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E4BP4 mediates glucocorticoid-regulated adipogenesis through COX2

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ABSTRACT

Adipogenesis is mediated by glucocorticoids via transcriptional regulation of glucocorticoid receptor (GR) target genes. However, the mechanism by which GR participates in adipogenesis has hitherto been poorly characterized. In this study, E4 promoter-binding protein 4 (E4BP4) was found to have a critical role in adipogenic differentiation of preadipocytes. Gain-of-function and loss-of-function studies revealed that E4BP4 acts as a positive regulator of adipogenesis in 3T3-L1 cells. *E4BP4* was markedly induced by glucocorticoid (dexamethasone) via GR and cAMP response element-binding protein (CREB) during adipogenesis. Knockdown of *E4BP4* abolished dexamethasone-induced adipogenesis, and overexpression of *E4BP4* partially accounted for the actions of dexamethasone in adipogenic differentiation. Promoter deletion analysis confirmed that E4BP4 transcriptionally represses *COX2* promoter activity, whereas *COX2* overexpression reversed the acceleration of E4BP4 in adipogenesis. Thus, E4BP4 acts as a key pro-adipogenic transcription factor by trans-repressing *COX2* in glucocorticoid-associated adipocyte differentiation.

1. Introduction

Adipose tissue is the main energy reservoir in the human body and a critical endocrine organ in whole-body metabolism, especially associated with obesity (Trayhurn, 2013). Adipogenesis, which plays a key role in adipocyte hyperplasia, is regulated by a number of extracellular cues, including nutrients, growth factors and glucocorticoid (GC) (Hauner et al., 1987; Rosen and Spiegelman, 2000). GC is well known to play a critical role in obesity, but the underlying mechanisms have not been clarified (Cha et al., 2013a). 3T3-L1 preadipocytes cannot be differentiated into adipocytes *in vitro* in the absence of the synthetic glucocorticoid dexamethasone (Pantoja et al., 2008). The adipogenic effect mediated by GC requires the transcriptional regulation function of glucocorticoid receptor (GR) target genes (Sargis et al., 2010). However, the downstream targets of GR in adipogenesis have yet to be fully identified. Recent studies have shown that E4 promoter-binding protein 4 (E4BP4) is a GC-regulated gene required for GC-induced apoptosis (Beach et al., 2011; Male et al., 2012). E4BP4 is regulated by GR, which directly binds to the promoter regions of *E4BP4* (Carey et al., 2013). In neuronal regeneration, GR also interacts with cAMP response element-binding protein (CREB) to promote the expression of E4BP4(MacGillavry et al, 2009). However, whether GC enhances E4BP4 in adipogenesis is unknown.

In our previous study, expression of *E4BP4* was found to be significantly upregulated during adipogenesis of porcine adipose SV cells by RNA-Seq (Jiang et al., 2013). This suggested its potential role in adipogenic differentiation. E4BP4, also called NFIL3, acts as both a transcriptional repressor and an activator, suggesting its role in multiple cellular functions, including IL-3-dependent B-cell survival, anti-inflammatory responses, cell apoptosis regulation, and regulation of COX2 by circadian rhythm (Ikushima et al., 1997; Chen et al., 2014; Wallace et al., 1997; Hirai et al., 2014; Ohno et al., 2007). It has been shown that COX2 functions as a vital molecular switch between osteogenesis and adipogenesis by inhibiting downstream target genes, PPARγ2 and C/EBPα(Hossain et al, 2010; Kellinsalmi et al, 2007, raising the possibility that E4BP4 may transcriptionally regulate COX2 in adipogenesis. Hence, it would be interesting to understand the roles of E4BP4 in GC-induced

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adipogenesis. In this study, we demonstrate for the first time that E4BP4 is a positive regulator of adipogenic differentiation. Specifically, we show that E4BP4 is induced by GR and CREB during GC-associated adipocyte differentiation and targets COX2 in promoting adipogenesis.

2. Materials and methods

2.1. Materials

Oil Red O, paraformaldehyde, insulin, dexamethasone, isobutylmethylxanthine, indometacin, BAPTA-AM, Dimethylsulfoxidewere (DMSO) were purchased from Sigma Aldrich. Rabbit anti-COX2, anti-PPARγ and anti-aP2 were purchased from Affinity biosciences Inc. Mouse anti-E4BP4 (A-9) was purchased from Santa Cruz.

2.2. Cell culture and differentiation

The 3T3-L1, C3H10T1/2, and HEK293T cells were cultured in DMEM (Gibco, San Diego, CA, USA) containing 10% fetal bovine serum (FBS, Gibco, San Diego, CA, USA) and 1% penicillin/streptomycin. For adipogenic differentiation, 2-day post-confluent (designated day 0) 3T3-L1 cells were treated with MDI (1 mM DEX, 0.5 mM isobutyl-methylxanthine, and 10 mg/mL insulin). After 2 days, the cells were transferred to 10% FBS medium containing only 10 mg/mL insulin and maintained in this medium for 2 days; subsequently, they were maintained in 10% FBS for another 2 days.

2.3. Plasmids and DNA transfection

E4BP4 and *COX2* cDNA was obtained from a mouse cDNA library and cloned into a pCDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) at the *Kpn*I and *Xho*I sites. *E4BP4* was also cloned into a pLVX-IRES-ZsGreen1 vector (Clontech h, Pato Alto, CA, USA) at the *Bam*HI and *Xho*I sites. To construct the plasmids pCMV-HA-E4BP4 CDS, pCMV-HA-E4BP4 ΔbZIP, and pCMV-HA-E4BP4 ΔTFD, the sequences of full-length E4BP4, with bZIP domain deletion and transcriptional repression domain (TRD) deletion, were amplified and cloned into a pCMV-HA vector (Clontech, Pato Alto, CA, USA) at the *Xho*I and *Not*I sites. A series of different COX2 promoter fragments was amplified by PCR from genomic DNA, sequence-verified, and cloned into a pGL3-basic vector (Promega, Madison, USA) at the *Kpn*I and *Xho*I sites. In this way, the following five promoter constructs were generated: COX2 (−2000 bp/−1 bp), P1 (−1260 bp/−1 bp), P2 (−837 bp/−1 bp), P3 (−545 bp/−1 bp), and P4 (−264 bp/−1 bp). The primers used for PCR amplification are listed in Supplementary Table 1. For DNA transfection, cells were plated into a 24-well plate and transfected with plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions.

2.4. RNA interference

Synthetic siRNA oligonucleotides specific for regions in the mouse *E4BP4*, *GR*, and *CREB* mRNA were designed and synthesized by GenePharma (Shanghai, China). The sequences that gave successful knockdown were *E4BP4* siRNA, 5′-GCAGGUGACGAACAUUCAATT-3′; *GR* siRNA, 5′-GCACCUUUGACAUCUUGCAGGAUTT-3′; and *CREB* siRNA, 5′-AAUACAGCUGGCUAACAAUGGTT-3′. The negative control (NC) siRNA was 5′-TTCTCCGAACGTGTCACGT-3′. 3T3-L1 cells were transfected at 50%–70% confluence with siRNA duplexes using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions.

2.5. Lentivirus generation and transduction

Lentivirus generation was performed as described in a previous report (Song et al., 2016). The lentiviruses were generated from HEK293T cells by Lipofectamine™ 2000-mediated cotransfection of the pLVX-IRES-ZsGreen1-E4BP4 or scramble plasmids, pMD2G and psPAX. Lentiviral supernatants were filtered through a 0.45 mM filter (Millipore, Billerica, MA, USA). For infection, 3T3-L1 cells were transduced with the lentiviruses in 5% FBS growth media supplemented with polybrene (8 mg/mL). The cells were incubated overnight with the lentiviruses and cultured in fresh 5% FBS growth media for another 3 days. Subsequently, the cells were observed for the expression of green fluorescent protein using a fluorescent inverted microscope.

2.6. Oil red O staining and triglyceride assay

Triglyceride accumulation was measured by oil red O (ORO) staining. The cells were washed twice with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 1 h at room temperature. The cells were then washed again with PBS and stained with freshly diluted ORO for 15 min. After staining, the cells were washed with PBS and photographed using a microscope (Nikon, Japan). For triglyceride assay, the concentrations of triglyceride were measured according to the manufacturer's instructions using a triglyceride measure kit (Applygen, Beijing, China) and normalized to the total protein level (n mol mg^{-1} protein) measured using the bicinchoninic acid protein assay kit (Beyotime, Nanjing, China).

2.7. RNA isolation and quantitative real-time PCR

Total RNA was extracted using an RNA extraction kit (GeneMark, China) and transcribed into cDNA using a first-strand cDNA synthesis kit (TOYOBO, Japan). Quantitation of the mRNA level by quantitative real-time (qPCR) was performed using a real-time PCR system (Bio-Rad, Richmond, CA, USA) and iTaq Universal SYBR Green Supermix (Bio-Rad, Richmond, CA, USA). The primers used are listed in Supplementary Table 2. The mean of the triplicate cycle thresholds (CT) of the target gene (*aP2, Adipo* etc) was normalized to the mean of triplicate CT of the reference *β-actin* gene using the formula "$2^{-\Delta\Delta Ct}$", which yielded relative gene expression level values.

2.8. Western blot

The cells were extracted with protein lysis buffer (Beyotime, China) supplemented with protease inhibitor cocktail. The samples were extracted at 4 °C for 30 min and then centrifuged at 10,000x*g* for 10 min, and the supernatant containing the total proteins was collected. Then, 20–30 mg of proteins/lane was separated on a 10% polyacrylamide precast SDS gel (Bio-Rad, Richmond, CA, USA) and then transferred onto a PVDF membrane (Millipore Billerica, MA, USA). After blocking with 5% skimmed milk powder (Sigma) for 3 h, the PVDF membrane was incubated overnight with the appropriate primary antibodies: against PPARγ, AP2, E4BP4, COX2 or β-actin. Then, mouse or rabbit secondary antibodies (Invitrogen, at a 1:10,000 dilution) were used to detect the primary antibodies. The membrane was exposed by using WesternBright™ Peroxide (Advansta, California, USA) in the imaging system (Carestream, New York, USA).

2.9. Luciferase reporter assays

Luciferase reporter assays were performed as described in a