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c-Jun regulates adipocyte differentiation via the KLF15-mediated mode

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ABSTRACT

Abnormal adipocyte differentiation is implicated in the development of metabolic disorders such as obesity and type II diabetes. Thus, an in-depth understanding of the molecular mechanisms associated with adipocyte differentiation is the first step in overcoming obesity and its related metabolic diseases. Here, we examined the role of c-Jun as a transcription factor in adipocyte differentiation. c-Jun overexpression in murine 3T3-L1 preadipocytes significantly inhibited adipocyte differentiation. In addition, the expression level of KLF15, an upstream effector of the key adipogenic factors C/EBPα and PPARγ, was decreased upon the ectopic expression of c-Jun. We found that c-Jun inhibited basal and glucocorticoid receptor (GR)-induced promoter activities of KLF15. c-Jun directly bound near the glucocorticoid response element (GRE) sites in the KLF15 promoter and inhibited adjacent promoter occupancies of GR. Furthermore, the restoration of KLF15 expression in 3T3-L1 cells with the stable ectopic expression of c-Jun partially rescued adipocyte differentiation. Our results demonstrate that c-Jun can suppress adipocyte differentiation through the down-regulation of KLF15 at the transcriptional level. This study proposes a novel mechanism by which c-Jun regulates adipocyte differentiation.

1. Introduction

Understanding the process of adipocyte differentiation and its transcriptional regulatory mechanisms is critical to improve several pathological problems caused by excessive fat deposition and insulin resistance [1–3]. The CCAAT/enhancer-binding protein (C/EBP) family and peroxisome proliferator-activated receptor gamma (PPARγ) are essential transcription factors that regulate adipocyte differentiation [3]. C/EBPβ and C/EBPδ are induced by a hormonal differentiation cocktail in the early stage of 3T3-L1 adipocyte differentiation [4]. Sequentially, C/EBPβ and C/EBPδ trigger the expression of C/EBPα and PPARγ, critical transcriptional regulators in the differentiation of the 3T3-L1 preadipocyte [5]. Transcription factor C/EBPα induces adipogenic gene expression for adipogenesis, and its growth-inhibitory and differential activities are supported by cyclin D3 [6]. Additionally, C/EBPα promotes adipogenesis in a PPARγ-dependent manner [7,8].

Recently, it was reported that Kruppel-like zinc finger transcription factors (KLFs) are critical molecules in adipogenesis and adipocyte biology [9]. KLFs play a role in both the promotion and inhibition of adipocyte differentiation. KLF4, KLF5, KLF6, KLF9 and KLF15 promote adipogenesis, while KLF2, KLF3 and KLF7 inhibit adipogenesis [9]. Among these, KLF15 is the first member of the KLF family reportedly linked to adipogenesis [10–12]. KLF15 is transcriptionally activated by GR during adipogenesis [13,14]. Sequentially, KLF15 can enhance the expressions of PPARγ and C/EBPα in adipogenic transcriptional circuit, and regulate expression of GLUT4 and acetyl-coA synthetase (AceCS) [12,15,16]. Furthermore, KLF15 can be a downstream target of C/EBPβ and C/EBPδ with regard to PPARγ induction during adipogenesis [12]. On the other hand, the *in vivo* expression level of KLF15 is decreased in white adipose tissue of a high fat diet-induced or genetically diabetic

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obese (*db/db*) mouse model. The overexpression of KLF15 in the adipose tissue of obese mice improves diet-induced obesity, insulin resistance, and glucose intolerance with a reduced mass of white adipose tissue [17]. Therefore, it is thought that KLF15 is an attractive target related to obesity and fat biology.

c-Jun promotes cell proliferation as a proto-oncogene that is overexpressed in many different types of tumors [18,19]. It is also a key target of c-Jun N-terminal kinase (JNK), which suppresses Glucocorticoid receptor (GR) transcriptional activity [20]. GR is a potent inducer of the differentiation of 3T3-L1 preadipocytes, as it activates the adipogenic transcription factors such as C/EBPβ, C/EBPδ, C/EBPα, and PPARγ during adipocyte differentiation [21–23]. c-Jun can occupy the promoter region (binding site: *tgactcagcc*) of Sirt1 that inhibits adipocyte differentiation by suppressing PPARγ [24,25]. Previously, it was reported that the expression of c-Jun is decreased by the adipocyte differentiation signal in 3T3 T cells [26]. Mariani et al. also reported that c-Jun overexpression blocks adipocyte differentiation in highly aggressive sarcomas through interaction with C/EBPβ [27].

Here, we show that the expression of c-Jun inhibits the GR-mediated stimulation of adipocyte differentiation through the suppression of KLF15 by binding to its promoter region. These data demonstrate a novel signal pathway during adipocyte differentiation and provide a deeper understanding of the molecular mechanisms associated with adipocyte differentiation.

2. Materials and methods

2.1. Generation of stable cell lines

The coding sequence of c-Jun was PCR-amplified from the cDNA of murine preadipocyte and was cloned into the pRetroX-IRES-ZsGreen1 vector with an N-terminal FLAG tag (Clontech). The full-length sequence of KLF15 was PCR-amplified from mouse cDNA and inserted into the pRetroX-IRES-DsRedExpress vector (Clontech). To generate a retrovirus expressing c-Jun and KLF15, retroviral constructs were individually co-transfected into GP2-293 cells with pVSV-G (Clontech) using Lipofectamine 2000 (Gibco-Invitrogen). At 48–72 h after transfection, media containing the retroviruses were collected and passed through a 0.45-μm filter. 3T3-L1 preadipocytes were co-infected with retroviruses expressing c-Jun and/or KLF15 in the presence of polybrene (8 μg/ml). Infected 3T3-L1 preadipocytes were enriched by determining the degree of GFP expression using a FACSAria cell sorter (BD Biosciences). The pRetroX-IRES-Zsgreen1 empty vector and the pRetroX-IRES-DsRed empty vector were used as control for the infection step.

2.2. Cell culture

3T3-L1 preadipocytes were obtained from the American Type Culture Collection (ATCC). Cells were maintained in a DMEM high-glucose medium (Gibco-Invitrogen) supplemented with 10% bovine calf serum and a 1% antibiotic-antimycotic solution at 37 °C in a humidified atmosphere of 5% CO_2. To initiate adipocyte differentiation, overconfluent 3T3-L1 preadipocytes were treated with an adipogenic induction medium containing a DMEM culture medium, 10% FBS, a 1% antibiotic-antimycotic solution, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), 1 μM dexamethasone (Sigma), and 5 μg/ml insulin (Sigma). After 48 h, the differentiation medium containing 10% FBS and 5 μg/ml insulin was changed every 2 days until the indicated times [28–30]. For a restoration experiment to assess the degree of KLF15 expression, 2 μM rosiglitazone was added to both the adipogenic induction and differentiation media.

2.3. Oil-red-O staining

Differentiated 3T3-L1 adipocytes were washed twice with PBS. Cells were fixed with 10% formalin in PBS for 30 min at room temperature. The cells were stained with a 0.5% filtered Oil-red-O solution (Sigma) in 60% isopropanol for 1 h at room temperature and were rinsed with distilled water. Images were captured with a Leica microscope with LED Illumination. To extract the incorporated Oil-red-O dye, 100% isopropanol was added to the stained cell-culture dish, after which the dish was shaken for 30 min at room temperature. Triplicate samples were read at 490 nm using a Victor™ X3 multilabel reader (Perkin Elmer, Waltham, MA) [31,32].

2.4. Quantitative PCR

Total RNA was isolated from undifferentiated or differentiated 3T3-L1 cells using the RNeasy® Lipid Tissue Mini Kit (Qiagen, Valencia, CA). 2 μg of total RNA was used to generate cDNA with the MMLV Reverse Transcriptase and a random primer (Promega). This was analyzed by quantitative PCR using a SYBR green PCR kit and the C1000 Touch™ Thermal Cycler (Bio-Rad). All data were normalized to the expression of TBP (TATA box-binding protein) in the corresponding sample.

2.5. Western blot analysis

A western blot analysis of 5–30 μg whole-cell extracts was performed. Proteins were separated by electrophoresis on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Pall Corporation). The membranes were then blocked with 5% skim milk and probed with primary antibodies. Anti-Flag M2 and anti-α-tubulin were obtained from Sigma. Antibodies against c-Jun, C/EBPα, PPARγ, and FABP4 were from Cell signaling technology. The KLF15 antibody was from Abcam. Antisera against HSP90 and C/EBPβ were purchased from Santa Cruz Biotechnology. The specific signals were amplified by horseradish peroxidase-conjugated secondary anti-rabbit IgG or anti-mouse IgG (Santa Cruz Biotechnology) and were visualized using an enhanced chemiluminescence system (Amersham).

2.6. Retroviral transfection

HEK293T cells were maintained with DMEM supplemented with 10% fetal bovine serum and a 1% antibiotic-antimycotic solution. For transfection, cells were seeded in triplicate in 24-well plates at 1×10^5 cells per well. Trans IT®-LT1 transfection reagents (Mirus Bio) was used in accordance with the manufacturer's instructions. Each transfection was performed with 200 ng of a KLF15 promoter reporter construct fused with the luciferase gene (pXP2-KLF15 promoter), 50 ng of a RSV-β-galactosidase plasmid, and 50 ng of a c-Jun expression plasmid (pcDNA3-c-Jun). After 24 h, cells were starved and stimulated with either 100 nM dexamethasone (Sigma) or EtOH vehicle for 12 h. Total cell lysates were prepared 48 h after transfection, and promoter activities were measured using the Dual-Luciferase reporter assay system (Promega, Madison, WI).

2.7. Chromatin immunoprecipitation

3T3-L1 cells were treated with 1% formaldehyde for 20 min to cross-link histones to DNA and were washed twice in PBS. Cross-linked cells were then lysed with cell lysis buffer (0.1% NP40, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 25 mM HEPES pH7.8, and a protease inhibitor cocktail). Subsequently, cell lysates were