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Activation of early phase of adipogenesis through Krüppel-like factor KLF9-mediated, enhanced expression of CCAAT/enhancer-binding protein β in 3T3-L1 cells

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ABSTRACT

In this study, we found that Krüppel-like factor (KLF) 9 activate the progression of the early phase of adipocyte differentiation in mouse adipocytic 3T3-L1 cells. KLF9 mRNA was detected in preadipocytes; and its level increased after the initiation of adipocyte differentiation, reached its maximum at 1 h, and gradually decreased thereafter. Functional suppression of KLF9 mRNA by its siRNAs repressed the accumulation of the intracellular lipids with a reduction in the expression of CCAAT/enhancer-binding protein (C/EBP) β, but not in that of C/EBPδ. In contrast, C/EBPβ and C/EBPδ did not affect the expression of KLF9 in 3T3-L1 cells. A chromatin immunoprecipitation assay revealed that KLF9 bound the KLF binding element at position −874 of the mouse C/EBPβ promoter. Moreover, the ability of KLF9 to bind to this element was enhanced, with a peak at 1–2 h after the initiation of adipogenesis, whose profile well resembled that of the expression of the C/EBPβ gene in 3T3-L1 cells. These results indicate that KLF9 activated the early phase of adipogenesis by enhancing the expression of the C/EBPβ gene in 3T3-L1 cells.

1. Introduction

Adipocytes play critical roles in the maintenance of energy balance, and these cells store energy in the form of lipids and release fatty acids in response to nutritional signals or energy insufficiency (Spiegelman and Flier, 2001). Adipocytes are also known as endocrine cells that secrete a number of adipocytokines (Antuna-Puente et al., 2008; Attie and Scherer, 2009; Galic et al., 2010; Rasouli and Kern, 2008). Adipogenesis is regulated through a complex process including coordinated alterations in hormone sensitivity and gene expression. Moreover, many transcription factors are involved in this regulation (Lefterova and Lazar, 2009; Rosen and Spiegelman, 2000; Rosen et al., 2000). Among them, CCAAT/enhancer-binding proteins (C/EBPs), peroxisome proliferator-activated receptors (PPARs), and sterol regulatory element-binding protein-1c (SREBP-1c) are key transcription factors in the regulation of adipogenesis (Lefterova and Lazar, 2009; Rosen and Spiegelman, 2000; Rosen et al., 2000). Studies to date suggest that C/EBPβ and C/EBPδ induce the expression of PPARγ and C/EBPα, which in turn initiates the adipogenesis (Lefterova and Lazar, 2009; Rosen and Spiegelman, 2000; Rosen et al., 2000) to regulate the expression of many other transcription factors (Lefterova and Lazar, 2009; Rosen et al., 2009; White and Stephens, 2010).

Krüppel-like factor (KLF), a member of the Sp/KLF family of transcription factors, is an evolutionally well-conserved member of the family of transcriptional regulators (Kaczynski et al., 2003; McConnell and Yang, 2010; Pearson et al., 2008; Suske et al., 2005). KLF has been identified in *Drosophila* as the "*krüppel*" protein. *Drosophila* embryos deficient in *krüppel* die with consequences of abnormal thoracic and abdominal segmentation (Wieschaus et al., 1984). The first mammalian *krüppel* homolog, termed erythroid KLF (EKLF/KLF1), was identified as a factor specifically expressed in erythrocytes (Pearson et al., 2008). At present, 17 mammalian KLFs have been identified (McConnell and Yang, 2010). KLF carries 3 highly conserved classical Cys2–His2 zinc finger motifs its C-terminal region and binds to consensus sequences known as GC- and CNCCCC-boxes in the promoter regions of various genes (Kaczynski et al., 2003; McConnell and Yang, 2010; Pearson et al., 2008; Suske et al., 2005). In contrast, the N-terminal portion is variable and is involved in transcriptional activation or repression as well as protein–protein interaction (Kaczynski et al., 2003; McConnell and Yang, 2010; Pearson et al., 2008; Suske et al., 2005). These KLFs are important in the regulation of various cellular events such as cell differentiation, development, and apoptosis, and stress responses in various types of cells (Kaczynski et al., 2003; McConnell and Yang, 2010; Pearson et al., 2008; Suske et al., 2005).

KLF9, previously referred to as basic transcription element binding (BTEB) protein 1, was firstly identified as a transcriptional repressor of

Abbreviations: KLF, Krüppel-like factor; C/EBP, CCAAT/enhancer-binding protein; PPAR, peroxisome proliferator-activated receptor; N.C., negative control; aP2, fatty acid binding protein 4; LPL, lipoprotein lipase; FAS, fatty acid synthase; SREBP, sterol regulatory element-binding protein; SCD, stearoyl-CoA desaturase; TBP, TATA-binding protein; ChIP, chromatin immunoprecipitation; KLFE, KLF-binding element.

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the rat liver CYP1A1 gene (Imataka et al., 1992). Subsequent work demonstrated that KLF9 activates the expression of the liver CYP7A gene (Foti et al., 1998). KLF9(−/−) mice exhibited deficits in motor learning, motor coordination and fear-conditioning (Morita et al., 2003), as well as uterine hypoplasia, smaller litter size, reduced numbers of implantation sites, and partial progesterone resistance in the uterus (Simmen et al., 2004). Moreover, Pei et al. reported that KLF9 is involved in the regulation of the middle phase of adipogenesis by activating PPARγ (Pei et al., 2011).

In the present study, we detected the expression of the KLF9 gene in preadipocytes, which expression increased with a peak at 1 h after the initiation of adipogenesis and then decreased. This temporal profile resembled that of the C/EBPβ gene, which is a key transcription factor in adipogenesis. The results of siRNA-mediated knockdown study and chromatin immunoprecipitation assay demonstrated that KLF9 acted as an activator of the C/EBPβ gene in the early phase of adipogenesis. Thus, KLF9 activates the progression of the early and middle phases of adipogenesis.

2. Materials and methods

2.1. Cell culture

Mouse adipocytic 3T3-L1 cells (Human Science Research Resources Bank, Osaka, Japan) were cultured in Dulbecco's modified Eagle medium (DMEM; Sigma, St. Louis, MO, USA) in a humidified atmosphere of 5% CO_2 at 37 °C.

Preadipocyte cells were caused to differentiate into adipocytes by incubation of the cells for 2 days in differentiation medium containing insulin (10 μg/ml; Sigma), 1 μM dexamethasone (Sigma), and 0.5 mM 3-isobutyl-1-methylxanthine (Nacalai Tesque, Kyoto, Japan). After 2 days, the medium was replaced with growth medium containing insulin (10 μg/ml) alone and changed every 2 days (Fujimori et al., 2010).

Oil Red O staining of the intracellular lipids was performed as described previously (Fujimori et al., 2012).

2.2. Preparation of RNA and quantification of RNA

Total RNA was extracted by the use of TriPure Isolation Reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. First-strand cDNAs were synthesized from 1 μg of total RNA with random hexamer (Takara-Bio, Kyoto, Japan) and ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan) by incubation at 42 °C for 60 min after initial denaturation at 72 °C for 3 min, followed by heat-denaturation of the enzyme at 99 °C for 5 min. The cDNAs were diluted and further utilized as the templates for quantitative PCR analysis. Quantification of mRNA levels was carried out by using a real-time PCR system (LightCycler, Roche Diagnostics) and THUNDERBIRD SYBR qPCR Mix (Toyobo) with the gene-specific primer sets shown in Table S1. The expression level of the desired genes was estimated by the use of concentration-known standard DNA, and normalized to that of TATA-binding protein (TBP).

2.3. Western blot analysis

Cells were lysed in RIPA buffer 50 mM Tris–Cl, pH 8.0, 150 mM NaCl, 0.1% [w/v] SDS, 0.5% [w/v] sodium deoxycholate, 1% [v/v] Nonidet P-40, and 1%[v/v] Triton X-100 with a protease inhibitor cocktail. After sonication, cell extracts were prepared by centrifugation of the lysates for 10 min at 12,000 ×*g* at 4 °C to remove the cell debris. Protein concentrations were measured with Pierce BCA Protein Assay Reagent (Thermo Scientific, Rockford, IL, USA). Proteins were separated on SDS-PAGE gels and then transferred onto Immobilon PVDF membranes (Millipore, Bedford, MA, USA) for Western blot analysis. The blots were first incubated with the desired primary antibodies, i.e., anti-KLF9 (BTEB1; C-17; 1:1000), anti-C/EBPβ (Δ198; 1:1000), anti-C/EBPδ (M17; 1:1000) polyclonal antibody (Santa Cruz Biotech., Santa Cruz, CA, USA) or anti-actin monoclonal antibody (AC-15; 1:5000, Sigma), followed by incubation with the appropriate secondary antibody, i.e., anti-rabbit, anti-goat or anti-mouse IgG antibody conjugated with horseradish peroxidase (Santa Cruz Biotech.). Immunoreactive signals were detected by the use of a Pierce Western Blotting Substrate (Thermo Scientific) and LAS-3000 UV Luminoimage Analyzer (Fujifilm, Tokyo, Japan), and analyzed with Multi Gauge software (Fujifilm).

2.4. Measurement of triglyceride level

Intracellular triglyceride level was measured by using a WAKO LabAssay Triglyceride Kit (Wako Pure Chemical, Osaka, Japan) according to the manufacturer's instructions. Protein concentrations were measured as described above. Absorbance was measured at 570 nm by the use of a Microplatereader Lucy2 (Anthos, Salzburg, Austria).

2.5. siRNA study

Stealth siRNA for KLF9 and Stealth Negative Control siRNA were obtained from Invitrogen: KLF9 siRNA#1, 5′-CCUACAGUGGCUGUGGGAAAGUCUA-3′; KLF9 siRNA#2, 5′-CCAUUACAGAGUGCAUACAGGUGAA-3′. Cells were transfected with each siRNA or Negative Control siRNA

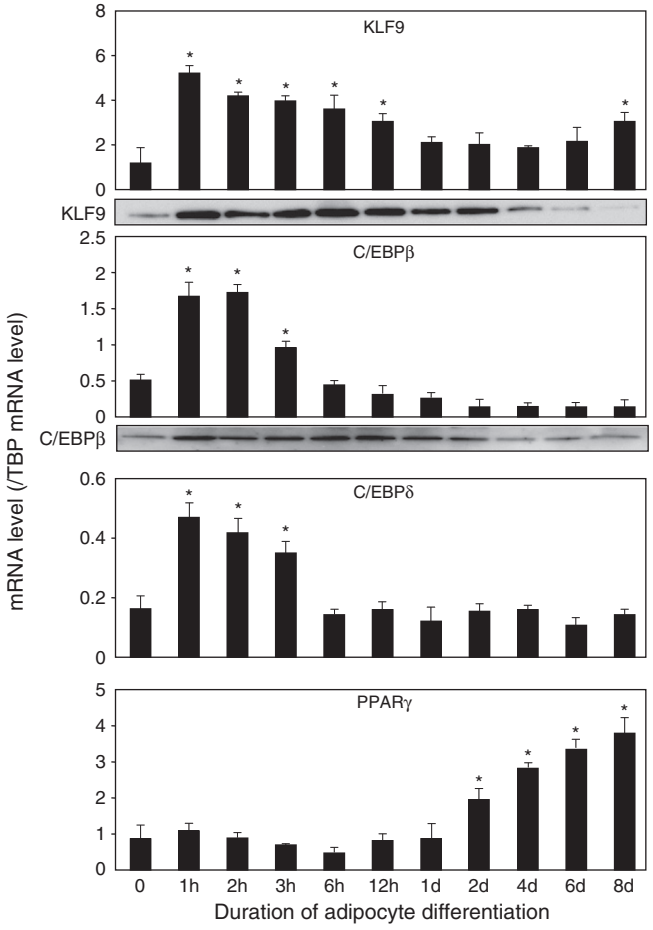

Fig. 1. Expression of KLF9, C/EBPs and PPARγ genes during adipogenesis of 3T3-L1 cells. Expression of the KLF9, C/EBPβ, C/EBPδ, and PPARγ genes during the adipocyte differentiation of 3T3-L1 cells. The expression level of each gene was measured by quantitative PCR. The data are presented as the mean ± S.D. from 3 independent experiments. *$p < 0.01$ as compared with value for undifferentiated cells. Protein levels were detected by Western blot analysis using crude cell extracts (20 μg/lane). Data are representative of 3 independent experiments.