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Fat mass and obesity associated (FTO) gene regulates gluconeogenesis in chicken embryo fibroblast cells

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ABSTRACT

Fat mass and obesity-associated (FTO) gene was found to be associated with energy homeostasis in mammals, yet the function of chicken FTO is less clear. In this study, chicken embryo fibroblast cells (DF-1) were transiently transfected to over-express (FTO^+) or to knockdown (FTO^-) the chicken FTO gene and were used for the functional analysis. FTO expression was significantly augmented in FTO^+ cells while depressed in FTO^- cells ($P < 0.05$). FTO^+ cells had significantly lower glucose yet higher lactic acid (LD) concentrations ($P < 0.05$) in the culture media, which was associated with significantly up-regulated ($P < 0.05$) mRNA expression of the rate-limiting gluconeogenic enzymes, glucose-6-phosphatase (G6PC) and the phosphoenolpyruvate carboxykinase-mitochondrial (PEPCK-m). The protein content and enzyme activity of G6PC were also significantly higher ($P < 0.05$) in FTO^+ cells. Moreover, CCAAT/enhancer-binding protein-beta (C/EBP-beta) and cAMP responsive element binding protein 1 (CREB1), which were found to transcriptionally regulate the expression of G6PC, were increased at the level of both mRNA ($P < 0.05$) and protein ($P < 0.05$) in FTO^+ cells. ChIP analysis revealed significantly higher ($P < 0.05$) binding of C/EBP-beta and phospho-CREB1 to G6PC gene promoter in FTO^+ cells. In addition, the interaction of FTO and C/EBP-beta was significantly enhanced ($P < 0.05$) in FTO^+ cells. Opposite changes in G6PC expression and regulation were observed in FTO^- cells. Our results indicate that chicken FTO regulates gluconeogenesis in DF-1 cells through enhanced transcriptional regulation of G6PC gene by C/EBP-beta and phospho-CREB1.

1. Introduction

Fat mass and obesity-associated (FTO) gene was the first gene found to be associated with the common forms of human obesity (Loos and Bouchard, 2008) in different populations all over the world (Scuteri et al., 2007; Al-Attar et al., 2008; Cha et al., 2008; Chang et al., 2008; Hotta et al., 2008; Cornes et al., 2009; Gonzalez-Sanchez et al., 2009; Zimmermann et al., 2009). Up to now, tremendous progress has been made to reveal the function of FTO in mammals (Fredriksson et al., 2008; Jia et al., 2008; Stratigopoulos et al., 2008; Fischer et al., 2009; Vujovic et al., 2013). However, knowledge of FTO in birds is limited. The full length chicken FTO cDNA was cloned in 2011 and was found to share 63–66% homology with the mammalian FTO sequences (Tiwari et al., 2011). Several regions including the substrate binding domains of chicken FTO protein were found to be identical to the FTO protein of mammals. Like mammals, chickens express FTO in high abundance in metabolically relevant tissues such as hypothalamus (Gerken et al., 2007; Fredriksson et al., 2008), adipose tissue (Qi et al., 2008; Stratigopoulos et al., 2008), skeletal muscle (Stratigopoulos et al., 2008; Wang et al., 2012) and liver (Wang et al., 2012).

In mammals, FTO is implicated to regulate energy metabolism in many tissues, mainly based on correlation analyses. For instance, hepatic FTO mRNA levels were positively associated with the mRNA expressions of glucose-6-phosphatase (G6PC) and phosphoenolpyruvate carboxykinase (PEPCK) (Poritsanos et al., 2010) in fasted mice, suggesting a possible role of FTO in gluconeogenesis.

Gluconeogenesis is a process by which glucose is generated from non-carbohydrate sources such as lactic acid (LD), glycerol, propionate and amino acids. As the rate-limiting enzymes of gluconeogenesis, G6PC and PEPCK are critical for the homeostatic regulation of blood glucose level. In mammals, many transcription factors, including cAMP response element-binding protein (CREB) and CCAAT/enhancer binding protein-beta (C/EBP-beta), can bind to the promoters of gluconeogenic genes to regulate their expression (Scott et al., 1998; Gautier-Stein et al., 2005, 2006; Vander Kooi et al., 2005). Moreover, an in vitro study revealed that mammalian FTO could act as a co-activator of C/EBP-beta to enhance the binding of C/EBP-beta to peroxisome proliferator-activated receptor gamma (PPARγ) gene promoter (Wu et al., 2010).

Abbreviations: C/EBP-beta, CCAAT/enhancer-binding protein-beta; ChIP, chromatin immunoprecipitation; CREB-1, cAMP-response element binding protein 1; EGFP, enhanced green fluorescent protein; FTO, fat mass and obesity-associated; G6PC, glucose-6-phosphatase; GR, glucocorticoid receptor; IP–IB, immunoprecipitation/immunoblot assay; LD, lactic acid; PCX, pyruvate carboxylase; PEPCK-m, phosphoenolpyruvate carboxykinase-mitochondrial.

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However, knowledge about the transcriptional regulation of chicken G6PC gene is lacking and it remains unknown whether and how chicken FTO participates in the transcriptional regulation of gluconeogenic genes.

Gain- and loss-of-functions are the most effective approaches for gene functional analysis. In this study, we constructed recombinant plasmids to over-express and to knockdown chicken FTO gene, respectively, and transfected these plasmids to chicken embryo fibroblast (DF-1) cells to explore the role of chicken FTO on gluconeogenesis. We tested the cell viability and metabolic parameters first, then detected the expression of the gluconeogenic genes, and finally explored the possible involvement of FTO in the transcriptional regulation of G6PC gene by C/EBP-beta and pCREB1.

2. Materials and methods

2.1. Plasmids construction

The recombinant plasmid expressing FTO was constructed to contain enhanced green fluorescent protein (EGFP) and full length chicken FTO cDNA. Briefly, chicken FTO cDNA was cloned by PCR with specific primers containing restriction sites (Xho I and EcoR I), and then was inserted into the multiple cloning site of the pEGFP-N1 expression vector (6085-1, BD Biosciences, USA).

The recombinant plasmid expressing FTO shRNA was constructed according to a previous publication (Dai et al., 2005). The pSilencer 3.1-H1 neo siRNA expression vector was purchased from Ambion (Texas, USA). The sequences of FTO-siRNA were: 5′-GATCC GCGGTATT TGAAATGCACTTTCAAGAGAAGTGCATTTCAAATACCGCTTTTTTGGAAA-3′ (forward), and 5′-AGCTTTTCCAAAAAAGCGGTATTTGAAATGCACTTC TCTTGAAAGTGCATTTCAAATACCGCG-3′ (reverse). The target position of siRNA sequence is from 1178 bp to 1197 bp downstream of the translation start site on chicken FTO gene. The linearized pSilencer 3.1-H1 neo plasmid and FTO-siRNA were ligated to form the recombinant chicken FTO knockdown plasmid.

2.2. Cell culture

Embryo fibroblast cells are widely used to study glucose metabolism and gluconeogenesis (Warrier et al., 2010), so in this study we chose chicken embryo fibroblast cell line (DF-1) as recipient cells. DF-1 cells were grown in Dulbecco's modified Eagle's Medium (DMEM, Gibco, UK) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) at 37 °C and 5% CO_2 in a CO_2 incubator. One day before transfection, 2×10^6 cells were plated in 15 mL of the growth medium without antibiotics, so the cells will be 90% confluent at the time of transfection. The transfection was conducted following the protocol of Lipofectamine® 2000 Transfection Reagent (11668-019, Invitrogen, US). Cells transfected with the empty pEGFP-N1 were named as pEGFP, and those transfected with the recombinant pEGFP-N1 plasmid expressing FTO named as FTO^+. Cells transfected with pSilencer 3.1-H1 neo expressing scramble siRNA were named as siR-sc, while those transfected with pSilencer 3.1-H1 neo expressing FTO-siRNA were named as FTO^-. 24 h after transfection, media were collected and the cells were fast frozen in liquid nitrogen, and then stored at −70 °C.

2.3. Cell viability assay

Cell viability assay was performed according to a previous publication (Chen et al., 2013). After transfection, cells in 96-well plate were cultured for 20 h, and then the cells were incubated with 10 μL of cell counting kit-8 (CCK-8, KeyGEN Biotech, Nanjing, China) for 4 h at 37 °C. Then the optical density at 450 nm was measured. The OD450 is proportional to the viability of the cells (BioTek, Winooski, VT, USA).

2.4. Analysis of media glucose and lactic acid concentrations and cell glycogen content

The media were collected and centrifuged to eliminate the cell debris. Supernatant was collected to measure glucose and lactic acid (LD) concentration in duplicate using commercial kits (A019-2, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the instruction of the manufacturer. Cells were lysed and glycogen was analyzed according to a previous publication (Bennett et al., 2007).

2.5. Quantitation of mRNA by real-time PCR

Total RNA was extracted from DF-1 cells with TRIzol total RNA Kit (Tiangen Biotech Co., Ltd., Beijing, China) and treated with DNase I (D2215, Takara, Japan) to eliminate possible contamination of genomic DNA. Two micrograms of total RNA was reverse transcribed and 2 μL of diluted cDNA (1:20) was used for PCR. All the primers (Table 1) were synthesized by Invitrogen Co. Shanghai, China. Real-time PCR was performed in Mx3000P (Stratagene, USA). Mock RT and no template controls (NTC) were set to monitor the possible DNA contamination at the stage of both RT and RCR. The pooled sample made by mixing equal quantity of cDNA from all samples was used for optimizing the PCR condition and tailoring standard curve for each target gene, and melting curves were performed to ensure a single specific PCR product for each gene. Chicken β-actin was selected as a reference gene, because β-actin mRNA abundance did not differ among groups. The mRNA levels were expressed as the fold change relative to the mean value of pEGFP group.

2.6. Western blotting

Protein extracts from DF-1 were prepared as previously described (Yuan et al., 2009). Protein concentration was determined by the BCA assay (Pierce, Rockford, IL, USA). Twenty micrograms of protein extract from each sample was subjected to electrophoresis on a 10% SDS-PAGE gel and then transferred onto the nitrocellulose membranes (BioTrace, Pall Co., USA). Western blot analysis for FTO, G6PC (ab83690, Abcam, USA; 1:1000), C/EBP-beta (sc-150 X, Santa Cruz, USA; 1:1000), and pCREB1 (sc-7978 X, Santa Cruz, USA; 1:1000) was performed with respective primary antibodies and corresponding HRP-conjugated secondary antibodies. GAPDH (AP0066, Bioworld, USA, 1:10,000) was used as a reference in the Western blot analysis. Finally, the nitrocellulose membranes were washed and visualized by enhanced chemiluminescence (ECL) detection (Super Signal West Pico Trial Kit, Pierce, USA). ECL signals were detected by an imaging system (Bio-Rad, USA) and analyzed with Quantity One software (Bio-Rad, USA). Protein contents were presented as the fold change relative to the average value of the pEGFP group.

The chicken FTO antibody was raised and verified in our lab. Briefly, the full length coding sequence of chicken FTO gene was amplified, and then the PCR product was inserted into pColdI plasmid (Takara, Japan) to construct the prokaryotic expression plasmid pColdI-FTO which was then transformed into *Escherichia coli* BL21 (DE3). The recombinant protein was expressed upon isopropyl β-D-1-thiogalactopyranoside (IPTG, SunShine Biotechnology, Nanjing, China) induction and was purified by Ni-NTA agarose chromatography column (30210, Qiagen, Germany). The antiserum against recombinant chicken FTO protein was raised in rabbits. The titer of the antibody detected by ELISA was 1:51,200. Western blot analysis proved that the specificity of the antibody was adequate.

2.7. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) analysis was performed according to our previous publication (Jia et al., 2012b). Briefly, cells were washed with PBS containing proteinase inhibitor cocktail