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Lactacystin inhibits 3T3-L1 adipocyte differentiation through induction of CHOP-10 expression ☆

Xi Li [a,b], Hai-Yan Huang [a,b], Jie-Gen Chen [a], Lin Jiang [a], Hong-Lei Liu [a,b], De-Guo Liu [a], Tan-Jing Song [a], Qun He [a], Chun-Gu Ma [a], Duan Ma [a,b], Hou-Yan Song [a], Qi-Qun Tang [a,b,c,*]

[a] *Key Laboratory of Molecular Medicine, Ministry of Education, Fudan University Shanghai Medical School, P.O. Box 238, 138 Yi Xue Yuan Road, Shanghai 200032, PR China*
[b] *Institute of Biomedical Sciences, Fudan University, Shanghai 200032, PR China*
[c] *Department of Biological Chemistry, Fudan University Shanghai Medical School, Shanghai 200032, PR China*

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Abstract

Hormonal induction triggers a cascade leading to the expression of CCAAT/enhancer-binding protein(C/EBP)α and peroxisome proliferator-activated receptor (PPAR) γ, C/EBPα, and PPARγ turns on series of adipocyte genes that give rise to the adipocyte phenotype. Previous findings indicate that C/EBPβ, a transcriptional activator of the C/EBPα and PPARγ genes, is rapidly expressed after induction, but lacks DNA-binding activity and therefore cannot activate transcription of the C/EBPα and PPARγ genes early in the differentiation program. Acquisition of DNA-binding activity of C/EBPβ occurs when CHOP-10, a dominant-negative form of C/EBP family members, is down-regulated and becomes hyperphosphorylated as preadipocytes traverse the G_1–S checkpoint of mitotic clonal expansion. Evidences are presented in this report that lactacystin, a proteasome inhibitor, up-regulated the CHOP-10 expression, blocked the DNA-binding activity of C/EBPβ, and subsequently inhibited MCE as well as adipocyte differentiation.

Keywords: DNA-binding activity; C/EBPα; C/EBPβ; PPARγ; Mitotic clonal expansion

Treatment of growth-arrested 3T3-L1 preadipocytes with differentiation inducers triggers a cascade in which CCAAT/enhancer-binding protein (C/EBP) β is rapidly expressed, followed by C/EBPα, and peroxisome proliferator-activated receptor (PPAR) γ, C/EBPα, and PPARγ transactivate several adipocyte genes that give rise to the adipocyte phenotype [1–5]. Upon induction, growth-arrested preadipocytes synchronously reenter the cell cycle and undergo mitotic clonal expansion (MCE), a process required for completion of the 3T3-L1 adipocyte differentiation program [6,7]. C/EBPβ, a transcriptional activator of the C/EBPα and PPARγ genes [8–11], is rapidly expressed and maintained at high levels throughout MCE [12,13]. However, expression of C/EBPα is delayed until the preadipocytes exit mitotic clonal expansion [5]. This is important that C/EBPα is anti-mitotic and thought to be responsible for terminating mitotic clonal expansion, its premature expression will interfere with the MCE and subsequently inhibit adipocyte differentiation. C/EBPβ is expressed immediately on induction [13], but lacks DNA-binding activity and therefore cannot activate transcription of the C/EBPα gene. Two mechanisms have been found to contribute to the delayed acquisition of DNA-binding activity

☆ *Abbreviations:* C/EBP, CCAAT/enhancer-binding protein; EMSA, electrophoretic mobility-shift analysis; MCE, mitotic clonal expansion; cdk, cyclin-dependent kinase; ALLN, *N*-acetyl-Leu-Leu-norleucine; PPARγ, peroxisome proliferator-activated receptor (PPAR) γ.

* Corresponding author. Fax: +86 21 64033738.
E-mail address: qqtang@shmu.edu.cn (Q.-Q. Tang).

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by C/EBPβ, one is due to hypophosphorylation of C/EBPβ [14] early in the differentiation program, another is due to transient interaction with CHOP-10, a dominant-negative member of the C/EBP family [15,16], which is down-regulated during the differentiation program.

CHOP-10 is a dominant-negative form of C/EBP family members: its C-terminus contains a leucine zipper region through which it can heterodimerize with other C/EBP family members (i.e., C/EBPβ), and it has two proline and one glycine residues instead of basic amino acids [15] in the region corresponding to DNA-binding domain in other C/EBP family members. Thus, heterodimers between CHOP-10 and other C/EBP family members cannot bind to C/EBP consensus sequence. Previous findings indicate that C/EBPβ transiently interacts with CHOP-10 as it expressed, upon induction of differentiation CHOP-10 is down-regulated by fetal bovine serum [17] and releases C/EBPβ from heterodimers, which leads to the acquisition of DNA-binding activity by C/EBPβ. Overexpression of CHOP-10 by ALLN [16] prevents the gain of DNA-binding activity by C/EBPβ. Concomitant with acquisition of DNA-binding activity, C/EBPβ localizes to centromeres by binding to the multiple consensus C/EBP-binding sites in centromeric satellite DNA [13]. It has been reported that lactacystin can induce CHOP-10 expression in another cell line [18].

In this report, we found that lactacystin, a proteasome inhibitor, up-regulated CHOP-10 expression, blocked acquisition of DNA-binding activity of C/EBPβ, and subsequently prevented MCE as well as adipocyte differentiation during 3T3-L1 adipocyte differentiation program.

Materials and methods

Cell culture and induction of adipocyte differentiation. The 3T3-L1 preadipocytes were maintained and propagated in DMEM containing 10% (vol/vol) calf serum. To induce differentiation, 2-day postconfluent 3T3-L1 preadipocytes (designated day 0) were fed DMEM containing 10% FBS, 1 μg/ml insulin (I), 1 μM dexamethasone (D), and 0.5 mM 3-isobutyl-1-methylxanthine (M) until day 2. Cells were then fed DMEM supplemented with 10% FBS and 1 μg/ml insulin for 2 days, after which they were fed every other day with DMEM containing 10% FBS. Adipocyte gene expression and acquisition of the adipocyte phenotype begin on day 3 and reach maximal by day 8.

Electrophoretic mobility shift assay (EMSA). Postconfluent growth-arrested 3T3-L1 preadipocytes (day 0) were induced to differentiation and treated with/out lactacystin (10 μM), nuclear extracts were prepared by NUN method as described [19] at the times indicated. Protein concentration was determined by Bradford method (Bio-Rad). C/EBP-binding site [9] in C/EBPα gene promoter was labeled with [γ-^{32}P]ATP. EMSA was performed as follow: ~0.25 ng ^{32}P-labeled probe, 10 μg nuclear extracts, and 2 μg poly[d(I–C)] in 30 μl buffer containing 100 mM NaCl, 0.3 M urea, 0.3% Nonidet P-40, 10 mM Hepes (pH 7.9), and 0.1 mM EDTA incubated for 15 min on ice and at room temperature for another 15 min; the mixture was then separated electrophoretically on 5% polyacrylamide gel. For the competition assays, 100-fold excess of unlabeled competitor was added before the reaction, for super-shift experiment 1 μl (~5 μg of IgG) C/EBPβ antibody was added before the probe was added. The sequence of the C/EBP-binding site in C/EBPα gene promoter is (−191)GCG TTG CGCCAC GAT CTC TC(−172) [9].

Analysis of CHOP-10 mRNA level by real-time PCR. Day 0 preadipocytes were induced to differentiate with/out lactacystin as described above. RNA was extracted with TRIzol reagent (Invitrogen) at times indicated according to the manufacturer's instructions. For real-time PCR, equal amount of RNA was reverse-transcribed and the fragment between 172 and 244 bp of CHOP-10 cDNA was amplified with primers designed by software PrimerExpress (Biosystem). The sequences of primers are 5′GTCCCTAGCTTGGCTGACAGA3′ and 5′TGGAG AGCGAGGGCTTTG3′. At the same time a fragment between 1151 and 1222 bp of GAPDH cDNA was amplified and used as loading control.

Immunoblotting. Postconfluent growth-arrested 3T3-L1 preadipocytes were induced to differentiation in the presence or absence of lactacystin (10 μM). At various time points, cell monolayers (6-cm dishes) were washed once with cold PBS (pH 7.4) and then scraped into lysis buffer (1% SDS, 60 mM Tris–Cl, pH 6.8). Lysates were heated at 100 °C for 10 min and clarified by centrifugation, and equal amount of protein was separated by SDS–PAGE. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes and immunoblotted with antibodies to CHOP-10, C/EBPβ, C/EBPα, PPARγ, 422/aP2, and cdk2 (antibodies to C/EBPβ, C/EBPα, and 422/aP2 were provided by Dr. M. Daniel Lane, Department of Biological Chemistry at Johns Hopkins University School of Medicine. Antibodies to CHOP-10, PPARγ, and cdk2 were purchased from Santa Cruz Biotechnology, Inc.).

Immunofluorescence microscopy. 3T3-L1 preadipocytes were plated onto coverslips in 3.5-cm dishes, grown to 2-day postconfluence, and then induced to differentiate with/out lactacystin (10 μM) as described above. At 4 and 24 h after induction, cells were washed with cold PBS and fixed for 20 min with 4% formaldehyde at room temperature. Cells were then

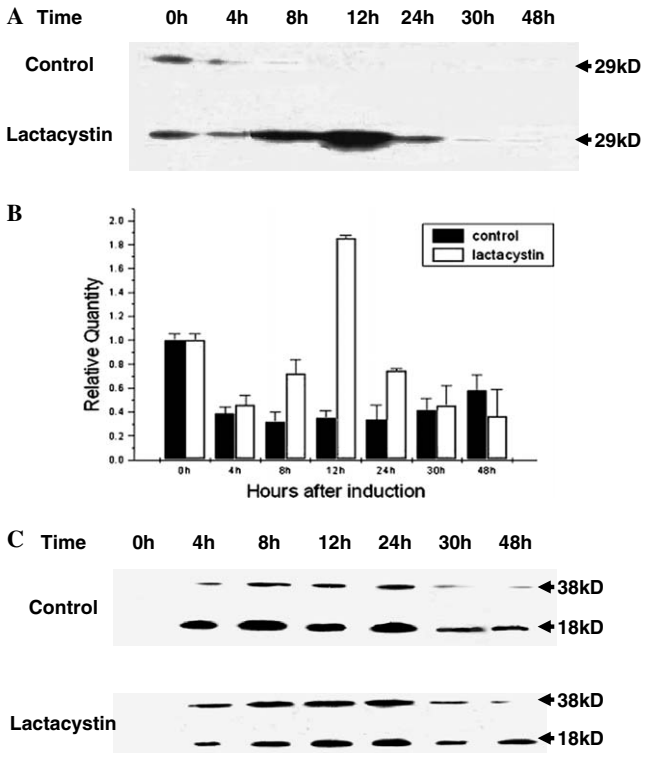

Fig. 1. Lactacystin induces CHOP-10 expression without effects on the expression of C/EBPβ during 3T3-L1 adipocyte differentiation. Postconfluent growth-arrested 3T3-L1 preadipocytes were induced to differentiation with/out lactacystin (10 μM), whole-cell extracts were prepared at the times indicated, equal amount protein was separated by SDS–PAGE, immunoblotted with anti-CHOP-10 (A) and anti-C/EBPβ (C) antibodies. Total RNA was also isolated, change of CHOP-10 mRNA was analyzed by quantitative real-time PCR (B).

permeabilized for 30 min with 0.075% Triton X-100 in 2 mg/ml BSA (in PBS) and blocked with 2 mg/ml BSA in PBS for 1–2 h at room temperature. Cells were incubated with C/EBPβ antibody (1:200 dilution in 2 mg/ml BSA) for 1–2 h and incubated for 1 h with FITC-labeled secondary antibody in the same buffer with 0.1 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI). After each step cells were washed with PBS three times. Antifade solution was added to coverslips and mounted on slides [13]. FITC-labeled C/EBPβ and DAPI-stained centromere were examined by microscope and photographed.

FACS analysis. 3T3-L1 preadipocytes were induced to differentiation as described above. At the times indicated, cells were washed with PBS, trypsinized, and fixed with 70% ethyl alcohol (vol/vol). After staining with 0.1 mg/ml propidium iodide, DNA content was determined by FACS analysis.

Oil red O staining. 3T3-L1 preadipocytes were induced to differentiation as described above, at day 8 differentiated adipocytes were washed three times with PBS and then fixed for 2 min with 3.7% formaldehyde. Oil red O (0.5% in isopropanol) was diluted with water (3:2), filtered through a 0.45 μm filter, and incubated with the fixed cells for 1 h at room temperature. Cells were washed with water, and the stained fat droplets in the adipocytes were visualized by light microscopy and photographed.

Results

Induction of CHOP-10 expression by lactacystin during 3T3-L1 adipocyte differentiation

To investigate whether lactacystin can also induce the expression of CHOP-10 during 3T3-L1 adipocyte differentiation, preadipocytes were induced to differentiate in the presence or absence of lactacystin, the expression of CHOP-10 was detected by Western blotting. Consistent with what we have found before [16], there is a significant amount of CHOP-10 expressed by growth-arrested preadipocytes, and it was down-regulated after preadipocytes exposed to differentiation inducers (Fig. 1A, top panel), our previous findings indicated that this is due to the change from calf serum to fetal bovine serum [17]. However, treatment with lactacystin markedly up-regulated CHOP-10 expression even in the presence of fetal bovine serum and differentiation inducers (Fig. 1A, bottom panel). Quantitative real-time RT-PCR analysis further confirmed that up-regulation of CHOP-10 by lactacystin happened in transcription level (Fig. 1B). It should be also noted that the expression of C/EBPβ is not affected by lactacystin (Fig. 1C). These findings indicate that lactacystin has similar effect of ALLN [16] on CHOP-10 expression during 3T3-L1 adipocyte differentiation.

Up-regulation of CHOP-10 by lactacystin inhibits DNA-binding activity of C/EBPβ

CHOP-10 is the dominant-negative form of C/EBP family members, heterodimers between CHOP-10 and C/EBP family members (i.e., C/EBPβ) cannot bind to C/EBP

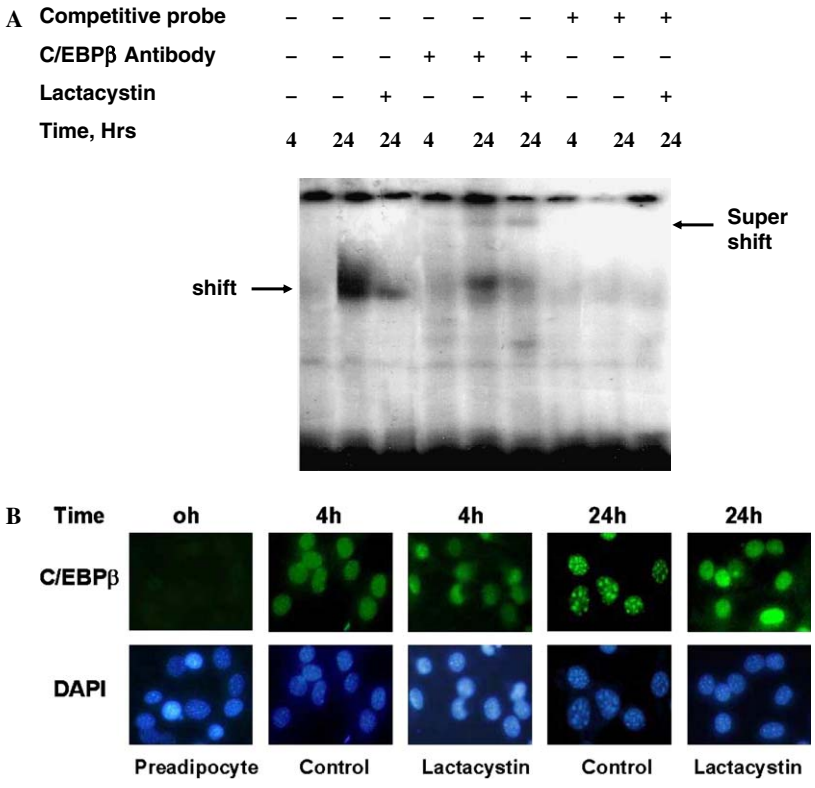

Fig. 2. Inhibition of DNA-binding activity of C/EBPβ by lactacystin treatment in the early stage of 3T3-L1 adipocyte differentiation. (A) Day 0 postconfluent 3T3-L1 preadipocytes were induced to differentiation using the standard differentiation protocol in the presence or absence of lactacystin. Nuclear extracts were prepared at 4, and 24 h, EMSA was performed with a ^{32}P-labeled oligonucleotide corresponding to the C/EBP regulatory element in the C/EBPα gene promoter. (B) Preadipocytes were propagated and induced to differentiation on coverslips in the absence or presence of lactacystin (10 μM), at 4 or 24 h immunofluorescence analysis was conducted with anti-C/EBPβ antibody and FITC-labeled secondary antibody.

consensus sequence. Lactacystin can induce CHOP-10 expression but has no effect on the expression of C/EBPβ (Fig. 1), whether lactacystin treatment prevents acquisition of DNA-binding activity by C/EBPβ was examined (Fig. 2A). Consistent with our previous findings [13], maximal expression of both the 38- and 18-kDa isoforms of C/EBPβ is achieved at 4 h and persists beyond 24 h (Fig. 1C), C/EBPβ fails to acquire DNA-binding activity (Fig. 2A), and does not become centromere associated at 4 h (Fig. 2B), however, C/EBPβ gains significant DNA-binding activity (Fig. 2A) and becomes centromere associated (as indicated by punctuate staining, Fig. 2B) at 24 h. Previous study has already shown that the gain of DNA-binding activity by C/EBPβ correlates with the down-regulation of CHOP-10 (Fig. 1A and Ref. [16]) and failure to gain DNA-binding activity of C/EBPβ early during adipocyte differentiation is at least partially due to the heterodimerization with CHOP-10. Consistent with these findings that induction of CHOP-10 expression by ALLN can prevent the DNA-binding activity of C/EBPβ, we found that treatment with lactacystin could also induce CHOP-10 expression (Fig. 1A and B) and block the DNA-binding activity of C/EBPβ as indicated by EMSA (Fig. 2A) and centromere association as indicated by punctuate staining (Fig. 2B).

Lactacystin treatment blocks mitotic clonal expansion as well as adipogenesis during 3T3-L1 adipocyte differentiation

C/EBPβ has been shown to be required for mitotic clonal expansion during 3T3-L1 adipocyte differentiation [20] and mitosis for other cells (hepatocytes) [21,22]. Lactacystin treatment can induce CHOP-10 expression (Fig. 1A) and prevent the DNA-binding activity of C/EBPβ (Fig. 2), question is raised whether lactacystin treatment can also affect the MCE. Our previous findings indicate that there is a special expression pattern of cdk2 in the MCE and specific inhibitor of cdk2 (Roscovitine) can block the MCE. We found that lactacystin treatment can totally block the induction of cdk2 expression (Fig. 3A) that normally happens during 3T3-L1 adipocyte differentiation, and MCE is almost totally prevented as indicated by both FACS analysis (Fig. 3B) and cell count (Fig. 3C).

Both C/EBPβ function and MCE are required for 3T3-L1 adipocyte differentiation [20], lactacystin treatment blocks DNA-binding activity of C/EBPβ and prevents MCE, whether lactacystin treatment inhibits adipocyte differentiation was also examined. As shown in Fig. 4, lactacystin almost totally blocked adipocyte differentiation as indicated by failure to accumulate cytoplasmic triglyceride (indicated by oil red O staining, Fig. 4A) as well as the expression of C/EBPα, PPARγ, and 422/aP2 which are adipocyte-specific markers [23] (Fig. 4B).

Discussion

Upon exposure to differentiation inducers, growth-arrested 3T3-L1 preadipocytes synchronously reenter the cell cycle and undergo mitotic clonal expansion (MCE), a process required for completion of the 3T3-L1 adipocyte differentiation program [6,7]. C/EBPβ, a transcriptional activator of the C/EBPα and PPARγ genes [8–11], is rapid-

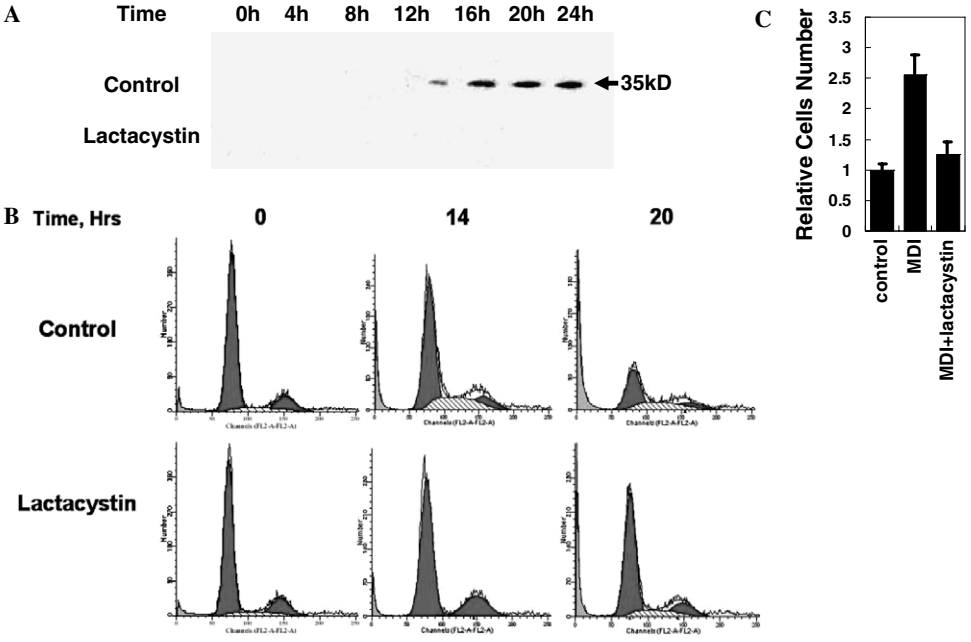

Fig. 3. Blocking the cdk2 expression and MCE by lactacystin treatment during 3T3-L1 adipocyte differentiation. Postconfluent 3T3-L1 preadipocytes were induced to differentiation with the standard differentiation protocol in the presence or absence of lactacystin (10 μM). (A) At times indicated, cell extracts were prepared, protein separated by SDS–PAGE and immunoblotted with anti-cdk2 antibody. (B) At the indicated times after induction, the cells were harvested and fixed, changes of DNA content were analyzed by FACS. (C) Four days after induction of differentiation, cells were counted and plotted.

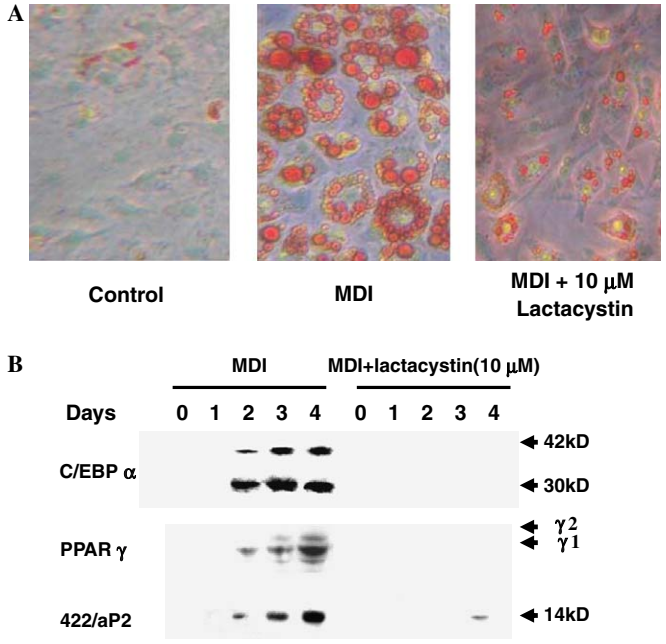

Fig. 4. Inhibition of adipocyte differentiation by lactacystin. Day 0 3T3-L1 preadipocytes were induced to differentiation with/out lactacystin (10 μM). (A) At day 8, cells were fixed and stained with oil red O, and cytoplasmic triglyceride accumulation was examined with microscope and photographed. (B) Whole cell extracts were prepared at times indicated, equal amount protein was separated by SDS–PAGE and subjected to immunoblotting with anti-C/EBPα, -PPARγ, and -422/aP2 antibodies.

ly expressed and maintained at high levels throughout MCE [12,13]. However, acquisition of DNA-binding activity by C/EBPβ is delayed until preadipocytes synchronously pass the G_1/S boundary of MCE and expression of C/EBPα is delayed until the preadipocytes exit mitotic clonal expansion. C/EBPβ has been shown to be required for both MCE and terminal adipocyte differentiation in the 3T3-L1 adipocyte differentiation program. Thus, overexpression of dominant-negative form of C/EBPβ (i.e., A-C/EBP [24]) in 3T3-L1 preadipocytes blocks both MCE and terminal adipocyte differentiation. Another evidence is from the experiment with mouse embryonic fibroblasts (MEFs): with the same differentiation inducers, ~15% of wild-type MEFs will undergo both MCE and terminal adipocyte differentiation, but almost none of C/EBPβ(−/−) MEFs can undergo MCE and terminal adipocyte differentiation, and overexpression of active form of C/EBPβ(LAP) can rescue both MCE and adipocyte differentiation in C/EBPβ(−/−) MEFs [20].

Previous findings in this group indicate that C/EBPβ is associated with CHOP-10, a dominant-negative form of C/EBP family members, and in a hypophosphorylation state when it is induced, when preadipocytes synchronously pass the G_1/S boundary of MCE, CHOP-10 is down-regulated and C/EBPβ is released, becomes hyperphosphorylated and activated. ALLN, a calpain inhibitor, can induce CHOP-10 expression and block the function of C/EBPβ [16]. In the present investigation, we found that lactacystin, a proteasome inhibitor, has the similar effect of ALLN: it can induce CHOP-10 expression but without any effect on the expression of C/EBPβ (Fig. 1C), the induction of CHOP-10 expression is through transcription level (Fig. 1C). Consistent with the fact that CHOP-10 is a dominant-negative form of C/EBP family members (i.e., C/EBPβ), treatment with lactacystin prevented the acquisition of DNA-binding activity as indicated by both EMSA (Fig. 2A), and punctuate staining associated with centromere (Fig. 2B). Lactacystin treatment can also prevent the S phase entry evidenced by inhibition of special expression of cdk2 during MCE (Fig. 3A), new DNA synthesis (Fig. 3B) and as a result there is no cell number increasing (Fig. 3C). Lactacystin treatment markedly inhibits 3T3-L1 adipocyte differentiation as evidenced by fat droplet accumulation (Fig. 4A) as well as the expression of adipocyte gene expression (C/EBPα, PPARγ, and 422/aP2) (Fig. 4B).

Acknowledgments

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