1 protein from the truncated pig gene using *E. coli* BL21. We used the expressed pig UCP1 protein as antigen for antibody production in a rabbit. We could not detect any UCP1 protein expression in different pig adipose tissues by the specific pig UCP1 antibody, while our antibody can detect the cloned pig UCP1 as well as the mice adipose UCP1 protein. This result shows although exons 1 and 2 of the pig *UCP1* gene were transcribed but not translated in the pig adipose tissue. Furthermore, we detected no uncoupled respiration in the isolated pig adipocytes. Thus, these results unequivocally demonstrate that pig has no UCP1 protein. Our results have resolved the controversy of whether pigs have the brown adipose tissue.

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1. Introduction

Mammals contain mainly two types of adipose tissues, white adipose tissue (WAT) and brown adipose tissue (BAT). WAT stores excess energy in the form of triglycerides when an animal's energy intake exceeds its energy expenditure. During starvation, WAT lipolysis provides energy to animals [1]. Brown adipose tissue contains multilocular fat droplets and many mitochondria, and is distinguished by its ability to dissipate energy in the form of heat [2]. BAT plays a significant role in newborns; they use this tissue for non-shivering thermogenesis to defend themselves against the cold environment [3].

BAT-dependent non-shivering thermogenesis is attributed to the uncoupling protein 1 (UCP1) [4–6]. UCP1 is a member of the of mitochondrial anion carrier proteins family and is a transmembrane protein with the size of approximately 35 KD. UCP1 disengages ATP synthesis from oxidative phosphorylation and dissipates energy as heat [7].

Although BAT has been found in many mammals such as mice, human, goat, and dog [8–11]. However, whether brown adipose tissue exists in the pig is still a controversy. Brown adipocytes were found in different pig adipose tissues by microscopic and electron microscopic methods [12]. But this result was not supported by the immunoblotting study in another laboratory using the rabbit anti-rat UCP1 antibody [13]. The authors concluded that pigs have no brown adipose tissue, and all adipose tissues in the pig appear to be exclusively “white” [13]. More recently, Berg et al. (2006) determined the complete genome sequence of pig *UCP1* gene by long-range PCR and genome walking. In this study, they found that exons 3 to 5 of the pig *UCP1* gene were lost 20 million years ago; more than twenty pigs from different breeds of domestic pigs and wild boar were included in their study [14]. Thus, pig *UCP1* gene contains only three exons: exons 1, 2 and 3, which are corresponding to the exons 1, 2 and 6 of the mouse *UCP1* gene, respectively. By using a goat UCP1 antibody in their immunoblotting assay Mostyn et al. (2014) showed that UCP1 protein is present in pig adipose tissue and is responsive to postnatal leptin treatment [15]. However, Jastroch & Anderson (2015) believe the increased UCP1 protein in Mostyn's study (2014) may be explained by non-specific hybridization with other mitochondrial anion carrier proteins, such

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as UCP2 or UCP3 by the goat UCP1 antibody [16]. Berg et al. (2006) only examined the *UCP1* gene at genome level; they did not measure the UCP1 protein. By contrast, Mostyn and his colleagues measured only UCP1 protein by a rabbit anti-goat UCP1 antibody; they did not study the *UCP1* gene structure or its transcript. Therefore, in this study, we examined the *UCP1* gene structure and determined whether pig *UCP1* gene is being transcribed, translated and functional.

2. Materials and methods

All animal use protocols were approved by the College of Animal Science, South China Agricultural University. All experiments were performed in accordance with relevant 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.

2.1. Tissue sampling

Male Landrace pigs (1 day and 7 days of age) were euthanized via intraperitoneal injection of pentobarbital sodium (40 mg/kg body weight) followed by exsanguinations. One gram of tissues from the fat pad in various locations, muscle, heart, liver, spleen, lung, and kidney were collected and stored at −80 °C until assayed.

2.2. RNA extraction and PCR analysis

Total RNAs were extracted from tissue or adipocytes using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Then total RNAs (0.5 μg) were reverse transcribed to cDNA by PrimeScript™ RT Master Mix (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's instructions.

General-PCR products were sequenced and blasted them with gene sequences deposit in NCBI to ensure the primers were specific. SYBR Green Real-time PCR Master Mix reagents (Toyobo Co., Ltd., Osaka, Japan) were used for real-time quantitative polymerase chain reaction (PCR), and PCR reactions were performed using a Mx3005p instrument (Stratagene, La Jolla, CA, USA). Relative expression of mRNAs was normalized to *β-actin* levels using the ΔΔCt method. Primers were designed using Primer Premier 5 according to the pig gene sequence obtained from NCBI. Primers used for PCR in this study were shown in Table 1.

2.3. Cell culture

The pig preadipocytes used in primary culture were isolated from the subcutaneous back and visceral fat pad of 1-day male Landrace pigs. 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 $CaCl_2$, 5 mM glucose, 100 mM HEPES, 4% BSA, 1.5 mg/mL Collagenase B). Digested tissue was filtered through a 70 μm cell strainer to remove large pieces, and the flow-through was centrifuged for 10 min at 500 × *g* to collect stromal-vascular fraction (SVF) cells in the pellet. The SVF cells were suspended in growth medium - 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). And they were plated in 75 cm^2 cell culture flasks. For plasmid transfection, primary adipocytes with 60% confluency were incubated with the plasmid for 6 h 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. Then the medium was replaced with new growth medium and cells were maintained in growth medium for an additional 12 h before adipogenic differentiation induction. Pre-adipocytes were induced to differentiation by the induction medium containing 10% FBS, 0.5 mM isobutylmethylxanthine, 0.25 μM dexamethasone, 1 μg/mL insulin, 1 nM T3 and 1 μM rosiglitazone for 48 h. Two days later, cells were switched to a different medium containing 10% FBS, 1 μg/mL insulin and 1 nM T_3. Differentiated cells were used for gene expression and uncoupling respiration assays. All chemicals for cell culture were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

Table 2
Antibody validation profile.

Primary antibody	Clone	Company	Catalog No.	Dilution
UCP1	Polyclonal	Bioss	bs-1925R	1:700
β-Actin	Monoclonal	Bioss	bsm-33036 M	1:1000
Secondary antibody	Conjugate Used	Company	Catalog No.	Dilution
Goat Anti-rabbit IgG	HRP	Bioss	bs-0295G	1:5000

2.4. Immunoblotting

Homogenized tissues were lysed in RIPA buffer containing protease inhibitors (1 μM PMSF). These protein lysates were separated with SDS-PAGE and then electroblotted to polyvinylidene fluoride. Electrophoresis supplies were purchased from Bio-Rad (California, USA). The membranes were blocked for 2 h at room temperature and incubated with different antibodies overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. The antibodies used in this study are listed in Table 2.

2.5. Preparation of pig UCP1 antibody

Pig *UCP1* gene exons 1 and 2 were cloned into pET-28a (+) recombinant vector. Recombinant pET-28a (+) vectors were transformed into *E. coli* BL21 (*E. coli* BL21-*UCP1*+). Isopropyl-β-*d*-thiogalactoside was used to induce *E. coli* BL21 to express truncated pig UCP1 protein (pig UCP1 protein). The pig UCP1 protein was purified by high-affinity Ni-NTA resin according to the manufacturer's instructions. We used the purified protein as antigen for antibody production. First, 1 mg antigen with Freund's complete adjuvant was injected into a 1.5 months old New Zealand rabbit. A second 1 mg antigen with Freund's incomplete adjuvant was injected four weeks later. One ml blood from the ear vein was collected every week to determine the UCP1 antibody titer in serum. At the eighth week, New Zealand rabbit was anesthetized via intraperitoneal injection of chloral hydrate (100 mg) followed

Table 1
Primers used for PCR analyses.

Gene name	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)
Primer 1	GGTCACCGCCAAAGTCCG	CAGCCCTCTGTAGTGCTTCATT
Primer 2	GCTGGCAAAGAGAGAAGGG	TGGATGGTAACATAGAGGCTGA
Primer 3	TCCTGCGAACAATCACTACTCT	TCCTGCGAACAATCACTACTCT
Primer 4	CGCACACCGCCAAAGTC	ATGCCAGTCACCAGAAGGAA

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