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Research paper

Characterization of fibronectin type III domain-containing protein 5 (*FNDC5*) gene in chickens: Cloning, tissue expression, and regulation of its expression in the muscle by fasting and cold exposure

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ABSTRACT

Irisin, a novel myokine encoded by fibronectin type III domain-containing protein 5 gene (*FNDC5*), is reported to stimulate brown fat-like development of white fat tissue and thermogenesis in mammals recently. However, information about the structure, tissue expression, and roles of FNDC5/irisin remains unknown in non-mammalian vertebrates including birds. In this study, we first cloned the *FNDC5* (*cFNDC5*) cDNA from chickens. *cFNDC5* is predicted to encode a 220-amino acid precursor containing the putative 'irisin peptide' of 112 amino acids, which shows high amino acid sequence identity with irisin of humans (97%), mice (97%), anole lizards (93%) and zebrafish (~80%). Using quantitative real-time PCR, we further examined c*FNDC5* mRNA expression in chicken tissues. The results showed that in adult chickens, c*FNDC5* is abundantly expressed in the muscle, heart, pituitary, ovary and various brain regions, and moderately expressed in adipose tissue, kidneys, lung, testes and small intestine. Moreover, *cFNDC5* is also abundantly expressed in the muscle, brain, hypothalamus and pituitary of developing embryos and post-hatching chicks. Interestingly, we noted that c*FNDC5* expression in the muscle of 3-week-old chicks could be induced by fasting and cold exposure, while its expression decreases during differentiation of pre-adipocytes cultured in vitro. Collectively, our data suggest that FNDC5/irisin is more than a 'myokine' and may be related to the development/functions of many tissues (e.g. muscle, brain, fat), as well as metabolic status of chickens.

1. Introduction

Irisin is a novel myokine recently identified in mice and humans (Bostrom et al., 2012). It is encoded by fibronectin type III domain-containing protein 5 gene (*FNDC5*), which is abundantly expressed in the muscle (Ferrer-Martinez et al., 2002; Hofmann et al., 2014; Huh et al., 2012). Proteolytically cleaved from FNDC5 protein, irisin is reported to be released into the circulation with heavy glycosylation (Bostrom et al., 2012; Lee et al., 2014). In addition to its abundant expression in the muscle, *FNDC5* mRNA or protein has also been found in other mammalian tissues including the brain, pericardium, intracranial artery, and adipose tissue (Huh et al., 2012; Piya et al., 2014; Teufel et al., 2002). Moreover, irisin-immunoreactivities have also been detected in human cerebrospinal fluid (Piya et al., 2014), breast milk of lactating women (Aydin et al., 2013b), and saliva (Aydin et al., 2013a).

Since its discovery in 2012 (Bostrom et al., 2012), irisin has been reported to be involved in the regulation of many physiological processes in mammals. In humans, irisin reduces pre-adipocyte differentiation, inhibits lipid accumulation in adipocytes, and accelerates muscle hypertrophy (Huh et al., 2014). Irisin also causes an increase in oxygen consumption, energy expenditure and mitochondrial content in myocytes (Vaughan et al., 2014). Most importantly, as an exercise-induced hormone, irisin is able to induce brown fat-like development of mouse white adipose tissue (WAT), including a pronounced expression of thermogenic uncoupling protein 1 (UCP1), a protein that uncouples mitochondrial respiration from ATP synthesis and results in heat production, and a higher density of mitochondria in the adipocytes (Bostrom et al., 2012). Exercise is widely recognized for its importance in improving the metabolic status in humans with obesity and type 2 diabetes. Muscle-derived irisin may then act as a critical signal to control a novel muscle-adipose crosstalk, which marks it as a potential target hormone for the treatment of obesity and metabolic disorders (Bostrom et al., 2012; Hofmann et al., 2014). Moreover, irisin has been viewed as a new diagnostic marker for diseases such as coronary heart diseases and macrovascular diseases (MVD) (Aronis et al., 2015; Zhang et al., 2014).

In contrast to the extensive investigation on FNDC5/irisin in mammals, little is known about the structure, expression, and physiological

Abbreviations: FNDC5, fibronectin type III domain-containing protein 5 gene; WAT, white adipose tissue; BAT, brown adipose tissue; UCP1, uncoupling protein 1; MVD, macrovascular disease; IBMX, 3-isobutyl-1-methylxanthine; mESCs, mouse embryonic stem cells; PGC1-α, peroxisome proliferator-activated receptor γ coactivator 1-α; CNS, central nervous system; RT, reverse transcription; PCR, polymerase chain reaction; qPCR, quantitative real-time PCR.

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roles of *FNDC5* in the non-mammalian vertebrates including birds. Therefore, using chicken as an experimental model, the present study aimed to: 1) clone the *FNDC5* and examine its tissue expression; 2) test whether *FNDC5* expression levels in the muscle and hypothalamus are regulated by fasting and cold, in the hope of revealing its possible association with energy balance and thermogenesis; and 3) examine the changes in *FNDC5* expression during pre-adipocyte differentiation. The results from the present study represent an initial step towards uncovering the roles of FNDC5/irisin in birds and provide invaluable comparative insights into the roles of *FNDC5* in vertebrates, such as its autocrine/paracrine actions on the brain, muscle and adipose tissue.

2. Materials and methods

2.1. Animals

Adult chickens and embryos (Lohmann layer chickens) were purchased from local markets or commercial companies. All animal experiments were performed according to the guidelines provided by the Animal Ethics Committee of Sichuan University. Chickens used in all experiments were selected from a 2-fold larger population in order to obtain chickens/chicks of uniform body weights. These experimental birds were kept in cages under conditions of controlled temperature (25 °C). All chickens were provided free access to water and commercial food before commencement of experiments. To study the fasting responses, a total of thirty 3-week-old male chicks were randomly assigned into three groups: a control group (free access to water and food) and two experimental groups (free access to water, and deprived of feed for 12 h or 24 h, respectively). For the cold exposure experiment, twenty 3-week-old male chicks were randomly divided into two groups: a control group and an experimental group. Both groups of chicks have free access to water and food. The control group was maintained at 25 ± 1 °C, while the experimental group was transferred to a cold environment (4 °C) for 24 h. At the end of these experiments, the *pectoralis* muscle and hypothalami of chicks were collected to examine target gene expression.

2.2. Total RNA extraction and reverse transcription

The tissues collected from adult chickens, post-hatching chicks (1-week-old and 3-week-old), and embryos [at embryonic day 12 (E12), day 16 (E16), and day 20 (E20)] were rapidly excised, frozen in liquid nitrogen, and stored at −80 °C for RNA extraction. Total RNA was extracted from the tissues by RNAzol (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions and dissolved in H_2O treated with diethyl pyrocarbonate.

Reverse transcription (RT) was performed at 42 °C for 2 h in a total volume of 10 μL consisting of 2 μg total RNA from different tissues, 1× single strand buffer, 0.5 mM each deoxynucleotide triphosphate, 0.5 μg oligo-deoxythymide, and 100 U MMLV reverse transcriptase (TaKaRa Biotechnology Co. Ltd, Dalian, China). These RT samples were then used for quantification of the mRNA levels of target genes.

2.3. Cloning the full-length cDNAs of cFNDC5

Since the predicted cDNA sequence of chicken *FNDC5* (*cFNDC5*) deposited in GenBank (XM_417814) is incorrect and lacks the 5′-cDNA end, thus, 5′-RACE was performed to amplify the 5′-cDNA ends of *FNDC5* from chicken brain using SMART-RACE cDNA amplification kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. The amplified PCR products were cloned and sequenced. Based on the cDNA sequences obtained by RACE-PCR, new primers were designed to amplify the full-length cDNA of *cFNDC5* using high-fidelity *Taq* DNA polymerase (Toyobo, Japan). The amplified PCR products were cloned into the pTA2 vector (Toyobo, Osaka, Japan) and then subjected to sequencing. The deduced amino acid sequence of *cFNDC5* was aligned with that of humans, mice, and other vertebrate species using the ClustalW program (Thompson et al., 1994).

2.4. Quantitative real-time polymerase chain reaction (qPCR)

According to our previously established method (Mo et al., 2014; Wang et al., 2007), quantitative real-time PCR (qPCR) assay was carried out to examine the mRNA levels of *FNDC5* in adult chicken tissues or chicken embryonic tissues (*β-actin* gene was used as an internal control). In this study, the *cFNDC5* mRNA levels in skeletal muscle (*pectoralis* muscle) after fasting and exposure to cold stress were also examined by qPCR. In brief, qPCR assay was performed with the use of CFX96 real-time PCR Detection System (Bio-Rad). The PCR condition is as follows: 2-min initial denaturation at 94 °C, and then followed by 40 cycles of amplification (94 °C for 20 s denaturation, 60 °C for 20 s annealing, and extension at 72 °C for 30 s). The primers used were listed in Table 1. All reactions were carried out in duplicate throughout the qPCR assay to check for reproducibility.

2.5. Induction of chick pre-adipocyte differentiation cultured in vitro

Chicken pre-adipocytes were cultured in vitro according to the method established by other laboratories (Matsubara et al., 2005; Ramsay and Rosebrough, 2003). Briefly, the subcutaneous adipose tissue was collected from 12-day-old chicks under sterile conditions, washed with PBS, minced into pieces, and then digested by 10 mL digestion buffer (PBS, 0.1% collagenase I, 2.8 mM glucose, 4% BSA) at 37 °C in a water bath for 1 h. After enzymatic digestion, the dispersed pre-adipocytes were filtered through a nylon mesh (40 μm pore size), and washed with medium 199. The filtered cells were centrifuged at 1000 *g* for 10 min to separate floating adipocytes from pellets of stromal-vascular cells. The stromal vascular cells were cultured in growth medium (Medium 199, 10% FBS, 100 U/mL penicillin and streptomycin) in a Corning CellBIND 48-well plates (Corning, Tewksbury, MA) at 37 °C with 5% CO_2 at a density of 1×10^5 cells/well. The growth medium was changed every other day.

To induce the differentiation of pre-adipocytes, the cells were cultured in differentiation medium (medium 199 containing 10% FBS, 500 nM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 20 μg/mL bovine insulin, and 150 μM oleate). Oil Red O staining was employed in this experiment to show the lipid droplets in cultured adipocyte cells as described by Liu et al. (2009). Then, the pre-adipocytes undergoing differentiation were used to examine the expression levels of *cFNDC5* and fatty acid-binding protein 4 (*FABP4*) genes using qPCR assay.

Table 1
Primers used.[a]

Gene name	Sense/antisense	Primer sequence (5′-to-3′)	Size (bp)
Primers for 5′-RACE			
cFNDC5	Antisense	TCACTGGCAGGGCTCTGGCCTTGGA	630
cFNDC5	Antisense	TGGATGCTGATGGCCTGGACATGCA	609
Primers for cloning full-length cDNA			
cFNDC5	Sense	CGCGGGGGATGGAGCCCTTCCT	927
	Antisense	ACCTCCCCGGTCATTGAGGCT	
Primers for quantitative real-time PCR			
cFNDC5	Sense	GTGACAATGAAGGAGATGGCGA	127
cFNDC5	Antisense	TGATGATGTCGTACTGCCTGCA	
FABP4	Sense	AGTTTGTGGGCACCTGGAAGCT	95
FABP4	Antisense	ACCAGCCATCTTCCTGGTAGCA	
β-Actin	Sense	CCCAGACATCAGGGTGTGATG	123
β-Actin	Antisense	GTTGGTGACAATACCGTGTTCAAT	

[a] All primers were synthesized by Beijing Genome Institute (Shanghai, China).

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