Transcriptional activation of histone H4 by C/EBPβ during the mitotic clonal expansion of 3T3-L1 adipocyte differentiation

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ABSTRACT **CCAAT enhancer binding protein β (C/EBPβ) is required for both mitotic clonal expansion (MCE) and terminal differentiation during the 3T3-L1 adipocyte differentiation program. Whereas the mechanism of C/EBPβ during terminal differentiation is well understood, the mechanism of C/EBPβ in MCE is not. We provide evidence that histone H4, the most conserved cell cycle–related histone, the change of which is strictly correlated with DNA content change during the cell cycle, is transcriptionally activated by C/EBPβ during MCE. Expression of histone H4 is increased at 16 h after induction when 3T3-L1 preadipocytes synchronously reenter S phase, which is correlated with the sequential phosphorylation and activation of C/EBPβ, and expression was partially suppressed when A-C/EBP (dominant negative for C/EBP protein) was overexpressed. One C/EBP-binding site was identified in one of the histone H4 gene promoters (hist4h4), confirmed by both electrophoretic mobility shift assay and chromatin immunoprecipitation assay. C/EBP-binding sites were also found in 9 of 11 other histone H4 promoters, which can also be transactivated by C/EBPβ. Knockdown of C/EBPβ by stealth small interfering RNA partially decreased H4 gene expression and arrested cells in G1 phase as indicated by bromodeoxyuridine incorporation and fluorescence-activated cell sorting analysis of DNA content. This study provides new insights into why C/EBPβ is required for MCE during 3T3-L1 adipocyte differentiation and why C/EBPβ plays important roles in the proliferation of other cell types.**

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INTRODUCTION

When induced to differentiate, growth-arrested 3T3-L1 preadipocytes synchronously reenter the cell cycle, undergo several rounds of division (mitotic clonal expansion [MCE]), and then express genes that produce adipocyte characteristics (Tang and Lane, 1999; Tang *et al.*, 2003a). CCAAT enhancer binding protein β (C/EBPβ) can bind directly to the promoters and activate the expression of CCAAT enhancer binding protein α (C/EBPα) and peroxisome proliferator–activated receptor γ (PPAR γ), transcriptional activators of most adipocyte genes that lead to the adipocyte phenotype (Christy *et al.*, 1991; Zhu *et al.*, 1995; Fajas *et al.*, 1997; Tang *et al.*, 2003b). Previous studies have indicated that C/EBPβ is potentially required for MCE, which is the necessary step for the terminal differentiation of 3T3-L1 preadipocytes (Tang *et al.*, 2003b; Zhang *et al.*, 2004b). MCE is blocked in C/EBPβ (−/−) mouse embryonic fibroblasts (MEFs), whereas wild-type MEFs can undergo both MCE and adipocyte differentiation upon induction with standard differentiation protocol (Tang *et al.*, 2003b); with overexpression of A-C/EBP, the dominant-negative form of C/EBP protein, which lacks functional DNA-binding and transactivation domains but can form stable inactive heterodimer with C/EBPβ (Greenwel *et al.*, 2000; Zhang *et al.*, 2004a), the S phase entry of MCE is almost totally blocked during the 3T3-L1 adipocyte differentiation program (Zhang *et al.*, 2004b). C/EBPβ also plays important roles in the proliferation of other cell types, such as

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Abbreviations used: C/EBPα, CCAAT enhancer binding protein α; C/EBPβ, CCAAT enhancer binding protein β; EMSA, electrophoretic mobility shift assay; MCE, mitotic clonal expansion; MEF, mouse embryonic fibroblasts; PPAR γ, peroxisome proliferator–activated receptor γ.

lobuloalveolar secretory cells (Seagroves *et al.*, 1998), epithelial tumor cells (Zhu *et al.*, 2002), hepatocytes (Trautwein *et al.*, 1996; Greenbaum *et al.*, 1998; Buck *et al.*, 1999), and mammary gland development (Robinson *et al.*, 1998). Although it has been reported that C/EBPβ can activate the promoter of cyclin D1 (Eaton *et al.*, 2001) and that C/EBPβ can also induce the expression of the cyclin-dependent kinase inhibitor p57 (Hirata *et al.*, 2009), the mechanism of its roles in the promotion of G1/S phase transition is not clearly understood.

The critical event for MCE during 3T3-L1 adipocyte differentiation is the transition from G1 phase to S phase. In this transition, histone replication is very important, as is the DNA replication during S phase (Borun *et al.*, 1972). Histone gene transcription occurs constitutively throughout the cell cycle but is up-regulated upon entry of S phase and down-regulated in quiescent cells or at the onset of terminal differentiation (Stein *et al.*, 2006). The changes in histone gene expression occurred concomitantly with modulation of their transcription factors (Pauli *et al.*, 1987). Histone H4, the most highly conserved and strictly cell cycle–regulated nucleosomal protein, has been extensively studied. Both mRNA stability and the transcription rate are involved in histone H4 gene regulation in mammalian cells (Sittman *et al.*, 1983; Heintz, 1991). H4 gene promoter is regulated by four principal protein–DNA interacting domains: two multipartite proximal promoter elements (sites I and II) and with two distal auxiliary domains (sites III and IV). The major cell cycle control proteins that regulate these four domains in histone H4 gene promoters were reported to contain SP1 (Birnbaum *et al.*, 1995), IRF-2 (Aziz *et al.*, 1998a; Vaughan *et al.*, 1998), CDP-cut/HiNF-D (Aziz *et al.*, 1998a, 1998b), HiNF-P (Aziz *et al.*, 1998; Miele *et al.*, 2005, 2007), and HiNF-M (Birnbaum *et al.*, 1995; Aziz *et al.*, 1998; Vaughan *et al.*, 1998).

Our previous findings indicated that C/EBPβ was induced almost immediately after growth-arrested 3T3-L1 preadipocytes were exposed to differentiation inducers. Furthermore, C/EBPβ undergoes sequentially phosphorylation and then becomes activated (Tang *et al.*, 2005). The sequential phosphorylation and activation occurs on the G1/S boundary, which is correlated with histone gene expression, as well as with DNA replication. In this article, we find that C/EBPβ can directly bind to the histone H4 promoters; 10 of 12 mouse histone H4 promoters have potential C/EBP-binding sites and can be transactivated by C/EBPβ. Knockdown of C/EBPβ expression partially decreased the induction of histone H4 expression and subsequently partially blocked MCE.

RESULTS

Inhibition of C/EBPβ by A-C/EBP reduced histone H4 expression and mitotic clonal expansion during 3T3-L1 adipocyte differentiation

After the induction of differentiation, growth-arrested 3T3-L1 preadipocytes synchronously reentered the cell cycle, underwent several rounds of MCE, and then underwent terminal adipocyte differentiation. Preadipocytes traversed the G1/S check point at ~16 h after induction, S phase reached maximum at ~20 h, and the first round cell division finished at ~28 h; this was evidenced both by the change in the amount of DNA (Supplemental Figure S1) and the expression of histone H4 (Figure 1A), which was consistent with a previous report (Tang *et al.*, 2003a).

To define the role of histone H4 induction during MCE, the expression of histone H4 was knocked down by specific RNAi. The knockdown effect was confirmed by quantitative real-time PCR (Figure 1B). Although 3T3-L1 preadipocytes treated with histone H4 small interfering RNA (siRNA) grew slowly than those treated with negative control RNAi, they could still reach postconfluence. When treated with histone H4 siRNA, more cells remained in G0/G1 phase and less cells transited to S phase, based on comparison of cells treated with negative control RNAi (Figure 1E). This indicated that the induction of histone H4 expression is required for MCE. We also found that knockdown of histone H4 expression partially suppressed the terminal differentiation of 3T3-L1 as indicated by oil red O staining of fat droplet accumulation as well as adipocyte gene expression (Figure 1, C and D).

C/EBPβ was previously found to be potentially required for MCE of 3T3-L1 adipocyte differentiation (Tang *et al.*, 2003b; Zhang *et al.*, 2004b). We then explored whether C/EBPβ regulated MCE through histone H4. A-C/EBP, the dominant-negative form of C/EBP protein, which can form a stable inactive heterodimer with C/EBPβ, was overexpressed in 3T3-L1 cells. It has been reported that A-CEBP repressed MCE as well as adipocyte differentiation (Zhang *et al.*, 2004b). Similar experiments were repeated here, and we found that overexpression of A-C/EBP could also partially reduce the induction of histone H4 expression, which indicated that the expression of histone H4 might be regulated by C/EBPβ (Figure 2, A and B). The proportion of cells entering S phase significantly decreased (Figure 2C), whereas more cells stayed in G1 phase (Figure 2D) when A-CEBP was overexpressed.

C/EBP-binding site was localized between −167 and −87 on the promoter of Hist4h4

In mammalian cells all histone genes are clustered in several groups in which more than one promoter decides histone expression (including histone H4). Data search from the PubMed website indicated that at least 12 different promoters regulate the transcription of mouse histone H4, and nothing has been reported about which promoters are more important in determining histone H4 expression during the cell cycle.

Hist4h4, the only histone H4 in histone cluster 4, was chosen to study the regulation of histones by C/EBPβ during MCE in the following experiments. From −1105 to +1 of hist4h4 promoter was amplified and cloned into pGL3.0 basic plasmid, and the cotransfection experiments indicated that forced expression of C/EBPβ can significantly transactivate the expression of hist4h4 (Figure 3A). Serial truncation assay indicated that the C/EBP response element was located between −167 and −87; the transactivation of hist4h4 by C/EBPβ did not change significantly when the promoter was longer than 167 base pairs, whereas the transactivation dramatically decreased to nearly the basal level when the promoter was truncated to less than 87 base pairs, as indicated in Figure 3C.

One specific C/EBP-binding site was identified in the hist4h4 promoter

To identify the C/EBP-binding site in the hist4h4 promoter, electrophoretic mobility shift assay (EMSA) was performed with the probe comprising base pairs −167 to −87 (Figure 4A, WT(a)) in the hist4h4 promoter. Two bands were detected. The lower band was significantly supershifted by C/EBPβ antibody but not by nonspecific immunoglobulin G (IgG) (Figure 4B), which indicated that there is a C/EBP-binding site in this 81–base pair probe. Aligning with the classic C/EBP-binding consensus reported before, we found that the region −125 to −117 of hist4h4 was similar to the C/EBP consensus sequence (Figure 4A). Probe Mut(a) was designed with the potential conserved TGG → AAA as underlined and italicized in Figure 4A. Competition experiments indicated that excess unlabeled WT(a) could easily compete with both of the two bands; however, unlabeled Mut(a) with the potential C/EBP-binding site

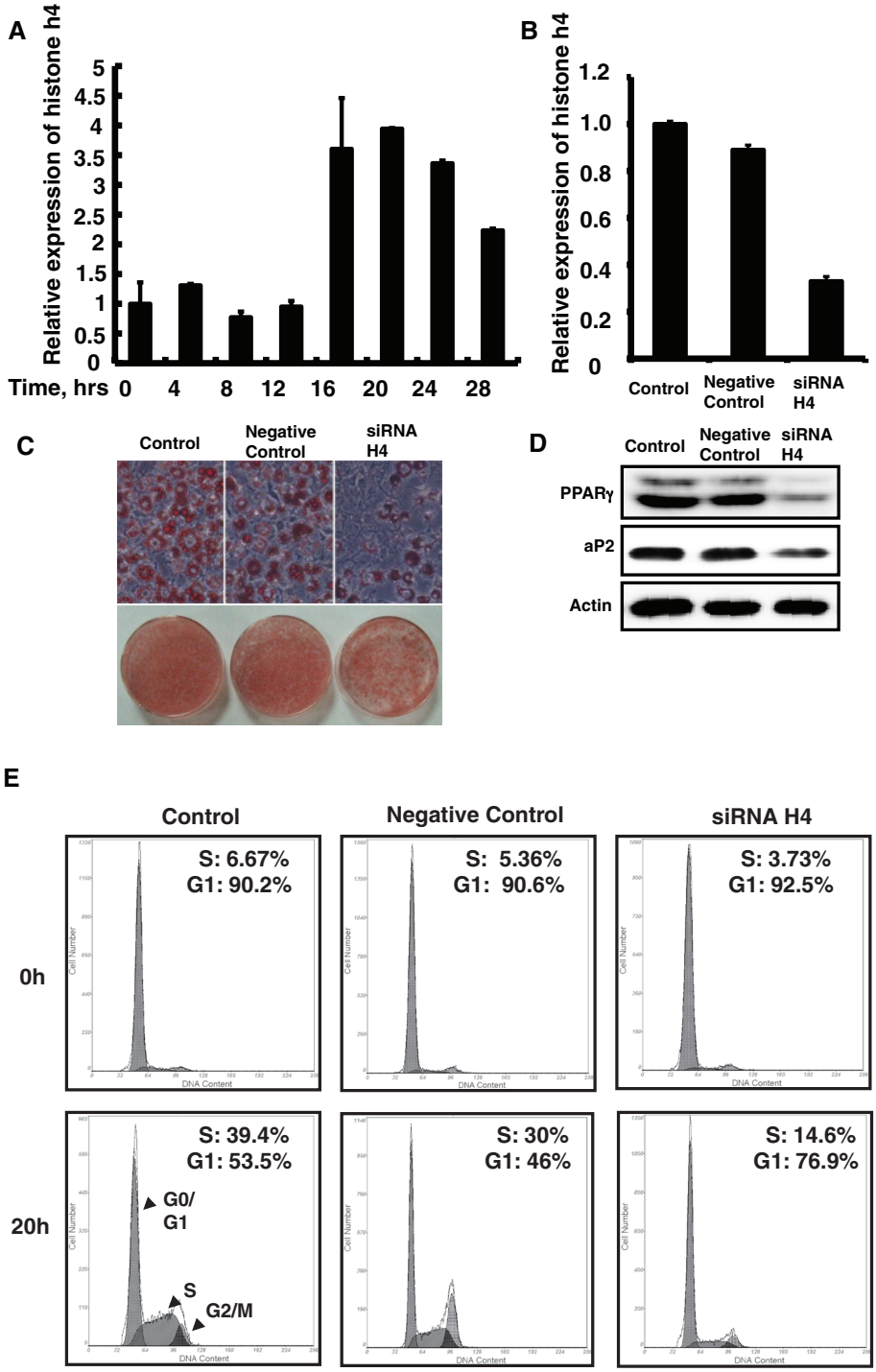

FIGURE 1: The induction of histone H4 expression was required for mitotic clonal expansion and terminal differentiation of 3T3-L1 preadipocytes. (A) Postconfluent 3T3-L1 preadipocytes were induced to differentiate as described, total RNA was isolated at different time points, and quantitative real-time RT-PCR was performed to detect histone H4 expression. (B–E) 3T3-L1 preadipocytes were transfected with histone H4 siRNA, and 24 h after reaching confluence, cells were induced to differentiate as described. Total RNA was isolated at 20 h after induction, the expression of total H4 was detected by real-time RT-PCR, and 18S rRNA was used as loading control (B). After 8 d of induction, fat droplet accumulation was detected by oil red O staining and photographed (C), and the expression of PPAR γ and 422/aP2 was detected by Western blotting; actin was used as loading control (D). DNA content was analyzed by fluorescence-activated cell sorting, and the percentage of cells in S phase or G0/G1 phase was calculated at 0- and 20-h time points (E).

mutated could compete with the upper nonspecific band, while the lower band was very hard to compete away (Figure 4C). To further identify the C/EBP-binding site, a probe WT(b) was designed that only comprised the region −135 to −106 of the hist4h4 promoter, whereas the mutant probe Mut(b) had the same mutation TGG → AAA as Mut(a) as indicated in Figure 4A. Only one band was detected by EMSA with labeled WT(b), and this band can be competed away by unlabeled WT(a) and WT(b) as well as the C/EBP-binding site (Figure 4A, c) in the C/EBPα promoter, but not by Mut(b) (Figure 4D), and the labeled WT(b) could form a specific DNA–protein complex, whereas labeled Mut(b) could not with the same amount of nuclear extract (Figure 4E).

Chromatin immunoprecipitation combined with quantitative PCR (ChIP-qPCR) was performed to further confirm that C/EBPβ could specifically be recruited to hist4h4 promoter before 3T3-L1 preadipocytes synchronously reenter the S phase of MCE (Figure 5A), and the cotransfection experiments indicated that the transactivation by C/EBPβ was significantly decreased (although not totally), whereas the C/EBP-binding site identified earlier was mutated (Figure 5B).

Further analysis indicated that there was more than one potential C/EBP-binding site in the hist4h4 promoter (Figure 6A), and more than one C/EBP-binding site was found in almost all 12 mouse histone H4 promoters as far upstream as 1000 base pairs starting from the transcription start point (Figure 6A). Cotransfection assays indicated that at least 10 of the 12 histone H4 promoters could be significantly transactivated by C/EBPβ (Figure 6B).

Knockdown of the expression of C/EBPβ reduced histone H4 expression and partially blocked mitotic clonal expansion, whereas overexpression of C/EBPβ promoted the expression of histone H4 and the terminal differentiation of 3T3-L1 cells

To clearly define the role of C/EBPβ in MCE during the 3T3-L1 adipocyte differentiation program, the expression of C/EBPβ was knocked down by stealth RNAi (Figure 7, A and E). Down-regulation of C/EBPβ reduced the expression of histone H4 both in mRNA level (Figure 7B) and in protein level at 20 h (Figure 7E). Consequently, the proportion of cells entering S phase significantly decreased when C/EBPβ was knocked down (Figure 7C), whereas more cells stayed in G1

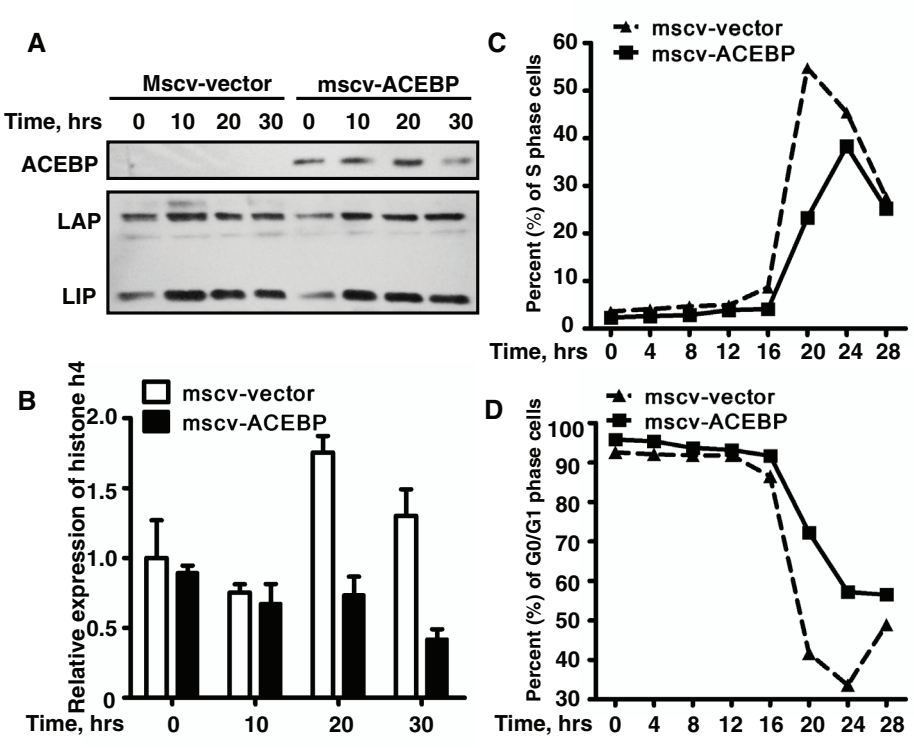

FIGURE 2: Overexpression of A-C/EBP partially suppressed the induction of histone H4 expression during MCE of 3T3-L1 adipocyte differentiation. (A, B) 3T3-L1 preadipocytes were infected with retrovirus expression flag–tagged A-C/EBP or with empty vector, cells were induced with differentiation media 2 d after reaching confluence, and the expression of A-C/EBP and C/EBPβ was detected by Western blotting (A); the effect of A-C/EBP on the expression of histone H4 was detected by quantitative real time RT-PCR as indicated in B. (C, D) DNA content was analyzed by fluorescence-activated cell sorting, and the percentage of cells in S phase or G0/G1 phase was analyzed and plotted.

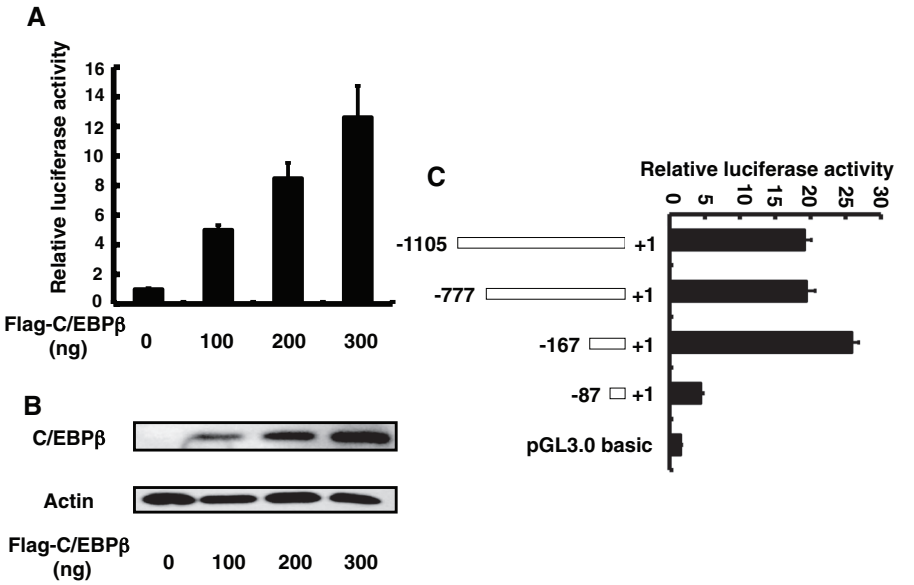

FIGURE 3: The C/EBP response element was localized between −167 and −87 of the hist4h4 promoter. (A) and (B) 3T3-L1 preadipocytes were transiently transfected with hist4h4 promoter reporter construct, along with different amounts of C/EBPβ expression vector, and pRL-TK plasmid was used as internal control; cell extracts were prepared, and luciferase activities were measured and normalized to *Renilla* activity. (C) 3T3-L1 preadipocytes were transiently transfected with reporter constructs having different truncated hist4h4 promoter, together with C/EBPβ expression vector (300 ng). pRL-TK plasmid was used as internal control, and 48 h later, cell extracts were prepared and luciferase activity was measured and normalized to *Renilla* activity. Data are presented as means ± SD of at least three independent experiments.

phase (Figure 7D), and bromodeoxyuridine (BrdU) labeling further confirmed the results (Figure 7F). Consistent with previous findings, we found that knockdown of C/EBPβ expression partially suppressed 3T3-L1 adipocyte differentiation, as indicated by oil red O staining of fat droplet accumulation (Figure 7G), as well as adipocyte gene expression (Figure 7H).

To further confirm the role of C/EBPβ in the induction of histone H4 expression and terminal differentiation of 3T3-L1 preadipocytes, C/EBPβ was overexpressed in 3T3-L1 preadipocytes. We found that overexpression of C/EBPβ promoted the induction of histone H4 expression (Figure 8B) and accelerated adipocyte differentiation, as indicated by oil red O staining of fat droplet accumulation (Figure 8C), as well as adipocyte gene expression (Figure 8A).

DISCUSSION

C/EBPβ plays an important role in the proliferation of several cell types, including MCE during 3T3-L1 adipocyte differentiation. We found that C/EBPβ was involved in MCE during 3T3-L1 preadipocyte differentiation by regulating the expression of histone H4 at the transcription level by directly binding to the C/EBP element of histone H4 promoter. We found the following: 1) Knockdown of histone H4 expression repressed the MCE and terminal differentiation of 3T3-L1 preadipocytes, and overexpression of A-C/EBP reduced the induction of histone H4 expression, as well as MCE (Figures 1 and 2). 2) One specific C/EBP-binding site was identified in the region –125 to –117 in the hist4h4 promoter by EMSA and ChIP assays (Figures 3–5), whereas 10 of 12 mouse histone H4 promoters have more than one potential C/EBP-binding site and can be transactivated by C/EBPβ (Figure 6). 3) Knockdown of the expression of C/EBPβ by specific stealth RNAi decreases histone H4 induction and partially blocks MCE as well as adipocyte differentiation of 3T3-L1 preadipocytes (Figure 7), whereas overexpression of C/EBPβ activates histone H4 expression (Figure 8).

The cell cycle is regulated by sequential interactions between cyclins and cyclin-dependent kinases (CDKs) (Tang and Lane, 1999), and cyclin A or E /CDK2 complex at the R-point is the initiator of the G1/S phase boundary (D'Urso *et al.*, 1990; Cardoso *et al.*, 1993; Tang and Lane, 1999). Evidence has been provided that activated CDK2 regulates both DNA replication and histone biosynthesis (Stein *et al.*, 2006). When stimulated by growth factors, activated CDK2 activates the transcription factor E2F by

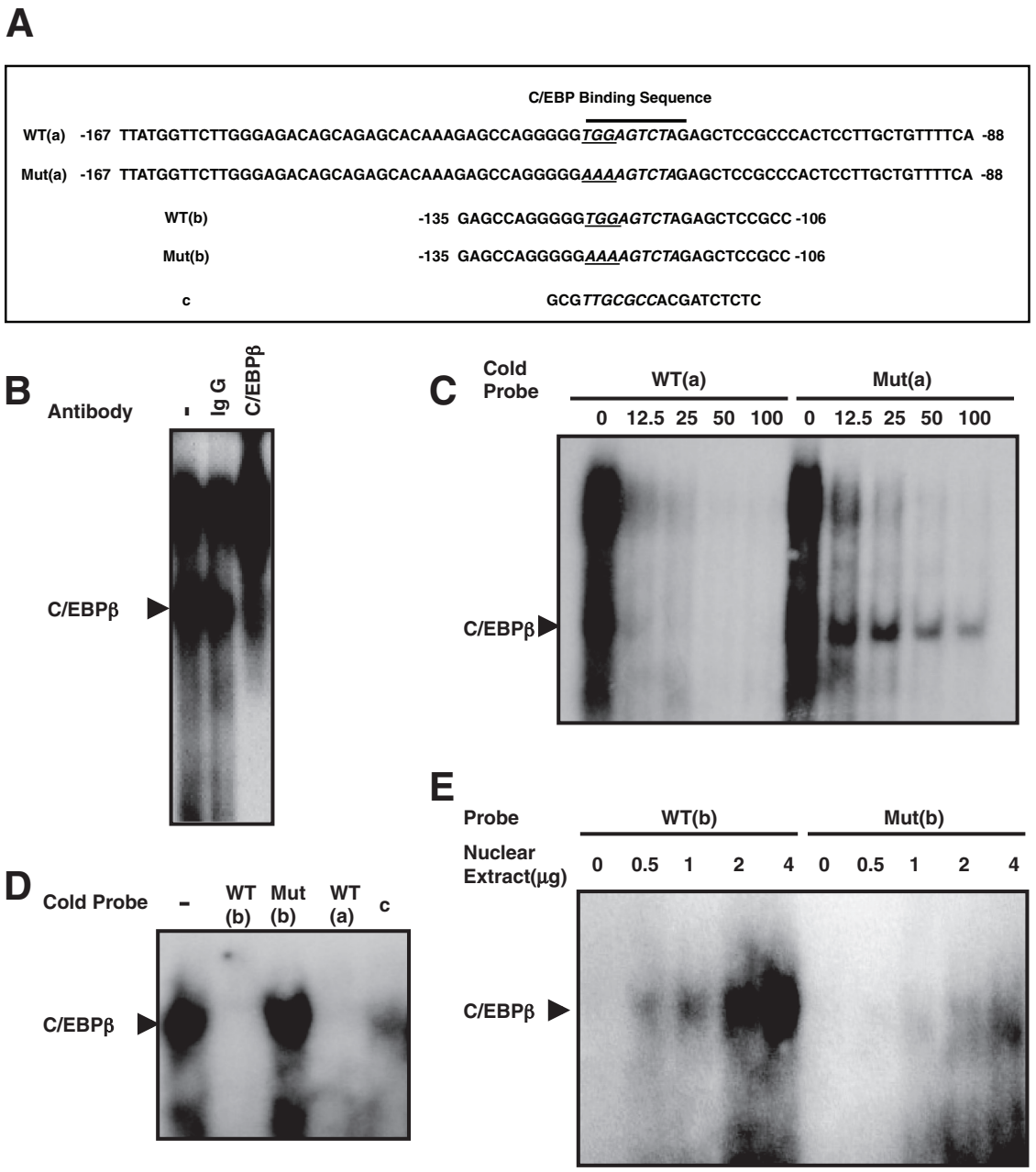

FIGURE 4: One specific C/EBP-binding site was identified in the hist4h4 promoter. (A) The sequence of five oligonucleotides was presented for following EMSA. Postconfluent 3T3-L1 preadipocytes were induced to differentiate, and nuclear extract was prepared at 20 h. (B) EMSA was performed with probe WT(a); the lower band in lane 1 can be supershifted with specific C/EBPβ antibody in lane 3 but not by nonspecific IgG in lane 2. (C) Both bands (including the lower C/EBPβ band and the upper nonspecific band) were with equal efficiently competed away by cold oligonucleotide WT(a), whereas the lower C/EBPβ band was much more difficult to compete away than the upper nonspecific band by cold mutant oligonucleotide Mut(a), in which the C/EBP-binding site was mutated. (D) WT(b) was used as probe, and in lanes 2–5, 100-fold of cold probe WT(b), Mut(b), WT(a), and c were used for competition. (E) WT(b) and Mut(b) were used as probes, and EMSA was performed with 0–4 μg of nuclear extract EMSA.

hyperphosphorylation and inactivates pRB, and E2F is a transcription activator for gene expression related to DNA replication; in another way, CDK2 stimulates the function of histone gene expression–related transcription factors, such as SP1 (Birnbaum *et al.*, 1995), IRF-2 (Aziz *et al.*, 1998a; Vaughan *et al.*, 1998), CDP-cut/HiNF-D (Aziz *et al.*, 1998a, 1998b), HiNF-P (Aziz *et al.*, 1998a; Miele *et al.*, 2005, 2007), and HiNF-M (Birnbaum *et al.*, 1995; Aziz *et al.*, 1998b; Vaughan *et al.*, 1998), leading to histone biosynthesis (Aziz *et al.*, 1998a, 1998b; Vaughan *et al.*, 1998; Miele *et al.*, 2005, 2007). Stringent coupling between histone biosynthesis and DNA replication is very important to normal G1/S phase transition. DNA and histone replication should be synchronous, and activated CDK2 is the key regulator (Borun *et al.*, 1972; Dou *et al.*, 1993).

C/EBPβ and C/EBPα play opposite roles in cell proliferation, including liver regeneration, mammary gland development, and MCE during 3T3-L1 preadipocyte differentiation. Whereas

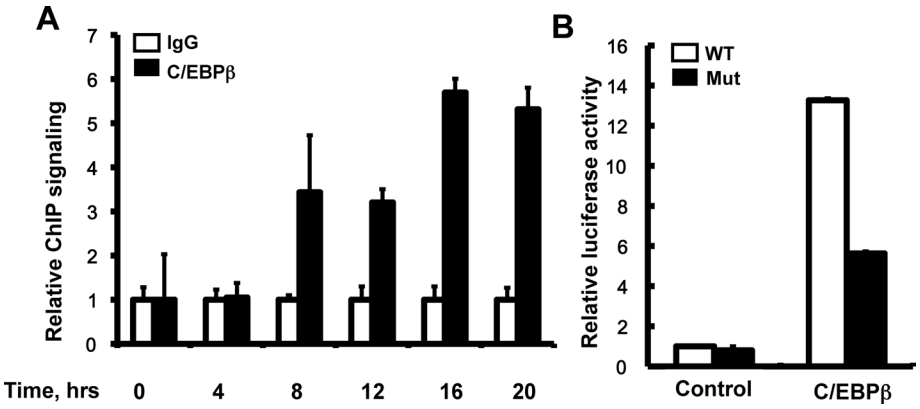

FIGURE 5: Binding of C/EBPβ to the hist4h4 promoter was confirmed by ChIP. (A) Postconfluent 3T3-L1 preadipocytes were induced to differentiate as described, ChIP-qPCR was performed at different time points after induction with anti-C/EBPβ antibody, and nonspecific IgG was used as negative control. Data are normalized to the IgG controls at each time point. An area of the insulin gene served as a negative control. (B) 3T3-L1 preadipocytes were transiently transfected with wt-1105 pGL3.0-hist4h4 promoter plasmid (open bar) and its mutant plasmid (closed bar) in which the C/EBP-binding site was mutated from TTG to AAA, together with pCDNA3.1-C/EBPβ plasmid. Forty-eight hours later, cell extracts were prepared, and luciferase activity was measured and normalized to *Renilla* activity. Data are presented as means ± SD of at least three independent experiments.

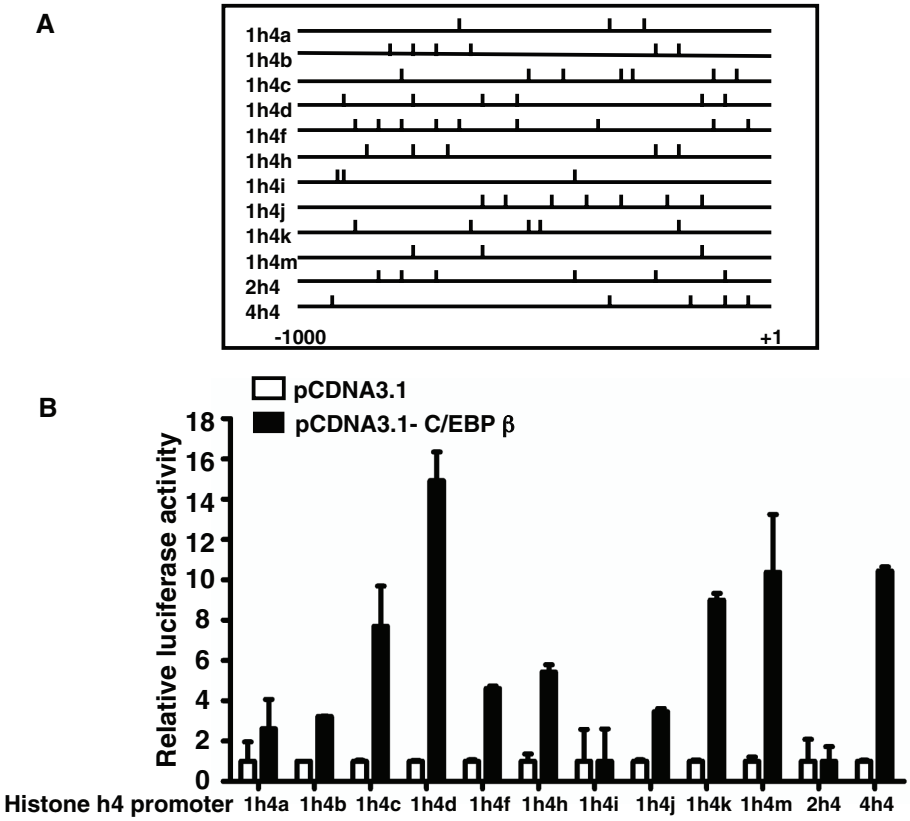

FIGURE 6: Ten of 12 histone H4 promoters could be transactivated by C/EBPβ. (A) Analysis of the potential C/EBP-binding sites and their relative positions in an ~1000–base pair length of all 12 histone H4 promoters, including hist4h4. (B) 3T3-L1 preadipocytes were transfected with pGL3.0-H4 promoter plasmids and pRL-TK plasmid, together with C/EBPβ expression plasmid (black bars) or empty vector (open bars). Forty-eight hours later, cell extracts were prepared, and luciferase activity was measured and normalized to *Renilla* activity. Data are presented as means ± SD of at least three independent experiments.

C/EBPβ is required for cell proliferation, C/EBPα is a strong inhibitor of cell proliferation (Greenbaum *et al.*, 1998; Robinson *et al.*, 1998; Seagroves *et al.*, 1998; Zhang *et al.*, 2004b). C/EBPα is a direct inhibitor of CDK2 by competing for the interaction between cyclin A and CDK2, and the expression of C/EBPα causes cell growth arrest (Wang *et al.*, 2001). Research from our group and Peter Johnson's group indicated that C/EBPβ is a downstream target gene for CDK2. Findings from Johnson's group indicated that phosphorylation of C/EBPβ by CDK2 is required to promote Ras-induced transformation of NIH 3T3 cells. Our previous study indicated that sequential phosphorylation on Thr-188/Ser-184/Thr-179 of C/EBPβ plays an important role in the acquisition of DNA-binding activity, and phosphorylation on Thr-188 by CDK2 is the priming event in the S phase of MCE (Tang *et al.*, 2005; Li *et al.*, 2007). In this article, we found that C/EBPβ is the transcription activator for the histone H4 gene, and CDK2 can regulate histone H4 expression through regulating the phosphorylation and activation of C/EBPβ. This will help in understanding why C/EBPβ is required for proliferation for many cell types.

MATERIALS AND METHODS

Cell culture and induction of differentiation

3T3-L1 preadipocytes were propagated and maintained in DMEM containing 10% (vol/vol) calf serum. Two-day postconfluent (designated day 0) cells were induced to differentiate with DMEM containing 10% (vol/vol) fetal bovine serum (FBS), 1 μg/ml insulin (I), 1 μM dexamethasone (D), and 0.5 mM 3-isobutyl-1-methyl-xanthine (M) until day 2. Cells were then fed with DMEM supplemented with 10% FBS and 1 μg/ml insulin for 2 d, after which they were fed every other day with DMEM containing 10% FBS. Expression of adipocyte genes and acquisition of adipocyte phenotype begins on day 3 and reaches maximum by day 8.

Electrophoretic mobility shift assay

Nuclei were isolated, and nuclear extracts were prepared by using 1× NUN buffer containing 0.3 M NaCl, 1 M urea, 1% Nonidet P-40, 25 mM HEPES (pH 7.9), and 1 mM dithiothreitol (DTT). Protein concentration was determined by the Bradford method (Bio-Rad, Hercules, CA). EMSA was performed essentially as described previously with the following

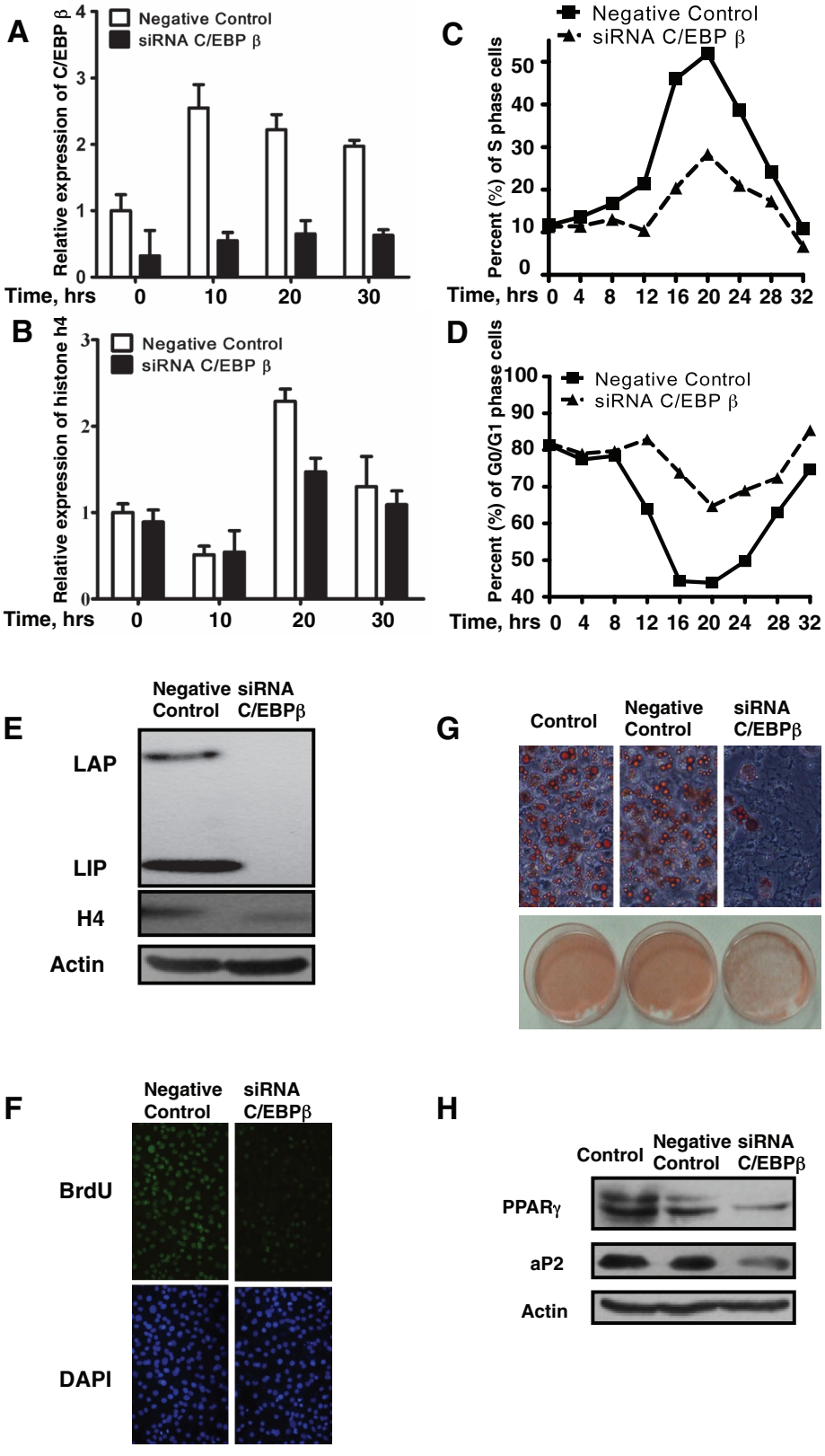

FIGURE 7: Knockdown of the expression of C/EBPβ with siRNA partially blocked the induction of histone H4, MCE, and adipocyte differentiation of 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were transfected with C/EBPβ siRNA; 24 h after reaching confluence, cells were induced to differentiate as described. (A, B) Total RNA was isolated at various time points after induction; the expression of C/EBPβ and histone H4 was detected by real-time RT-PCR, with 18S rRNA used as loading control. (C, D) At different time points the DNA content was analyzed by fluorescence-activated cell sorting, and the percentages of cells in S phase or G0/G1 phase were calculated and plotted. (E) The expression of C/EBPβ and histone H4 was detected by Western blotting. (F) At 18 h after induction, a BrdU labeling experiment was performed and visualized with fluorescence microscopy. (G, H) After 8 d of induction, fat droplet accumulation was detected by oil red O staining, and the expression of PPAR γ and 422/aP2 was detected by Western blotting. Actin was used as loading control.

modification. Reaction mixtures containing ~0.25 ng of ^{32}P-labeled oligonucleotide, 2 μg of poly(dI-dC), and 10 μg of nuclear extract protein in 30 μl of buffer (10 mM HEPES, 0.1 mM EDTA, 5% glycerol, 100 mM NaCl, 0.3 M urea, 0.3% Nonidet P-40) were incubated on ice for 15 min and at room temperature for another 15 min and were then separated on 5% polyacrylamide gels in 0.5× TBE (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA [pH 8.3]). For competition experiments, a 0- to 100-fold excess of unlabeled competitor was added to reaction mixtures before the addition of labeled probe. For supershift experiments, 1 μl of anti-C/EBPβ monoclonal antibody (mAb) (clone H-7, ~2 μg of IgG protein; Santa Cruz Biotechnology, Santa Cruz, CA,) was added to the reaction mixture before the addition of labeled probe. The sequences for the oligonucleotide probes are as follows:

WT(a): −167TTATGGTTCTTGGGAGACAGCAGAGCACAAAGAGCCAGGGGG *TGGAGTCTA*GAGCTCCGCCCACTCCTTGCTGTTTTCA −88; WT(b): −135 GAGCCAGGGGG*TGGAGTCTA*GAGCTCCGCC −106. Mut(a): −167 TTATGGTTCTTGGGAGACAGCAGAGCACAAAGAGCCAGGGGGAAA*AGTCTAGA*GCTCCGCCCACTCCTTGCTGTTTTCA −88. Mut(b): −135 GAGCCAGGGGGAAA*AGTCT A*GAGCTCCGCC −106. The C/EBP-binding site is indicated in italics, and the mutant nucleic acids are underlined.

Transfection analysis of histone H4 promoter

The 12 different histone h4 promoter fragments were amplified with the following primers and cloned into the *Kpn*I and *Mlu*I sites of the pGL3-Basic luciferase vector (Promega, Madison, WI):

Hist1h4a (NP_835499)
Upstream: TACCAGAAGTCGACCGAGCTGCTGATC

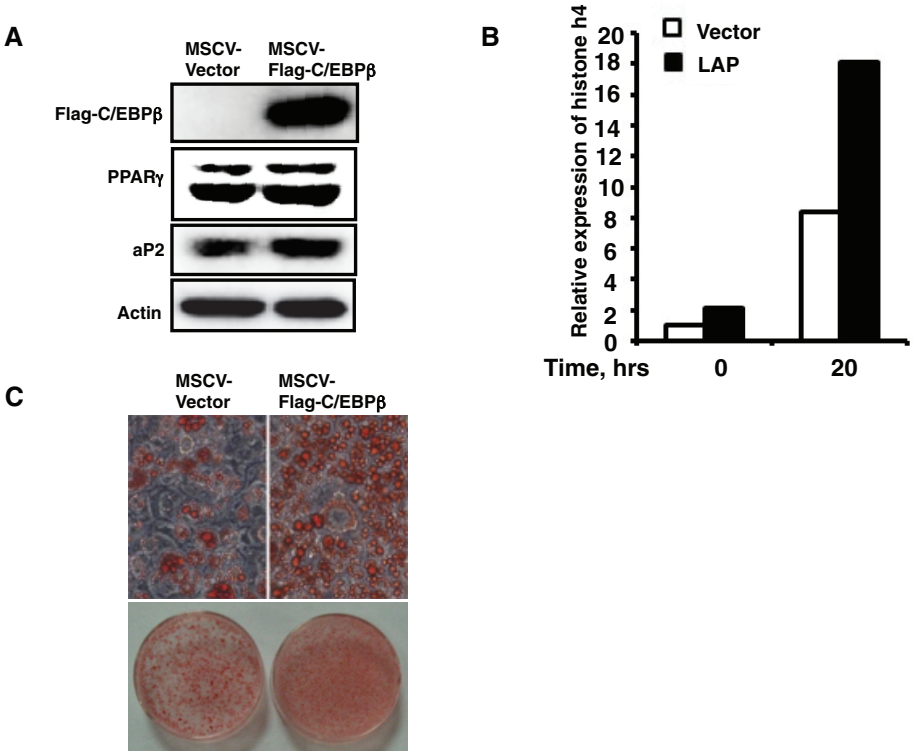

FIGURE 8: Overexpression of C/EBPβ promoted histone H4 expression and accelerated 3T3-L1 adipocyte differentiation. 3T3-L1 preadipocytes were infected with retrovirus expression flag–tagged C/EBPβ or with empty vector; cells were induced with differentiation media 2 d after reaching confluence, and the expression of C/EBPβ was detected by Western blotting (A); the effect of C/EBPβ on the expression of histone H4 was detected by quantitative real-time RT-PCR (B). Eight days after induction, fat droplet accumulation was detected by oil red O staining (C), and the expression of PPAR γ and 422/aP2 was detected by Western blotting; actin was used as loading control (A).

Downstream: GTCTTAGGAAGAATGGAAAATGGAGTCA
Hist1h4b (NP_835500)
Upstream: AAAGGTGCTGCTTACAGACTTGCTCTAT
Downstream: AGTTAAATTCTTACAAGCTTTCAGTAGG
Hist1h4c (NP_835515)
Upstream: TGCATTCATGGGTGCCATTGAGATCAG
Downstream: GGTTATGACAGCAGGGAGACAGCAAT
Hist1h4d (NP_783585)
Upstream: CAGAAACTCAGAGTCTTTGAATTCTGT
Downstream: GTTAGCAAACTAGGAAGGAAGCTCACT
Hist1h4f (NP_783586)
Upstream: CCTTAAGTGACCTTTGAGTTCCTTGG
Downstream: GGCTATGGAGCACTGAGTTGAAATTC
Hist1h4h (NP_694813)
Upstream: cagagatcgatttacctcacgaactta
Downstream: GGTGTACTTGCTGAAGAAAAAACCTCA
Hist1h4i (NP_783587)
Upstream: GTAGGTTTCCTTTTTAGCAAGTTAGGC
Downstream: GATGAAGACAAGATTGTACTGCTCTAA
Hist1h4j (NP_835582)
Upstream: GGAGACCCAGTGATTCAGATGACGGT
Downstream: GACTGCACTCAGGACAGCTCAGAACA
Hist1h4k (NP_835583)
Upstream: GACCAAGAAGACAACCAGCATTAGAACCT
Downstream: GACTGCACTCAGGACAGCTCAGAACAG
Hist1h4m (NP_783588)
Upstream: AACGACGAGGAGCTCAACAAGCTG
Downstream: GATGAAGACAAAACTGTAC-TGCTCTAA
Hist2h4 (NP_291074)
Upstream: GCCTAAGGTGACTCAAAACCT-ACATCT
Downstream: CTGAAACACCAGCTGGTG-AGGCATTGGA
Hist4h4 (NP_783583)
Upstream: ACTACAAACTAAACGCATTTA-TCCTGCT
Downstream: TCCTCGTCCTGACATATTAG-TAAACAAC

All 12 promoters have been sequenced and authenticity verified. The hist4h4 promoter and its truncated forms were generated by PCR with following upstream primers and the downstream primer used for hist4h4 as described earlier:

5′- CCATGTACTTCTATCTATGTACATCTT-3′ for 777-Luc
5′- TTATGGTTCTTGGGAGACAGCAGAG-CAC -3′ for 167-Luc
5′- TCAGGTCCGCTCCCAGGAAATATAA-GCT -3′ for 87-Luc

*Kpn*I and *Mlu*I restriction sites were added to the upstream and downstream primers, respectively, when designing the primers. The mutant hist4h4 promoter containing a 3–base pair substitution in the C/EBP-binding element (*AAA*AGTCTA) was generated with a KOD-Plus-Mutagenesis Kit (Toyobo, Osaka, Japan). Transfection experiments were performed with the transfection kit Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's instruction; luciferase activities were normalized to internal control *Renilla* luciferase activity.

Western blotting

Cells were lysed with lysis buffer containing 2% SDS, 10 mM DTT, 50 mM Tris-HCl, pH 6.8, 10% glycerol, 0.002% bromophenol blue, and 1× protease inhibitor mixture. Equal amounts of protein were separated by SDS–PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA), immunoblotted with antibodies (anti-H4 mAb was from Millipore, anti-actin mAb from Sigma-Aldrich [St. Louis, MO], anti-BrdU mouse antibody from Sigma-Aldrich), and visualized with horseradish peroxidase–coupled secondary antibodies.

RT-PCR and real-time quantitative PCR

Total RNA was isolated using the TRIzol reagent (Invitrogen) according to the manufacturer's instruction. First-strand cDNA synthesis was performed using a RevertAid First Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD) with specific RT primer for all histone h4 genes: CCTGGCGCTTGAGCGCGT. PCR reactions were performed using the synthesized cDNA as the template in a 25-μl reaction mixture containing specific primers for all mouse h4 genes (upstream, 5′-gtgatccgcgacgccgtca-3′; downstream, 5′-CTGGCGCTTGACGCGTAC-3′). Mouse 18S rRNA (NR_003278.1) (upstream, 5z-CGCCGCTAGAGGTGAAATTCT-3′; downstream, 5′-CATTCTTGGCAAATGCTTTCG-3′) was used as

control. The PCR was carried out following a cycling protocol: an initial denaturation step at 95°C for 2 min followed by 21 cycles each of a denaturation at 94°C (30 s), annealing at 58°C (20 s), and elongation at 72°C (15 s). The reaction products were resolved by electrophoresis on a 1.5% agarose gel and visualized with ethidium bromide. Real-time quantitative PCRs were performed with 2× PCR Master Mix (Power SYBR Green; Applied Biosystems, Foster City, CA) on a Bio-Rad Q5 instrument (Bio-Rad). The threshold cycles (Ct) for the histone h4 gene and 18S rRNA control signals were determined in triplicate experiments, and the relative RNA quantity was calculated using the comparative Ct method. Primers used for real-time quantitative PCR were the same as that used for RT-PCR.

Knockdown of expression of C/EBPβ and histone H4 with siRNA

Synthetic siRNA oligonucleotide specific for C/EBPβ (NT_039207) (5′ to 3′: CCCUGCGGAACUUGUUCAAGCAGCU) and histone H4 (5′ to 3′: gauccgcg acgccgucaccuacatt) were designed and synthesized by Invitrogen. 3T3-L1 preadipocytes at 30–50% confluence were transfected with the siRNA oligonucleotide by using Lipofectamine RNAiMAX (Invitrogen). Thirty-six hours after cells reached confluence, they were subjected to the standard differentiation protocol as described earlier, and at various times thereafter cells were prepared for the test. Stealth siRNA Negative Control Duplexes (Invitrogen) were used as a negative control.

BrdU labeling and immunofluorescence microscopy

For BrdU labeling, 3T3-L1 preadipocytes plated on glass coverslips were induced to differentiate by using the standard differentiation protocol, and 18 h after induction (during S phase) cells were pulse labeled with 30 μg/ml BrdU for 2 h and then shifted to normal medium. On day 3, coverslips were fixed in 70% ethanol for 30 min and incubated in 100% methanol for 10 min at room temperature, after which they were treated with 1.5 M HCl, blocked with 0.2% Triton in phosphate-buffered saline (PBS) for 5 min, incubated with anti-BrdU primary antibody (1:500) (Sigma-Aldrich) in the same buffer for 2 h at room temperature, and incubated with fluorescein isothiocyanate–conjugated secondary antibody (1:200) with 0.1 μg/ml 4′,6-diamidno-2-phenylindole for 1 h at room temperature. After each step, cells were washed with PBS three times, and the coverslips were mounted on slides and were visualized by fluorescence microscopy.

Oil red O staining

Cells 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 1h at room temperature. Cells were washed with water, and the stained fat droplets in the cells were visualized by light microscopy.

Fluorescence-activated cell sorting analysis

3T3-L1 preadipocytes were transfected with Stealth siRNA Negative Control Duplexes (Invitrogen) or C/EBPβ siRNA as described earlier. After they were treated with differentiation inducer, 3T3-L1 cells were harvested at every 4 h until 28 h. Cells were further trypsinized, washed with 1× PBS, and fixed with 2% (wt/vol) paraformaldehyde in 1× PBS. They were treated with 0.5 mg/ml RNase A for 1 h at room temperature and incubated with 0.1 mg/ml propidium iodide (Sigma-Aldrich) for 45 min at 37°C. DNA content was determined by flow cytometry (Bio-Rad).

Chromatin immunoprecipitation analysis

Cells were fixed with 1% formaldehyde for 10 min at room temperature with swirling. Glycine was added to a final concentration of 0.125 M, and the incubation was continued for an additional 5 min. Cells were washed twice with ice-cold PBS, harvested by scraping, pelleted, and resuspended in 1 ml of SDS lysis buffer (50 mM Tris-HCl, pH 8.0, 1% SDS, 10 mM EDTA, and protease inhibitors). Samples were sonicated eight times for 30 s each with an interval of 30 s with a Bioruptor sonicator (Diagenode, Denville, NJ). Samples were centrifuged at 14,000 *g* at 4°C × for 10 min. After removal of an input aliquot (whole-cell extract), supernatants were diluted 10-fold in ChIP dilution buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% TritonX-100, and complete protease inhibitor tablets). Samples were immunoprecipitated using C/EBPβ antibodies (sc7962; Santa Cruz Biotechnologies) or the nonspecific IgG control (Abcam, Cambridge, MA). Immunoprecipitated samples were eluted and reverse cross linked by incubation overnight at 65°C in elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS). Genomic DNA was then extracted with a PCR purification kit (Qiagen, Valencia, CA). Purified DNA was subjected to quantitative PCR amplification using the specific primer for the C/EBP-binding element present in the hist4h4 gene promoter (sense primer, 5-TTATGGTTCTTGGGAGACAG-3; antisense primer, 5-ATTGAAAACAGCAGGAGTG-3). Primers (sense primer, CTTCAGCCC AGTTGACCAAT; antisense primer, AGGGAGGAGGAAAGCAGAAC) in an area of the insulin gene promoter serve as a negative control.

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