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Transcriptional activation of pref-1 by E2F1 in 3T3 L1 cells

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The E2F gene family appears to regulate the proliferation and differentiation of events that are required for adipogenesis. Pref-1 is a transmembrane protein that inhibits adipocyte differentiation in 3T3-L1 cells. In this study, we found that the expression of pref-1 is regulated by the transcription factor E2F1. The expression of pref-1 and E2F1 was strongly induced in preadipocytes and at the late differentiation stage. Using luciferase reporter assay, ChIP assay and EMSA, we found that the −211/−194 region of the pref-1 promoter is essential for the binding of E2F1 as well as E2F1-dependent transcriptional activation. Knockdown of E2F1 reduced both pref-1 promoter activity and the level of pref-1 mRNA. Taken together, our data suggest that transcriptional activation of pref-1 is stimulated by E2F1 protein in adipocytes. [BMB reports 2009; 42(10): 691-696]

INTRODUCTION

Preadipocyte factor-1 (Pref-1) is an EGF-like transmembrane protein that contains many repeat domains and is encoded by 385 amino acids (1). Although pref-1 regulates the differentiation of several cell lineages including skeletal stem cells, thymocytes and adrenal gland cells, its major function is the regulation of preadipocyte differentiation (2). Pref-1 activates the MEK/extracellular signal-regulated kinase pathway, thereby inhibiting adipocyte differentiation (3-5). Moreover, transcriptional repression of pref-1 by glucocorticoids promotes differentiation in 3T3-L1 preadipocytes (6).

E2F1 is a member of the E2F family of transcription factors (7). In mammalian cells, the E2F family plays a pivotal role in regulating the expression of target genes involved in the G1/S transition of the cell cycle as well as in DNA synthesis. Loss of E2F function results in the elevation of p21 protein, leading to a decrease in cyclin-dependent kinase activity and Rb phosphorylation (8). Repression of E2F1 expression induces transcriptional activation of C/EBPα during adipocyte differentiation (9). Transcription of PPARγ is stimulated by E2F1 during clonal expansion but is repressed by E2F4 during terminal differentiation of adipocytes (10, 11). In addition, E2F1/E2F2 activity negatively regulates the growth of mature pancreatic cells (12). Reduction of E2F1 is critical for cell cycle arrest and the subsequent differentiation of many cell types. However, the role of E2F1 in mouse pref-1 gene expression in adipocytes remains unknown. In this study, we therefore examined the response of the mouse pref-1 promoter to E2F1.

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RESULTS

The expression of pref-1 during adipocyte differentiation

To identify the potential regulation of pref-1 in adipocytes, we examined the expression pattern of E2F1 in 3T3-L1 cells after induction of differentiation. The expression of E2F1 mRNA was strong in preadipocytes, but decreased gradually during early differentiation. This was followed by a rapid increase in E2F1 expression at day 6 after induction of differentiation. As expected, these expression patterns were similar to those of pref-1 (Fig. 1). These results suggest that E2F1 may regulate the transcriptional activation of pref-1 in 3T3-L1 cells.

The promoter activity of pref-1 stimulated by E2F1

A luciferase reporter assay was performed in order to measure pref-1 promoter activity in the presence of E2F1. Cos-7 cells were co-transfected with pCDNA3.1-E2F1 and pGL3-Pref-1, a luciferase reporter vector. In addition, several deletion mutants (pGL3-pref-1-1950, −928, and −315) were constructed. As shown in Fig. 2A, we observed relatively enhanced luciferase activity in COS-7 cells transfected with pGL3-pref-1-315 in the presence of E2F1 compared with that of pGL3-pref-1-1950 and pGL3-pref-1-928. This suggests that the −211/−194 region of the pref-1 promoter contains putative E2F1 binding sites. The luciferase activity of pref-1 in E2F1-expressing cells was increased 6-7 fold compared to cells not expressing E2F1. To identify the active E2F1 binding site (EBS) within the pref-1 promoter, we conducted site-directed mutagenesis of conserved, putative E2F1 binding sites (Fig. 2B). As shown in Fig. 2C, the activity of the EBS mutant [pref-1(Mu)] was eliminated in the presence of E2F1 protein, whereas intact EBS [pref-1(WT)] exhibited high promoter activity. These results suggest that E2F1 regulates the transcriptional activation of pref-1 by

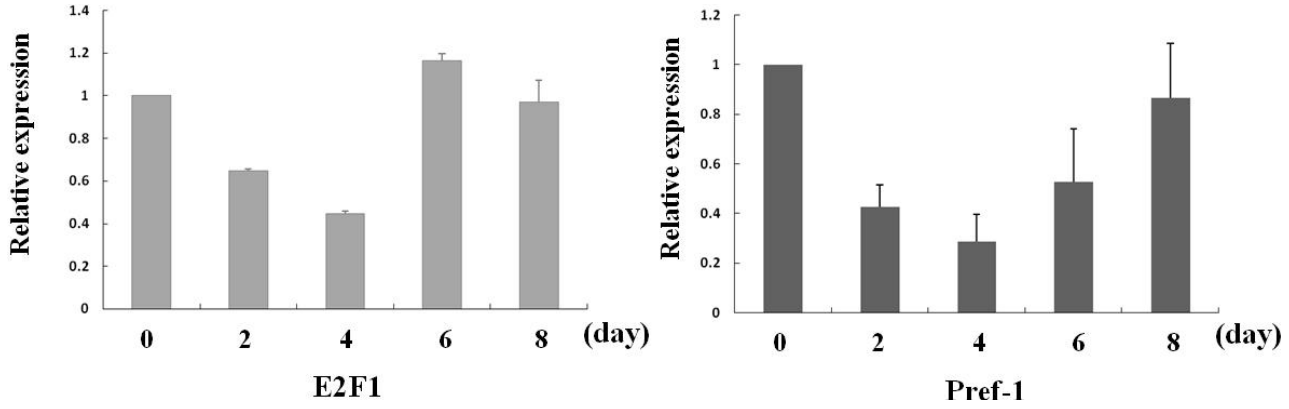

Fig. 1. Levels of pref-1 and E2F1 mRNA during 3T3-L1 differentiation. Expression of pref-1 and E2F1 mRNA during 3T3-L1 cell differentiation was quantified by real-time PCR. Quantities of mRNA were normalized with that of GAPDH mRNA. Experiments were conducted independently in triplicate. The data are presented as mean ± S.E. Day 0 indicates the day of induction for adipocyte differentiation.

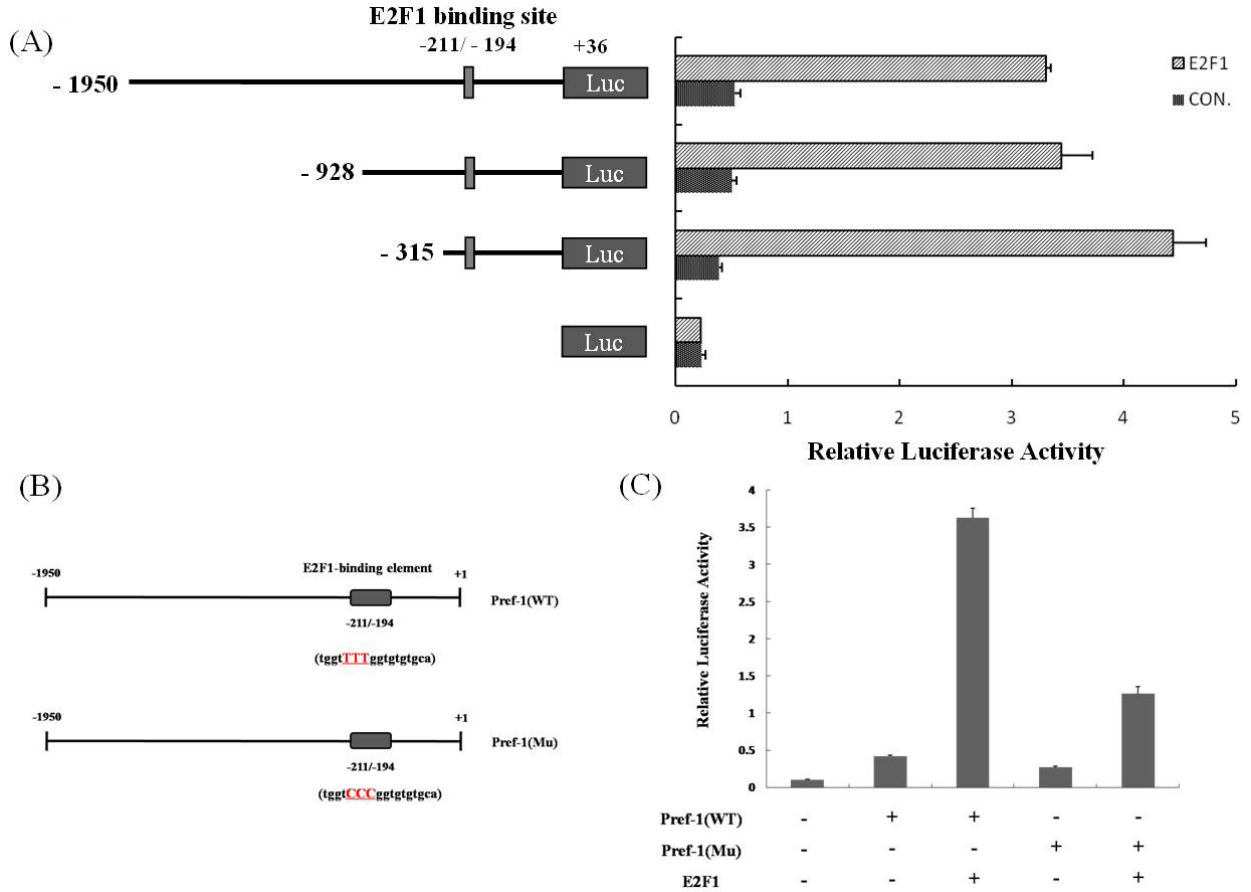

Fig. 2. E2F1 transactivates the pref-1 promoter. (A) COS-7 cells were transiently transfected with a series of mouse pref-1 promoter deletion mutants (pGL3-pref-1-1950, pGL3-pref-1-928, pGL3-pref-1-315) and the pcDNA3.1-E2F1 vector. The pGL3-Basic vector was used as a control. After 24 h, luciferase reporter activity was measured. (B) The sequences are shown in alignment with the consensus E2F1 binding site (EBS) of the pref-1 promoter. The conserved EBS region was mutated as indicated. (C) Luciferase reporter vectors were co-transfected into cells containing the E2F1 expression vector. Reporter activity was measured 48 h later. Experiments were conducted independently in triplicate. The data are presented as mean ± S.E.

binding to the −211/−194 region of the pref-1 promoter.

Sequence-specific binding of E2F1 to the putative pref-1 promoter region

The binding affinity of E2F1 to the pref-1 promoter was examined by EMSA. As shown in Fig. 3A, the formation of a major DNA-protein complex was detected using labeled oligonucleotide. To further define the sequence within the E2F1 binding element required for DNA-protein complex formation, unlabeled oligonucleotides were used as competitors in gel mobility shift assays. A specific DNA-protein complex was observed with nuclear extracts derived from preadipocytes (Fig. 3A, lane 2), but disappeared in the presence of competitors (Fig. 3A, lanes 3 and 4). A ChIP assay was performed in order to verify that E2F1 binds the EBS site of the pref-1 promoter. As shown in Fig. 3B, we observed PCR products corresponding to the −211/−194 region. These results indicate that the −211/−194 region of the pref-1 promoter could be the requisite binding element in E2F1-dependent transcriptional activation of pref-1.

Knockdown of E2F1 inhibits the expression of pref-1

To determine the transcriptional regulation of pref-1 by E2F1, the expression of E2F1 was knocked down in adipocytes using shRNA and the transcriptional activity of pref-1 was examined. As shown in Fig. 4A, the level of pref-1 mRNA was decreased in preadipocytes infected with shE2F1 lentiviral vector. Moreover, the transcriptional activity of pref-1 in Cos-7 cells expressing E2F1 protein was decreased by 50% upon infection with shE2F1 lentiviral vector (Fig. 4B). These results suggest that E2F1 stimulates the transcriptional activation of pref-1 during adipocyte differentiation.

DISCUSSION

In the present study, E2F1 expression was highly stimulated in preadipocytes and at day 6 after induction of differentiation. Consistent with our results, the phosphorylation of Rb during clonal expansion causes the release of Rb-associated E2F1, which forms the E2F1/DP complex and stimulates the transcriptional activity of PPARγ (10, 13). In addition, E2F1 inhibits the expression of the PAI-1 gene, a member of the serine proteinase inhibitor family, resulting in the induction of PPARγ, C/EBPα and AP2 (14). During differentiation, the progressively reduced levels of pRb cause a concomitant increase in both free E2F1 and its transcriptional capacity (15). In the present study, we found that pref-1 is an E2F-responsive gene. Although, pref-1 is a well-known inhibitor of adipogenesis in preadipocytes, its E2F1-stimulated expression during late differentiation has no clear function. Therefore, pref-1 may have a distinct role in mature adipocytes. Further study should focus on the role of pref-1 in the late differentiation stage of adipocytes.

Previous studies have shown that the Hes-1 gene, a target of Notch signaling, acts as a transcriptional repressor of both E2F1 and pref-1 (16). Hes-1 directly down-regulates E2F1 ex-

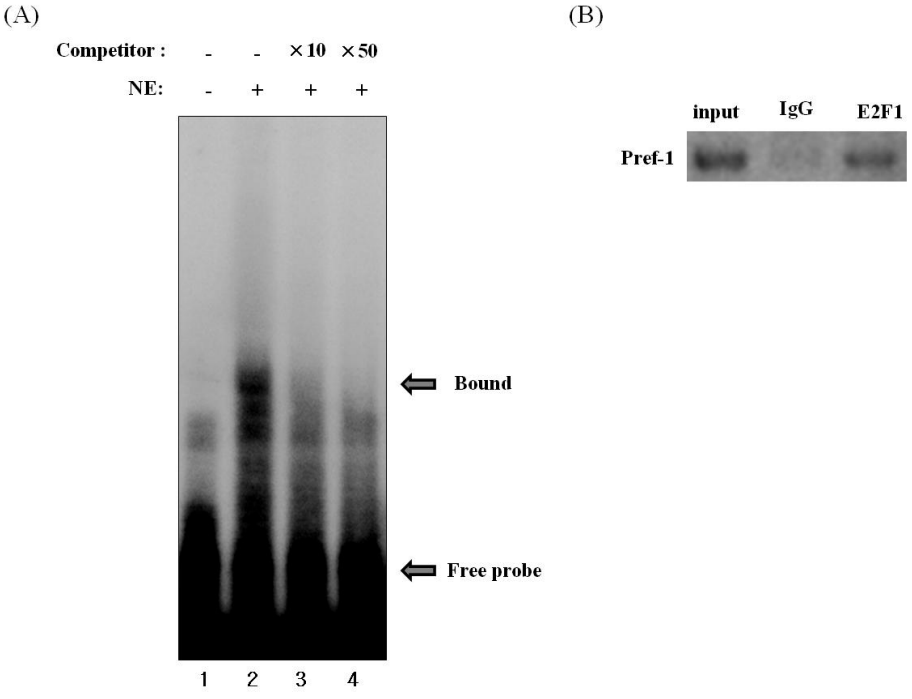

Fig. 3. E2F1 binds to the −211/−194 region of the pref-1 promoter. (A) EMSA was performed with 2.5 μg cell extracts from 3T3-L1 cells (day 0). Extracts were incubated with a ^{32}P-labeled oligonucleotide corresponding to the consensus E2F1 binding site. For competition assays, the reaction was preincubated with either 10-fold or 50-fold molar excess of cold oligonucleotide before the addition of labeled oligonucleotide probe. Lanes 3 and 4 show the competition between unlabeled oligonucleotides. The arrow indicates the major DNA-protein complex. (B) The binding of E2F1 to the −211/−194 region of the pref-1 promoter was analyzed by ChIP assay. After immunoprecipitation, the EBS region was amplified by PCR reaction. Total chromatins were indicated as 'input'. Pre-immune IgG was used as a negative control.

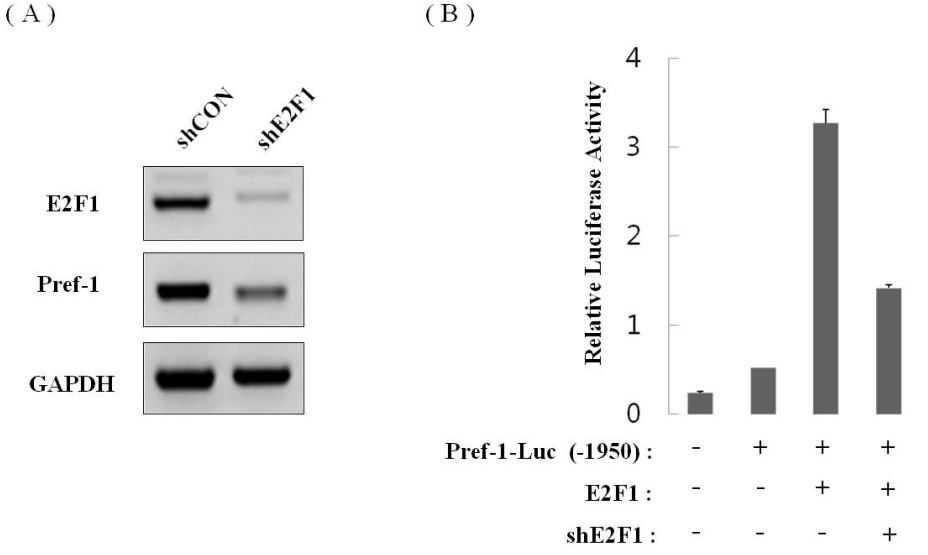

Fig. 4. Knockdown of E2F1 inhibits the expression of pref-1. (A) 3T3-L1 cells were infected with a shRNA negative control (shCON) or the shE2F1 lentivirus. Expression of E2F1 and pref-1 mRNAs was observed by RT-PCR. GAPDH was used as the internal control. (B) To assess the effect of the E2F1 depletion on the transcriptional activity of pref-1, COS-7 cells were transfected with the indicated vectors. After 24 h, relative luciferase activities were assayed. All data represent the mean ± SE of three independent experiments.

pression by binding a specific CACGAG-site within the E2F1 promoter (17, 18). Notch signaling is essential for adipogenesis in 3T3-L1 cells, promoting differentiation through the down-regulation of pref-1 transcription and the activation of Hes-1 expression (19, 20). In our study, the levels of E2F1 and pref-1 were decreased during the early differentiation of adipocytes. Therefore, Notch may be involved in adipogenesis by inducing Hes-1 homodimers, which repress the expression of both E2F1 and pref-1. In conclusion, our results demonstrate that E2F1 could play a critical role in adipogenesis by regulating the transcription of target genes such as pref-1. These observations may aid the understanding of the functional mechanisms of adipogenesis in obesity.

MATERIALS AND METHODS

Construction of the pref-1 promoter-reporter

To generate the 2 kb pref-1 promoter-reporter, a MluI-BglII fragment of the mouse pref-1 promoter comprising nucleotides −1950 to +36 was cloned into the pGL3-Basic luciferase reporter vector (Promega- Madison,WI, USA). pGL3 vectors containing pref-1 promoter deletion mutants were also constructed. Vectors pGL3-pref-1-1950, pGL3-pref-1-928 and pGL3-pref-1-315 contain 1950 bp, 928 bp and 315 bp from the translation start site of the pref-1, respectively. These fragments were generated by PCR using specific primers: for 1950 bp, forward primer: 5'-CGACGCGTCTGCCCGAGAGGTTCC CA-3', for 928 bp, forward primer: 5'-CGACGCGTCACACTCC CATCCCATCC-3' and for 315 bp, forward primer: 5'-CGCGT GCCGAAAGGTGTGTTTGG-3'; for +36, reverse primer: 5'-G AAGATCTAGCACGCGGCTGGAG-3'. PCR conditions for each construct were: 96°C for 3 min, 30 cycles of 96°C for 1 min, 55°C for 1 min 40 sec and 72°C for 1 min, along with a final extension of 72°C for 10 min. Site-directed mutagenesis of the luciferase promoter was performed using the Quik Change site-directed mutagenesis kit following the instructions provided by the supplier. The putative E2F1 binding site sequence TGGT*TTT*GGTGTGTGCA in the pref-1 promoter was changed to TGGT*CCC*GGTGTGTGCA. Successful mutagenesis was confirmed by sequence analysis.

Cell culture

3T3-L1 preadipocytes were plated and grown for two days post-confluence in Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Grand Island, NY, USA) with 10% bovine serum. To induce differentiation, cells were cultured in differentiation medium containing 10% fetal bovine serum, 0.5 mM 3-isobutyl-1-methylxanthine, l M dexamethasone and 10 μg/mL insulin. Medium was replaced with DMEM containing 10% fetal bovine serum and 10 μg/mL insulin after 48 h. Adipocytes were maintained in DMEM with 10% fetal bovine serum for at least 8 days.

Real-time PCR

Total RNA was isolated from 3T3-L1 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The synthesis of cDNA was performed using MMLV-reverse transcriptase (Takara, Shinga, Japan) and oligodT primers (Invitrogen, Carlsbad, CA, USA). Real-time quantitation was performed using the BIO-RAD iCycler iQ system (BioRad, Hercules, CA, USA) according to the manufacturer's protocol. The fluorescence threshold value was calculated using the iCycle iQ system software. The reverse transcription reaction mixture was incubated with iQ SYBR Green supermix (BioRad, Hercules, CA, USA). Data were processed by the comparative cycle threshold method and expressed as fold increase relative to the basal tran-

scription level. The amount of target mRNA was normalized by determining the level of GAPDH mRNA.

Luciferase reporter assays

Cos-7 cells (ATCC, CRL-1651, USA) in 24-well plates were transfected with a combination of pRC-E2F1 and pRL-TK (*Renilla* control vector) at a final DNA concentration of 1 μg by using ExGene 500 reagent (Fermentas, Hanover, MD, USA). At 24 h post-transfection, luciferase activities were measured using the Dual-Luciferase assay kit (Promega, Madison, WI, USA) and a GloMax 20/20 luminometer (Turner Biosystems, Sunnyvale, CA, USA). Data were normalized to a *Renilla* reporter signal.

Chromatin immunoprecipitation (ChIP) assay

3T3-L1 adipocytes were fixed in 18.5% formaldehyde for 10 min at room temperature and neutralized with 125 mmol/L glycine for 5 min. After washing with PBS, cell lysates were sonicated to produce chromatin fragments averaging 500 bp in size. Fragmented chromatins were added to the ChIP dilution buffer [50 mM Tris-HCl (pH8.1), 10 mM EDTA, 1% SDS and inhibitor cocktail]. After blocking with Staph A cells, samples were incubated with anti-E2F1 antibody (Abcam, Cambridge, UK) at 4°C. The DNA-protein complexes were precipitated with Staph A cells and then applied to mini columns (BioRad, Hercules, CA, USA) for washing. Finally, the beads were eluted using TE buffer. DNA-protein crosslinks were treated with proteinase K (Roche, Indianapolis, IN, USA) at 55°C for 30 min. Precipitated chromatins were used as the template. PCR was performed using the following conditions: 5 min at 95°C followed by 32 cycles of 30 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C.

Knock-down of E2F1 mRNA using lentiviral vector

For construction of E2F1-specific shRNA, the pLKO.1 lentiviral vector was digested with AgeI and EcoRI and was subsequently subcloned with target sequences: sense: 5'-CCGGGGAGGA GTCACGCTATGAAACCTCGAGGTTTCATAGCGTGACTTCT CCTTTTTG-3 and anti-sense: 5'-AATTCAAAAAGGAGAAGTC ACGCTATGAAACCTCGAGGTTTCATAGCGTGACTTCTCC-3'. For lentivirus packaging, sub-confluent HEK 293T cells (ATCC, CRL-11268) in 10 cm culture dishes were co-transfected with E2F1 lentiviral vector (1 μg), lentiviral packaging vector (pCMV-dR8, 750 ng) and envelope vector (pCMV-VSVG, 250 ng) using FuGENE 6 reagent. The viruses were collected from culture supernatants 2 days after transfection and were subsequently transfected into COS-7 or 3T3-L1 cells. Infected cells were selected by treating with puromycin (200 ng/mL; Sigma-Aldrich, St. Louis, MO) for 3 days to eliminate uninfected cells. The shRNA-mediated down-regulation of E2F1 expression was confirmed by RT-PCR analysis.

Electrophoretic mobility shift assay

Nuclear extracts were prepared from 3T3-L1 cells. For annealing of the EMSA probe, double-stranded oligonucleotides with GG overhangs were end-labeled by a fill-in reaction using a DNA polymerase I Klenow fragment in the presence of [α-^{32}P]dCTP, as previously described (21). The sequence of the pre-1 promoter region used as the probe was as follows: 5′-GTTTTCGTGGTGGTTTTCGTGTGTGCATCTGTGATC-3′. The ^{32}P-labeled probe was incubated with 2 μg nuclear extract in 20 μL binding buffer containing 2.5 mg/mL BSA, 20 pg/μL Poly (dI-dC) and 1 mM DTT. For cold competition analysis, nuclear extracts were incubated on ice for 15 min with unlabeled oligonucleotides before the addition of [^{32}P]-labeled probe. The competition assay was performed by the addition of 10-fold and 50-fold excess of unlabeled probe to the reaction mixture. DNA-protein complexes were resolved on 5.0% polyacrylamide gels at 180 V for 2 h. Gels were then dried using a vacuum gel dryer while protein-DNA complex formation was analyzed using a Personal Molecular Imager FX system (Bio-Rad Laboratories, Cambridge, USA).

Statistical analysis

Data are presented as the mean ± SE of three independent experiments. Comparisons were analyzed by a one-way ANOVA using SPSS software package (SPSS Inc.). The value of $P < 0.05$ was considered for statistical significance.

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