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Crucial roles of D-type cyclins in the early stage of adipocyte differentiation

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ABSTRACT

Cyclin D2 was isolated as one of the genes expressed early in adipogenesis. The expression of cyclin D2 increased temporarily early on and then again late in the differentiation process. The expression of cyclin D1 and cyclin D3, the other D-type cyclins, was also transiently induced early during adipocyte differentiation. RNAi (RNA interference)-mediated knockdown of cyclin D1, D2, or D3 inhibited the differentiation of 3T3-L1 cells into lipid-laden adipocytes. Moreover, the knockdown of cyclin D1 or D3 significantly inhibited mitotic clonal expansion (MCE), while silencing of the cyclin D2 gene had a milder effect on MCE. Each of the D-type cyclins seems to play a crucial role in adipocyte differentiation by regulating MCE.

Adipose tissue is an inevitable organ for energy homeostasis as well as energy storage [1]. It secretes numerous kinds of cytokines, called adipocytokines, such as leptin, TNF-α, and resistin, which are known to influence food intake, insulin resistance, and arteriosclerosis [2,3]. Obesity, a pathological accumulation of adipose tissue, results in an imbalance in the secretion of several adipokines, which can lead to diseases such as type 2 diabetes mellitus, hypertension, hyperlipidemia, and cardiac infarction [1,4,5].

Obesity results from an increase in the size and number of individual adipocytes. Therefore, in the context of the treatment of obesity-related diseases, it is important to elucidate, not only the mechanisms of hypertrophy of adipocytes, but those of adipogenesis which leads to an increase in the number of mature adipocytes. Scientific studies have demonstrated that transcription factors such as peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein α (C/EBPα) are important for the differentiation of preadipocytes into lipid-laden adipocytes [2,6]. These transcription factors are expressed midway through the differentiation and up-regulate the expression of many adipogeneic genes. However, little is known about the mechanisms underlying the early stage of adipocyte differentiation.

In order to elucidate the molecular mechanisms of adipocyte differentiation, we previously isolated, using a PCR-subtraction system, 102 genes which were induced to express at the early stages of the differentiation process. These include unidentified genes as well as genes for transcription factors and signaling molecules, some of which are crucial to adipogenesis [7–10]. Herein, we newly identified cyclin D2 from unknown genes of 102 genes. D-type cyclins, such as cyclin D1, cyclin D2, and cyclin D3, are known to function as key sensors for mitogenic stimuli and control G1–S progression [11].

Recent studies have showed that D-type cyclins function not only as components of the cell-cycle core machinery but also as co-factors for several transcription factors. In fact, it has been reported that cyclin D1 inhibits adipocyte differentiation through the repression of the expression and transactivation of PPARγ, while cyclin D3 promotes adipocyte differentiation as the coactivator of PPARγ [12,13]. Moreover, these studies seemed to focus on the mid- and late stages of the differentiation process, and the functions of D-cyclins early on remain unknown. We investigated the roles of cyclin D2 as well as cyclin D1 and cyclin D3 at the early stage of adipogenesis, and found that they contribute to adipocyte differentiation, partly by acting on mitotic clonal expansion (MCE), which is requisite for adipogenesis.

Materials and methods

RNA isolation and real-time quantitative RT-PCR (Q-PCR). RNA isolation and Q-PCR were performed as described previously [10]. For Q-PCR, the pre-designed primers and probe sets for cyclin D1, cyclin D2, cyclin D3, cyclin E1, cyclin A2, cyclin

Abbreviations: CDK, cyclin-dependent kinase; C/EBP, CCAAT/enhancer-binding protein; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PPARγ, peroxisome proliferator-activated receptor γ; RNAi, RNA interference; RT-PCR, reverse transcription-coupled polymerase chain reaction; shRNA, short-hairpin RNA; TNFα, tumor necrosis factor α; UTR, untranslated region.

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B2, PPARγ, C/EBPα, C/EBPβ, and 18S rRNA were obtained from Applied Biosystems (Applied Biosystems, Foster City, CA, USA). Relative standard curves were generated in each experiment to calculate the input amounts of the unknown samples. The relative abundance value obtained is divided by the value derived from the control sequence (18S rRNA) in the corresponding PCR. The normalized values were shown as relative expression in Q-PCR results.

Cell culture and differentiation. Mouse 3T3-L1 preadipocyte cells were cultured and induced to differentiate as described previously [10]. Mouse NIH-3T3 (clone 5611, JCRB 0615) fibroblastic cells were maintained in DMEM containing 10% calf serum.

Western blot analyses. For Western blot analyses, post-confluent 3T3-L1 cells were treated with differentiation inducers. The cells at different time-points after the induction were rinsed twice with phosphate-buffered saline (PBS), and then lysed in RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, and 1 mM EDTA) freshly supplemented with protease inhibitor mixture, mildly sonicated, and clarified by centrifugation at 15,000 rpm for 10 min. Protein content of each supernatant was quantified using Bradford assay. Equal amounts of protein were separated using SDS–PAGE, transferred to the polyvinylidenedifluoride membrane. Western blot analyses were performed with the following antibodies: cyclin D1 (C-20), cyclin D2 (M-20), and cyclin D3 (C-16) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

RNAi experiment. The target regions in the open reading frame of mouse cyclin D1 (GenBank Accession No. NM_007631), cyclin D2 (NM_009829), and cyclin D3 (NM_007632); the region of cyclin D1 at 523–543 bp (nucleotide A in the translation initiation codon is the first nucleotide), that of cyclin D2 at 361–381 bp, and that of cyclin D3 at 148–168 bp, were selected according to the Qiagen siRNA online design tool (http://sirna.qiagen.com/) for the RNAi of cyclin D1, cyclin D2, and cyclin D3, respectively. A 19-nucleotide short-hairpin RNA (shRNA)-coding fragment with a 5′-TTCAAGAGA-3′ loop was subcloned into the ApaI/EcoRI site of pSilencer 1.0-U6 (Ambion, Inc. Austin, TX, USA). As a negative control, the scrambled fragment 5′-GTAAGATGAGGCAATGGAG-3′ which does not have similarity with any mRNA listed in GenBank was generated. Methods for the transfection of shRNA expression plasmids into 3T3-L1 cells and cell-counts were described previously [10].

Results

Time course of mRNA expression of D-type cyclins

We previously isolated 102 genes, which were induced to express early on in adipocyte differentiation [9,10]. Of these, almost half were unknown genes at that time. However, one of the unknown genes was identified as cyclin D2, because the isolated fragments showed identity to the 3′-UTR of mouse cyclin D2, which was later listed in updated databases. Cyclin D2 belongs to the D-type cyclins, key cell-cycle factors that function as the regulatory subunits of CDK4 and CDK6, which regulate G1 progression [11,14]. Therefore, we investigated the functions of cyclin D1 and cyclin D3 as well as cyclin D2 at the early stage of adipocyte differentiation.

We first determined the expression profiles of cyclin D1, cyclin D2, and cyclin D3 during the differentiation process by Q-PCR (Fig. 1). Early on, the mRNA expression of all D-type cyclins was up-regulated, reaching a peak at 12 h after the differentiation was induced. Later, the expression of cyclin D1 was dramatically decreased, that of D2 was significantly increased, and that of D3 was sustained as the differentiation progressed (Fig. 1A). To determine the protein expression patterns of each of D-cyclins during adipogenesis, we performed Western blot analyses using antibodies against each of D-cyclins. Consistent with mRNA expression patterns, the protein expression levels of all D-cyclins were elevated early in the differentiation processes, although the expression pattern of each was different later (Fig. 1B). The upper band observed in Western blotting using anti-cyclin D2 antibody corresponded to cyclin D1, because this antibody recognizes not only cyclin D2 but also, to a lesser extent, cyclin D1, and the electrophoretic mobility of this band corresponded to that of cyclin D1. We also confirmed that the intensity of this band was reduced by the knockdown of cyclin D1 (data not shown). These results imply that D-cyclins play some functional roles in the early stage of adipogenesis, although each might have different functions in the late stage.

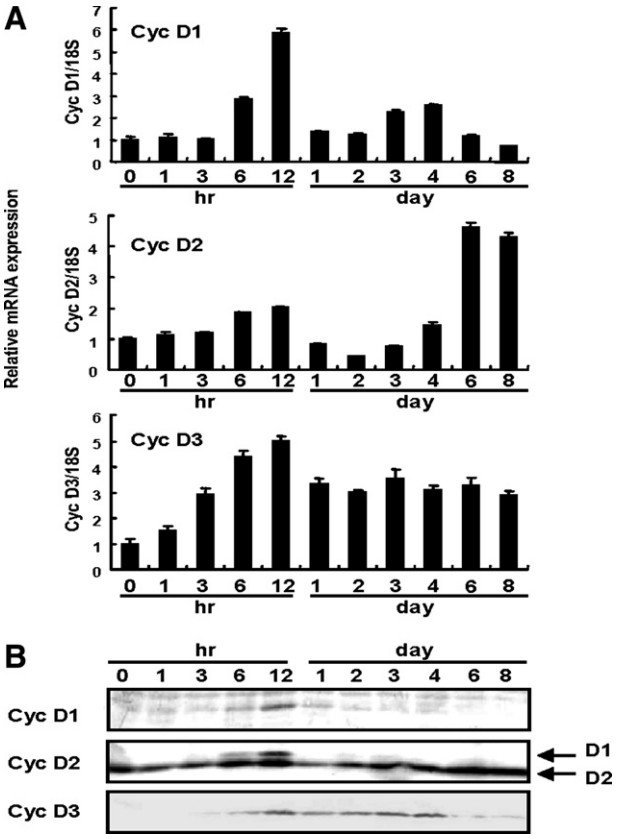

Fig. 1. Time course of the mRNA and protein expression of D-type cyclins during adipocyte differentiation. (A) Total RNA was prepared from 3T3-L1 cells at various time-points after treatment with inducers and was subjected to Q-PCR. The levels of D-type cyclins were normalized with 18S rRNA expression determined by Q-PCR. Data are expressed as inductions relative to levels before the induction (0 h). Each column represents the mean with standard deviation ($n = 3$). (B) Western blot analyses of whole cell lysate prepared at the indicated time-points of adipocyte differentiation. The proteins detected by cyclin D1, D2, and D3 antibodies are indicated.

Expression profiles of D-type cyclins in growth-arrested or proliferating 3T3-L1 and NIH-3T3 cells

The D-type cyclins function as key sensors for mitogenic growth factors, and their expression is controlled by the extracellular environment [11]. To investigate whether the transient expression of D-cyclins is limited to adipocyte differentiation or not, we next determined the expression profiles of D-cyclins in differentiating and non-differentiating cells. It is reported that mouse 3T3-L1 cells enter a temporary quiescent state arresting at the G0/G1 cell-cycle boundary 2 days after they reach confluence, and growth arrest at confluence seems to be required for adipocyte differentiation [15]. Namely, they differentiate fully into lipid-laden adipocytes only when exposed to differentiation induction in growth-arrested state, not proliferating state. NIH-3T3 cells did not differentiate when in either a growth-arrested or a proliferating state in the presence of inducers. These two cell lines were treated with inducers in a post-confluent state described above (growth-arrested) or proliferating state (proliferating), and then total RNAs, extracted from these cells, were subjected to Q-PCR (Fig. 2). The expression of cyclin D1 and cyclin D2 was remarkably induced when growth-arrested 3T3-L1 cells were exposed to adipogenic inducers. Although the expression of cyclin D1 and cyclin D2 was observed in the proliferating 3T3-L1 cells, their expression was not up-regulated by inducers. Also, the up-regulation of cyclin D1 and cyclin D2 expression was not observed in either the growth-arrested or