Biochemical and Biophysical Research Communications 430 (2013) 1132–1139

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Active form Notch4 promotes the proliferation and differentiation of 3T3-L1 preadipocytes

Peng-Yeh Lai [a], Chong-Bin Tsai [a,b], Min-Jen Tseng [a,*]

[a] Institute of Molecular Biology and Department of Life Science, National Chung Cheng University, Chiayi 621, Taiwan, ROC
[b] Department of Ophthalmology, Chiayi Christian Hospital, Chiayi 600, Taiwan, ROC

ARTICLE INFO

Article history:
Received 19 November 2012
Available online 10 December 2012

Keywords:
Notch
3T3-L1
Adipogenesis

ABSTRACT

Adipose tissue is composed of adipocytes, which differentiate from precursor cells in a process called adipogenesis. Many signal molecules are involved in the transcriptional control of adipogenesis, including the Notch pathway. Previous adipogenic studies of Notch have focused on Notch1 and HES1; however, the role of other Notch receptors in adipogenesis remains unclear. Q-RT-PCR analyses showed that the augmentation of Notch4 expression during the differentiation of 3T3-L1 preadipocytes was comparable to that of Notch1. To elucidate the role of Notch4 in adipogenesis, the human active form Notch4 (N4IC) was transiently transfected into 3T3-L1 cells. The expression of HES1, Hey1, C/EBPδ and PPARγ was up-regulated, and the expression of Pref-1, an adipogenic inhibitor, was down-regulated. To further characterize the effect of N4IC in adipogenesis, stable cells expressing human N4IC were established. The expression of N4IC promoted proliferation and enhanced differentiation of 3T3-L1 cells compared with those of control cells. These data suggest that N4IC promoted proliferation through modulating the ERK pathway and the cell cycle during the early stage of 3T3-L1 adipogenesis and facilitated differentiation through up-regulating adipogenic genes such as C/EBPα, PPARγ, aP2, LPL and HSL during the middle and late stages of 3T3-L1 adipogenesis.

1. Introduction

Notch is a transmembrane receptor that functions in diverse developmental events to control cell fates [1]. After Notch activation, the Notch intracellular domain (NICD) enters the nucleus, where it interacts with transcription factor CSL to activate the transcription of downstream genes, such as HES and Hey, to regulate cell differentiation [2].

The process of adipogenesis includes five steps: cell proliferation, cell contact inhibition/growth arrest, clonal expansion, permanent growth arrest and lipid accumulation [3]. The transcriptional control of adipogenesis involves the activation of several families of transcription factors, such as CCAAT/enhancer binding protein family proteins (C/EBPs) and peroxisomal proliferator-activated receptor family proteins (PPARs). Adipogenic stimuli induce the expression of C/EBPβ and C/EBPδ, followed by an increase in PPARγ and C/EBPα expression. Subsequently, C/EBPα directly binds to PPARγ promoter and induces its expression [4,5]. Finally, terminal differentiation genes, such as adipocyte protein 2 (aP2), stearoyl-CoA desaturase, glucose transporter isoform 4, phosph*enol*pyruvate carboxykinase, leptin and insulin receptor are up-regulated through C/EBPα and PPARγ. During late stage of adipogenesis, cAMP-dependent protein kinase activates hormone-sensitive lipase (HSL) [6–8].

Previous studies have shown that Notch signaling is involved in adipogenesis. The ectopic overexpression and siRNA knockdown of presenilin enhancer-2 (PSENEN), a component of the γ-secretase complex, inhibited and induced 3T3-L1 adipogenesis, respectively [9]. The overexpression of constitutively active Notch1 also inhibits adipocyte formation and reduces the expression of PPARγ, aP2 and adiponectin genes in human bone marrow-derived stromal cells [10]. However, impaired Notch1 expression by antisense constructs prevented 3T3-L1 adipogenesis and reduced PPARγ and PPARδ expression [11]. The constitutive expression of a Notch downstream target gene, HES1, inhibited adipogenesis at a step prior to the induction of C/EBPα and PPARγ [12,13]. However, the siRNA-mediated reduction of HES1 mRNA in 3T3-L1 cells also inhibited differentiation. This observation might reflect that HES1 promoted adipogenesis through down-regulating Pref-1, an adipogenic inhibitor [13].

Studies concerning the involvement of Notch signaling in adipogenesis have focused on Notch1 and HES1; however, the

Abbreviations: N4IC, active form Notch4; Pref-1, preadipoctye factor 1; C/EBP, CCAAT/enhancer binding protein; PPAR, peroxisome-proliferator activated receptor; aP2, adipocyte protein 2; LPL, lipoprotein lipase; HSL, hormone-sensitive lipase.

* Corresponding author. Fax: +886 5 2722871.
E-mail address: biomjt@ccu.edu.tw (M.-J. Tseng).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.12.024

differential function of other Notch receptors in adipogenesis remains unknown. In this study, we analyzed the transcripts of all Notch receptors on different days during 3T3-L1 differentiation, and observed that the level of increment of Notch4 transcripts was comparable to that of Notch1. To determine the role of Notch4 during the process of 3T3-L1 adipogenesis, we characterized the effects of the transient and stable expression of N4IC. Our data provide further insight into the mechanisms underlying the regulation of adipogenesis.

2. Materials and methods

2.1. Cell lines and culture

3T3-L1 preadipocytes were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% calf serum and penicillin- streptomycin-amphotericin. To establish stable 3T3-L1 cells expressing human c-myc-N4IC (3T3-L1 N4IC), the cells were transfected with pcDNA3-N4IC using Turbofect™ reagent (Fermentas). After 48 h, the cells were replated in selection medium containing 1000 μg/ml G418. Stable clones were screened for the human Notch4 transcripts using RT-PCR. Control stable cells (3T3-L1 GFP) were also established through transfection with pcDNA3-GFP.

2.2. Adipogenesis assay

The differentiation of 3T3-L1 preadipocytes was induced by growing the cells to confluence (Day -2), the medium was replaced 48 h later with differentiation medium (DMI) (Day 0). The DMI contained 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), and 10 μg/ml of insulin in DMEM supplemented with 10% fetal bovine serum (FBS). After DMI treatment for 48 h (Day 2), the medium was replaced with DMEM supplemented with 10% FBS and 10 μg/ml of insulin. The medium was replenished every day for 6 days (Day 8).

2.3. RT-PCR and Q-RT-PCR

Total RNA was isolated from cells using REzol C&T reagent (PROTECH) and the RNA was reverse transcribed into cDNA using MMLV High Performance Reverse Transcriptase (Epicentre). The obtained cDNA was used directly as a template for PCR or diluted 30-fold for Q-PCR. The Q-PCR reactions were performed using the KAPA SYBR® FAST qPCR kit (KAPA Biosystems). Each reaction was performed in duplicate and run in an ABI StepOne detection system using analysis software (Applied Biosystems). All samples were run in triplicate for each PCR reaction. The levels of the test genes were normalized to β-actin mRNA level using a comparative threshold cycle ($2^{-[\mathrm{delta}]\ Ct}$) method which converts the differences in the cycle numbers to test gene/β-actin ratios. The primer sequences are listed in Supplementary Table 1.

2.4. Cell proliferation assay

The cells (1.5×10^3) were plated in triplicate into each well of 96-well plates and grew for 24, 48, 72 or 96 h. For drug treatment, the cells were treated with DMSO or U0126 (1 or 5 μM) and the effect of compounds on cell growth was measured at 3 days after treatment. The MTT solution (2 mg/ml) was added at the indicated times for 3 h at 37 °C. The reactions were terminated upon the addition of solubilization solution (DMSO) and the absorbance was measured at 490 and 595 nm.

2.5. Oil Red O stain

At the indicated times, the cells were washed with PBS and fixed with 3.7% formaldehyde for 1 h at room temperature followed by staining with 0.3% Oil Red O in isopropanol for 3 min. The images of Oil Red O staining cells were acquired using a Nikon microscope. Subsequently, the stained oil droplets were treated with isopropanol to elute Oil Red O dye and the absorbance was quantified at 490 nm.

2.6. Immunofluorescence staining

3T3-L1 cells grew on cover slips and were transfected with pcDNA3-N4IC or pcDNA3-GFP for 2 days. Cells were fixed in ice-cold methanol for 5 min at −20 °C. After blocking with 1% BSA and 0.0375% saponin in PBS, the cover slips were incubated with mouse anti-c-myc antibody (1:200, Santa Cruz) for 1 h at room temperature, followed by incubation with Alexa Fluor® 555 goat anti-mouse antibody (1:400, Molecular Probes) for 30 min at room temperature. The cells were subsequently counterstained with DAPI, and the cover slips were mounted onto microscope slides. The expression of N4IC protein in 3T3-L1 cells was examined using a confocal microscope (Olympus).

2.7. Immunoblot

To prepare whole cell lysates, the cells were lysed with lysis buffer containing 20 mM HEPES pH 7.6, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1 mM NaF, 1 mM Na_3VO_4, and protease inhibitor cocktail (Roche). Added Laemmli's sample buffer to the cell lysates (50 μg) and boiled for 5 min. The samples were subsequently resolved using SDS–PAGE and electro-transferred onto a PVDF membrane. After blocking with skim milk, membranes were incubated with primary antibodies (1:1000) overnight at 4 °C, followed by incubation with HRP-conjugated secondary antibodies (1:5000) for 1 h at room temperature. The immunoreactive proteins were visualized using an enhanced chemiluminescent reagent (PerkinElmer).

2.8. Cell cycle analysis

The cells (1.5×10^5) were cultured in medium containing 10% calf serum for overnight followed by serum starvation treatment for 36 h. Subsequently, the serum was restored and cultured the cells for 0, 4, 8, 12 or 24 h. To perform cell cycle analyses during cell differentiation, the cells grew to confluence followed by DMI treatment for 0, 4, 8, 12, 16 and 24 h. The treated cells were fixed with ice-cold 70% ethanol overnight at −20 °C. The fixed cells were centrifuged and resuspended the cell pellets in 4 μg/ml propidium iodine solution (containing 100 μg/ml RNase A) for 30 min at room temperature. The populations of treated cell at G1, S and G2/M stages were analyzed using a flow cytometer (Becton Dickinson) and the distribution was analyzed using the ModFit LT software (Becton Dickinson).

2.9. Statistical analysis

Samples were analyzed in duplicate and the data of three independent experiments were presented as the means ± standard deviation (SD). The statistical analyses were performed using GraphPad Prism 5 software. Student's *t* test was used to compare quantitative data among the groups.