Biochemical and Biophysical Research Communications 492 (2017) 419–424

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

E3 ubiquitin ligase DTX4 is required for adipogenic differentiation in 3T3-L1 preadipocytes cell line

Zonggui Wang [a], Zhong Dai [b], Yaqiong Pan [b], Simin Wu [b], Zhengli Li [b], Changqing Zuo [b, c, *]

[a] Department of Biochemistry and Molecular Biology, Guangdong Medical University, Dongguan, Guangdong, 523808, China
[b] Department of Pharmocology, Guangdong Medical University, Dongguan, Guangdong, 523808, China
[c] Guangdong Key Laboratory for Research and Development of Natural Drugs, Guangdong Medical University, Zhanjiang, Guangdong, 524023, China

ARTICLE INFO

Article history:
Received 9 August 2017
Accepted 21 August 2017
Available online 23 August 2017

Keywords:
DTX4
Adipogenic differentiation
Mitotic clonal expansion
3T3-L1 cells
Wnt signaling pathway

ABSTRACT

Deltex4 (DTX4) is a member of the Deltex family of proteins. To date several lines of evidences suggest that Deltex family of proteins is closely linked to cell development and cell differentiation. However, little is known about the role of DTX4 in adipogenic differentiation. In this study, we assessed the impact of DTX4 on adipogenic differentiation *in vitro*, we found that DTX4 protein expression gradually increased during adipogenic differentiation of 3T3-L1 preadipocytes cell line. While DTX4 stable knockdown by recombinant shRNA lentivirus (sh-DTX4) notably reduced the number of lipid droplets and down-regulated the expression of adipogenic transcription factors C/EBPα and PPARγ and adipogenic markers gene FABP4 and Adipsin. Besides, cell numbers and incorporation of 5-Ethynyl-2′-deoxyuridine (EdU) into cells were significantly decreased during mitotic clonal expansion (MCE) in sh-DTX4 cells postinduction. Furthermore, compared to recombinant shRNA lentivirus control group (sh-CON), the mRNA levels of Wnt signaling genes such as Wnt6, Wnt10b and β-catenin, were obviously elevated in sh-DTX4 group at day 3 of postinduction. Taken together, our results indicate that DTX4 stable knockdown inhibits adipogenesis of 3T3-L1 cells through inhibiting C/EBPα and PPARγ, arresting mitotic clonal expansion and regulating Wnt signaling pathway.

1. Introduction

Adipogenic differentiation is a complex and multi-step biological process modulated by a cascade of transcription factors such as Peroxisome proliferators activated receptor γ (PPAR-γ) and CCAAT/enhancer binding protein α (C/EBPa) [1]. PPAR-γ and C/EBPa are thought to be master transcription factors of adipogenic differentiation [2,3]. PPAR-γ activates expression of C/EBPa in the early phase of adipogenesis and then C/EBPa further increases PPAR-γ expression [4,5]. At last, PPARγ and C/EBPα mutually regulate each other and cooperate to contribute to adipose specific gene expression such as fatty acid binding protein 4 (FABP4, also named aP2), adipsin and adiponectin [6,7]. Recently, many efforts have been devoted to explore and elaborate the precise regulation of adipogenic differentiation. In addition to the above important factors, a series of novel genes and signaling molecules have been demonstrated to be involved in adipogenic differentiation [8–10]. However, the precise molecular mechanism of adipogenic differentiation is far from complete.

Deltex family of proteins, which consists mainly of four members such as DTX1, DTX2, DTX3 and DTX4, has been identified as cytoplasmic downstream elements of the notch signaling pathway [11,12]. Several lines of evidences suggest that Deltex family of proteins is closely linked to cell development and cell differentiation. For example, DTX1 is involved in regulating neural differentiation [13,14] and B cell development [15]. Overexpression of DTX2 reduces the expression of myogenic transcriptional regulator myogenin [14]. Nowadays, there are actually very few reports about physiological roles of DTX4. Cui et al. [16] have demonstrated that NLRP4 recruited the E3 ubiquitin ligase DTX4 to TBK1 and led to degradation of TBK1 to restrict the induction of virus-induced type I IFN. A recent research found that, upon ligand binding, notch1 at the cell surface was ubiquitylated by DTX4 [17]. Our lab has identified that DTX4 regulate the C2C12 myoblast differentiation [18]. However, little is known about the role of DTX4 in adipogenic differentiation.

In this study, we investigated the role of DTX4 in adipogenic

* Corresponding author. Department of Pharmocology, Guangdong Medical University, Dongguan, Guangdong, 523808, China.
E-mail address: zuochangqing@gdmu.edu.cn (C. Zuo).

http://dx.doi.org/10.1016/j.bbrc.2017.08.083
0006-291X/

differentiation of 3T3-L1 preadipocytes. We first demonstrated that DTX4 protein expression was gradually increased after inducing adipogenic differentiation. Then, we analyzed the effects of DTX4 knockdown with lentivirus on the differentiation of 3T3-L1 preadipocytes and elucidated that silencing of DTX4 blocked adipogenic differentiation. In addition, silencing of DTX4 strongly decreased expression of adipocyte master transcription factors PPARγ and C/EBPα, drastically inhibited mitotic clonal expansion (MCE) during the early stage of adipogenic differentiation and obviously elevated the expression of Wnt signaling genes. To our knowledge, the present study is the first to show a linkage between DTX4 and adipogenic differentiation.

2. Materials and methods

2.1. Cell line, reagents and antibodies

3T3-L1 preadipocytes were provided by the Cell Resource Center of Peking Union Medical College (Beijing, China). Fetal bovine serum (FBS) and newborn calf serum (NBCS) were purchased from Life technologies. Isobutylmethylxanthine (I5879), dexamethasone (D2915) and rosiglitazone (R2408) were obtained from Sigma Aldrich. Insulin (I8040) and puromycin (P8230) were obtained from Solarbio (Beijing, China). Luminata™ Crescendo Western HRP Substrate was obtained from Merck Millipore (USA). PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time) and PrimeScript™ RT-PCR Kit II (Tli RNaseH Plus) were obtained from TaKaRa Clontech (Kyoto, Japan). Antibodies to aP2, PPAR-γ were obtained from Santa Cruz Biotechnology. Anti-DTX4 was obtained from Proteintech Group, Inc. Anti-tubulin and HRP-labeled goat anti-mouse IgG (H+L) were obtained from Beyotime Biotechnology.

2.2. Cell culture and adipogenic differentiation

3T3-L1 preadipocytes were cultured in Dulbecco's modified Eagle's medium containing 10% newborn calf serum at 37 °C in humidified atmosphere of 5% CO_2 in air. Adipogenic differentiation was performed as described previously with minor modification [19,20]. In brief, Forty-eight hours postconfluence (designated day 0, D0), cells were treated with differentiation medium containing 500 μmol/L isobutylmethylxanthine, 1 μmol/L dexamethasone, 10 μg/mL insulin, 1 μmol/L rosiglitazone (MDIR) and 10% fetal bovine serum. Two days later (D2) the differentiation medium was changed to DMEM containing 10% FBS and 10 μg/mL insulin. After D4, the media were replaced every other day with 10% FBS-DMEM without insulin until cells were harvested at the indicated days.

2.3. Oil Red O staining

For Oil Red O staining, cells were washed three times with phosphate buffered saline (PBS) and then fixed with 3.7% formaldehyde for 20 min at room temperature. Oil Red O (ORO, Sigma) (0.5% in isopropanol) was diluted with water (3:2), filtered through a 0.45 μm filter, and incubated with the fixed cells for 30 min at room temperature. Cells were washed with PBS, and then ORO-stained lipid droplets were visualized by light microscopy and photographed. To quantify the degree of lipid droplets formation, ORO was extracted with 100% isopropanol solution and the absorbance reading was measured at a wavelength of 520 nm.

2.4. Lentivirus transduction

The Lentivirus for shRNA-mediated knockdown of DTX4 (sh-DTX4, 5′-CCAACATGTAAGACCATTT-3′) and non-silenced control lentivirus (sh-CON, 5′-TTCTCCGAACGTGTCACGT-3′) were purchased from Shanghai Genechem Co. Ltd. (Shanghai, China). In order to establish the stable cell line, 3T3-L1 preadipocytes were infected with lentivirus in the presence of 8 μg/mL polybrene (Sigma) for 12 h. Three days after infection, the growth medium containing 3 μg/mL of puromycin was used to select the infected cells for 1 week before used for next experiments. The expression efficiency was evaluated by western blot analysis.

2.5. Cell counting and EdU incorporation assay

3T3-L1 preadipocytes were induced to differentiation with differentiation medium in 6-well plates. After induction for 0, 1, 2, and 3 days, cells were trypsinized and counted using a hemacytometer. EdU incorporation assay was performed using the Cell-Light™ EdU Apollo®643 In Vitro Imaging Kit according to the manufacturer's protocol (RiboBio). Briefly, 18 h after induction, cells were labeled for 2 h with 10 μM EdU before fixation with 4% paraformaldehyde. Cell nuclei were stained with DAPI for 30 min. After incorporation, EdU was visualized by fluorescence microscopy. The ratio of EdU positive cells (EdU staining cells/the total of cells) was calculated.

2.6. RNA extraction and quantitative real-time PCR

Total RNA was extracted with the Trizol Reagent (Life Technology, Rockville, MD, USA) and reverse-transcribed into cDNA using PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time, Takara Bio Inc., Otsu, Japan). And then the cDNA was used as a template for PCR. Real-time PCR was performed in PikoReal Real-Time PCR System using one step SYBR® PrimeScript™ RT-PCR Kit II (Takara Bio Inc., Otsu, Japan) according to the manufacturer's protocols. The housekeeping gene 36B4 was used as an internal normalization control to obtain the relative fold changes using the comparative CT method. All primers for qPCR were as following: PPAR-γ, 5′-CACAAGAGCTGACCCAATGGT-3′(F) and 5′-GATCGCACTTTGGTATTCTTGGA-3′(R); C/EBPa, 5′-CGCAAGAGCCGAGATAAAGC-3′(F) and 5′-CGGTCATTGTCACTGGTCAACT-3′(R); aP2, 5′-GATGCCTTTGTGGGAACCTG-3′(F) and 5′-CAGTTTGAAGGAAATCTCGGT-3′(R); Adipsin, 5′-CTGAACCCTACAAGCGATGG-3′(F) and 5′-ACCCAACGAGGCATTCTG-3′(R); Wnt6, 5′-ATGGACTGTGGTGGGCCG-3′(F) and 5′-AACTGACATTCTCGAACCCCC-3′(R); Wnt10b, 5′-CCACTACAGCCCAGAACCTC-3′(F) and 5′-GGAGAGACCCTTTCAACAACTG-3′(R); β-catenin, 5′- ATGGAGCCGGACAGAAAAGC-3′(F) and 5′-CTTGCCACTCAGGGAAGGA-3′(R); 36B4, 5′-GCTTCATTGTGGGAGCAGAC-3′(F) and 5′-ATGGTGTTCTTGCCCATCAG-3′(R).

2.7. Protein extraction and Western Blot analysis

Cells were lysated with RIPA lysis buffer containing 1 mM PMSF (Beyotime Institute of Biotechonogy, Haimen, China) and then centrifuged at 12,000 rpm for 15 min at 4 °C. The protein concentrations were measured with BCA protein Assay Kit and then equivalent amounts of proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Polyvinylidene Fluoride (PVDF) Membranes. After blocking in 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h at room temperature, membranes were incubated overnight at 4 °C with primary antibodies. After being washed 3 times with TBST, detection was performed using HRP-labeled goat anti-mouse IgG (Beyotime Institute of Biotechonogy, Haimen, China) or HRP-labeled goat anti-rabbit IgG (Abcam, Cambridge, MA, USA) for 1 h at 37 °C. Antigen-antibody interactions were visualized by incubation with the Luminata™ Crescendo Western HRP Substrate (Millipore, Billerica, USA).