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# Peroxisome Proliferator-Activated Receptor Gamma/Signal Transducers and Activators of Transcription 5A Pathway Plays a Key Factor in Adipogenesis of Human Bone Marrow-Derived Stromal Cells and 3T3-L1 Preadipocytes

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Adipogenesis is largely dependent on the signal transducers and activators of transcription (STAT) pathway. However, the molecular mechanism of the STAT pathway in the adipogenesis of human bone marrow-derived stromal cells (hBMSCs) remains not well understood. The purpose of this research was to characterize the transcriptional regulation involved in expression of STAT5A and STAT5B during adipogenesis in hBMSCs and 3T3-L1 cells. The expression of STAT5A and STAT5B increases with the onset of adipogenesis in hBMSCs and 3T3-L1 cells. The PPAR response elements regulatory element of STAT5A exists at a promoter region ranging from −346 to −101, and the CCAAT/enhancer-binding protein (C/EBP) regulatory element is located at −196 to −118 of the STAT5B promoter. C/EBPβ and C/EBPα bound to the STAT5B promoter region, whereas peroxisome proliferator-activated receptor γ (PPARγ) bound to STAT5A. RNA interference of STAT5A completely blocked differentiation, whereas the inhibition of STAT5B only partially blocked differentiation. We propose that C/EBPα, C/EBPβ, and PPARγ control adipogenesis by regulating STAT5B and STAT5A and that STAT5A is necessary, whereas STAT5B plays a supplementary role during adipogenesis. Further, the regulation of PPARγ-STAT5 by C/EBPβ signaling seems to be the crucial adipogenesis pathway-initiating cascade of the various adipogenic genes.

## Introduction

Human bone marrow-derived stromal cells (hBMSCs) have the capacity to differentiate into multiple lineages and the ability for self-renewal. Specifically, BMSCs are well known for their potential to differentiate into adipocytes, osteoblasts, chondrocytes, myocytes, and neurocytes [1–6]. Imbalances in osteogenic and adipogenic differentiation are related to aging and diseases. In aging patients or people with osteoporosis, decreased bone density is observed along with reduced osteoblast numbers and increased adipocyte numbers, which suggest that balanced adipogenesis and osteogenesis are important for the maintenance of bone structure and volume [7–9]. Therefore, a comprehensive understanding of the complicated network of various transcription factors and genes involved in the transdifferentiation of hBMSCs into adipocytes and osteoblasts is crucial for developing and improving the treatment of osteoporosis.

Previous research has shown that adipogenesis is a complex process involving the proliferation of precursor cells, adipocyte formation, and terminal differentiation [10]. Various gene cascades and signaling pathways are involved in the process. CCAAT/enhancer-binding protein (C/EBP) family members belong to the basic leucine zipper class of transcription factors and they bind to specific DNA sequences as dimers with other C/EBPs. The transcriptional activity of peroxisome proliferator-activated receptor γ (PPARγ) is dependent on both heterodimerization with retinoid X receptor α (RXRα) and the regulation of fatty acid binding protein 4 (FABP4), leptin, and adiponectin by the heterodimers [10–15].

The introduction of adipogenic hormonal stimuli activates 2 transcription factors crucial for the initiation of adipogenesis, C/EBPβ and C/EBPδ. They continue to be expressed for approximately 2 days, and they induce the expression of C/EBPα and PPARγ [10,13,15]. Activated C/EBPα and PPARγ upregulate the expression of one another in a positive feedback loop. Simultaneously, C/EBPα and PPARγ regulate the expression of adipocyte genes necessary for the development of functional, mature adipocytes [11,13–17]. Despite detailed

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knowledge about the molecular pathways involved in adipogenesis, we do not yet have a complete understanding of the signaling pathways and functional mechanisms behind each process, and the potential mediators between C/EBPα and PPARγ and terminal differentiation markers have not been yet identified.

Signal transducers and activators of transcription (STATs) are a family of latent transcription factors of cytokine-mediated signaling events in cell growth and differentiation [18–21]. STAT1–6 comprise a group of latent cytoplasmic transcription factors that are activated by cytokines, peptides, and growth factors. STAT proteins form homo- or heterodimers at tyrosine phosphorylation sites, and dimerized STAT proteins immediately enter the nucleus, where they bind to specific DNA sequences in the promoters of various genes and mediate transcriptional regulation. STATs are involved in a variety of cellular processes such as innate and acquired immunity as well as cell proliferation, mammary cell differentiation, lymphoid development, and cell survival [20]. Studies have shown increased expression levels of STAT genes during adipogenesis [22–27], and it has been reported that ectopic expression of STAT5A in NIH3T3 cells induces adipogenesis [22]. In primary rat preadipocytes, GH-mediated inhibition of FABP4 gene expression was shown to be regulated by activation of the STAT5A signaling pathway [28]. Collectively, these studies have shown that STATs and their target genes play a specific role in adipogenesis; however, the regulators of STAT remain unknown.

In this study, we sought to identify the potential regulators of STAT5A and STAT5B expression with a particular interest in the transcription factors already shown to display a distinct expression pattern during adipogenesis. Specifically, we focused on the transcriptional regulation of STAT5A and STAT5B by C/EBPα, C/EBPβ, and PPARγ and confirmed the effect of STAT5A and STAT5B on adipogenesis using an siRNA silencing technique. We demonstrated that inhibition of STAT5A expression completely blocks adipogenesis; however, inhibition of STAT5B only partially inhibits adipogenesis. Additionally, we demonstrated that C/EBPα and C/EBPβ directly regulate the transcriptional activity of STAT5B and that PPARγ regulates STAT5A.

## Materials and Methods

### *hBMSCs and 3T3-L1 cells cultures and differentiation*

Bone marrow aspirates were obtained from the posterior iliac crest of 7 healthy volunteers aged 13–69 years after approval from the institutional review board. hBMSCs were purified from the marrow using the Percoll density gradient centrifugation method. hBMSCs were cultured in Dulbecco's modified Eagle's medium (DMEM)-low glucose (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Welgene, Daegoo, South Korea) and 1% antibiotic–antimycotic solution; confluence was achieved within 7 days. The cells were cultured for 14 days in an adipogenic medium consisting of DMEM-low glucose supplemented with 1% antibiotic–antimycotic solution plus MDI (0.5 mM isobutylmethylxanthine, 1 μM dexamethasone, and 200 μM indomethacin; Sigma, St. Louis, MO) and 5 μM insulin (Sigma). Next, 3T3-L1 preadipocytes were propagated and maintained in DMEM-high glucose (Gibco) containing 10% calf serum (Gibco). To induce adipogenic differentiation, the cells were treated with DMEM-high glucose containing 10% FBS, 1 μg/mL insulin, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine until day 2. The cells were then treated with DMEM supplemented with 10% FBS and 1 μg/mL insulin for 2 days and were finally treated every other day with DMEM containing 10% FBS.

### *Plasmid constructs*

Expression plasmids for pCMV-C/EBPα, pCMV-C/EBPβ, pCMV-PPARγ, and pCMV-RXRα were constructed. Expression plasmids for pcDNA-STAT5A and pcDNA-STAT5B were gifts from Dr. H. Yamashita (Nagoya City University Graduate School of Medical Science, Nagoya, Japan). The mouse STAT5A promoter region spanning −1500 to +57 bp was cloned into the pGL3-basic vector and named pGL-1500. Constructs pGL-766, pGL-346, and pGL-101 containing 5′ serial deletions of the STAT5A promoter reporter were constructed by amplifying the STAT5A promoter regions from −766 to +57, −346 to +57, and −101 to +57 bp, respectively. The STAT5B promoter region spanning −1443 to +45 bp was cloned into the pGL3-basic vector and named pGL-1443. Constructs pGL-600, pGL-196, and pGL-118 containing 5′ serial deletion of the STAT5B promoter reporter were constructed by amplifying mouse STAT5B promoter regions from −600 to +45, −196 to +45, and −118 to +45 bp, respectively, and subcloning into the pGL3-basic vector.

### *Transient transfection and luciferase reporter assays*

3T3-L1 and NIH3T3 cells in 6-well plates were cultured in DMEM supplemented with 10% bovine calf serum (BCS) (Gibco) , 100 U/mL penicillin, and 100 μg/mL streptomycin. Transient transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, 500 ng of the luciferase reporter construct, 200 ng of the mouse C/EBPα and mouse C/EBPβ expression vectors, 100 ng of the pCMV-β-galactosidase expression vector, and 4 μL of Lipofectamine 2000 were independently mixed with 250 μL of opti-MEM and incubated for 5 min. To transfect constant amounts of DNA, sample DNAs were supplemented with the control vector pcDNA3. After 5 min of incubation, diluted DNA samples were mixed with Lipofectamine 2000 and incubated for 20 min at room temperature. During this period, the cells were washed twice with phosphate-buffered saline, and 2 mL of opti-MEM was added. After 20 min of incubation, transfection mixtures were added to the cells and incubated at 37°C in 5% $CO_2$ and 95% air for 12 h. Next, the medium was replaced with DMEM containing 10% BCS (Gibco). After 24 h, the cells were harvested and lysed with 200 μL of reporter lysis buffer (Promega, Madison, WI), and cell debris was removed by centrifugation. Luciferase activities were measured using 20 μL of cell extract and 50 μL of luciferase assay reagent (Promega). β-Galactosidase activity was measured for 50 μL of cell extract with 50 μL of 2× β-galactosidase assay reagent containing *O*-nitrophenyl-β-galactopyranoside. Luciferase activities were normalized to β-galactosidase activities to adjust for transfection efficiency. Lucifease activities from the wild-

type promoter constructs in the absence of any reporters were used as controls. The assays were performed in triplicate.

### Chromatin immunoprecipitation analysis

Chromatin immunoprecipitation (ChIP) analysis was performed, as described in the protocol included in the ChIP assay kit (Upstate Biotechnology, Charlottesville, VA). Briefly, hBMSCs and 3T3-L1 preadipocytes were maintained and induced to differentiate as described earlier. At various time points, the cells were cross-linked with 1% formaldehyde in PBS buffer. The cross-linked cells were harvested, lysed in sodium dodecyl sulfate lysis buffer, and sonicated. After incubation with salmon sperm DNA/protein A at 4°C overnight, the DNA–protein complexes in the supernatant were immunoprecipitated with antibodies against C/EBPβ, C/EBPα, and PPARγ (Santa Cruz Biotechnology, Santa Cruz, CA), and the immune complexes were recovered by adding protein A–agarose. After washing the DNA–protein A complex with saline, DNA was extracted with phenol/chloroform, precipitated, redissolved, and used as a template for polymerase chain reaction (PCR). We used the mouse STAT5A promoter primers (sense, AAAAGGTTCAGGTTTT CCCC; and antisense, GGCTTCCTCTCTTACTAAT), the mouse STAT5B promoter primers (sense, GCTTCAGCCGA GACCCTTCA; and antisense, GGTCAGCTGCTTCAGAC AGA), and the human STAT5B promoter primers (sense, CTCCCTGAACCTACTCTGTG; and antisense, CACTCTCC TTTCCTCTGCTA).

### Nuclear extract preparations and $T_NT$ reactions

For nuclear extract preparations, the cells were harvested in a 10-cm plate. Hypotonic lysis buffer containing 20 mM Tris-Cl (pH 7.5), 10 mM NaCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 30 mM β-glycerophosphate, and 1×PIC I and II. Igepal CA-630 was added to a final concentration of 0.1%, and the cells were homogenized. The homogenates were centrifuged at 2,000 rpm for 10 min. The pellet of intact nuclei was resuspended in NUN buffer containing 0.3 M NaCl, 1 M urea, 1% NP-40, 25 mM HEPES (pH 7.6), 1 mM dithiothreitol, 1 mM sodium orthovanadate, 30 mM β-glycerophosphate, and 1×PIC I and II and incubated for 30 min on ice. The samples were centrifuged at 12,000 rpm at 4°C for 10 min. Supernatants containing nuclear extracts were analyzed for protein content using the BCA assay kit. To produce PPARγ and RXRα protein, we used the $T_NT$ kit (Promega). Reaction mixtures containing $T_NT$ T7 quick master mix, 0.5 μg of DNA template, and 1 mM [$^{35}$S]methionine were incubated at 30°C for 90 min.

### Electrophoretic mobility shift analysis

Electrophoretic mobility shift analysis (EMSA) was performed using the following method. Reaction mixtures containing ~0.25 ng of the appropriate $^{32}$P-labeled oligonucleotide probe, 2 μg of poly[d(I-C)], and 10 μg of nuclear extract protein (for C/EBPα and C/EBPβ protein) or 2 μL of $T_NT$ mixtures (for PPARγ and RXRα protein) in 30 μL of buffer (10 mM HEPES, 100 mM NaCl, 0.3 M urea, 0.3% NP-40, 0.1 mM EDTA, and 5% glycerol) were incubated on ice for 15 min and then at room temperature for 15 min. Proteins and labeled oligonucleotide probes were separated by electrophoresis on 5% polyacrylamide gels in 0.5×TBE. For supershift experiments, 1 μL of antiserum was added to the reaction mixture before the addition of labeled probe. The labeled oligonucleotide probes included double-stranded oligonucleotides corresponding to the C/EBP regulatory element in the STAT5B promoter, (−153) AATGTGATTGCGT ACTAGCTGC (−132), and a cold probe based on the C/EBP binding site of the C/EBPα promoter. The PPRE regulatory element in the STAT5A promoter was used as probe 1, (−301) CAGCCCAGACATTTTCAACTTCTGC (−277), probe 2, (−206) AAAGACCAGAAAGAAGGATCAGG (−183), and probe 3, (−140) CCTCCAGGCCACTGGGTCTTGGCC (−117). The positive control (CNTL) was the PPRE regulatory element in the FABP4 promoter, TTTGCCTTCTTACTGGATCAGAG TTCAC.

### RNAi of STAT5A, STAT5B, PPARγ, and C/EBPβ

Synthetic siRNA oligonucleotides specific for regions in STAT5A, STAT5B, PPARγ, and C/EBPβ mRNA were designed and synthesized by Bioneer (Daejeon, South Korea). The silencing effects of several siRNA oligonucleotides were first tested for their ability to silence STAT5A, STAT5B, PPARγ, and C/EBPβ expression. 3T3-L1 preadipocytes in 6-cm dishes at 90% confluence were transfected with siRNA oligonucleotides using Lipofectamine 2000, and the cells were subjected to the standard differentiation protocol at 24 h later. Finally, cell extracts were prepared for analysis at various time points. Control transfections were performed with Negative Control Duplexes (Bioneer).

### Oil Red O staining for adipogenesis

Monolayer cultured cells were rinsed with PBS, fixed in a 3.7% solution of formaldehyde buffer for 3 min, washed with distilled water, and stained with an Oil Red O solution for 1 h, followed by repeated washing with 70% ethanol. After staining, the cells were photographed. Assays were performed in triplicate in at least 3 independent experiments.

### Reverse transcription-polymerase chain reaction

Total RNA was isolated from 3T3-L1 cells using the RNeasy kit (Qiagen, Valencia, CA). The total RNA was reverse-transcribed using the Omniscript kit (Qiagen); all experiments were performed with Taq DNA polymerase (Qiagen). The reaction products were subjected to 1.5% agarose gel electrophoresis. Reverse transcription-polymerase chain reaction (RT-PCR) was used for amplification using the following primers: STAT5A 1F-ATGGGGA CTATGATC CAGGC, STAT5A 2F-CGTCAGGAGCCGTCA GAAGC, STAT5A 3R-CCCAGCTTGATCTTCAGCAA, STAT5B 4F-GAGGACTCAAGACGGTCCCT, STAT5B 5F-T GCGGATG AGAAAACTGAGG, and STAT5B 6R-AAGTA GTGCCG GACCTCGAT.

### Immunoblotting

Cell extracts were prepared by adding 1×passive lysis buffer (Promega) and a cocktail of protease and phosphatase inhibitors (Sigma). Aliquots (20 μg) of cell lysates were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions. Separated

proteins were transferred to a PVDF membrane (Amersham Pharmacia, Piscataway, NJ) at 50 V for 2 h in transfer buffer. Membranes were blocked with 5% skimmed milk dissolved in 1×TBST buffer at room temperature for 1 h. The membranes were probed with the following primary antibodies: STAT5A, STAT5B, pSTAT5 (BD, Franklin Lakes, NJ), FABP4, C/EBPα, C/EBPβ, PPARγ, and GAPDH (Santa Cruz Biotechnology). Immunoblots were visualized using the ECL Plus detection kit (Amersham Pharmacia).

## Results

### *The expression of STAT5 and phosphorylated STAT5 is induced during hBMSC adipogenesis*

We observed the expression of STATs and phosphorylated STATs during the adipogenesis of hBMSCs. The expression levels of STAT1, STAT2, and STAT4 decreased in a time-dependent manner after the induction of adipogenic differentiation, whereas the expression levels of STAT3 and STAT6 did not change during adipogenesis. Interestingly, STAT5 expression increased in a time-dependent manner (Fig. 1A). Phosphorylated STAT1 (p-STAT1) was expressed until day 1, p-STAT4 was transiently activated on adipogenesis day 1, and p-STAT5 was activated in a time-dependent manner. The expression of p-STAT2 and p-STAT6 was not detected (Fig. 1B). Using the TESS program, we predicted the presence of a C/EBP binding site within the STAT5B promoter region. Using ChIP assays, we detected the binding of C/EBPα to the STAT5B promoter, which indicated that the transcription of STAT5B was regulated by C/EBPα during adipogenesis (Fig. 1C).

### *The expression of STAT5A and STAT5B is induced during the adipogenesis of 3T3-L1 preadipocytes*

hBMSCs have characteristically high heterogeneous phenotypes, which makes it extremely difficult to correctly assess the expression of different gene cascades in multiple donors. Therefore, we used the 3T3-L1 preadipocyte cell line to examine our hypothesis. During the adipogenesis of 3T3-L1 preadipocytes, the expression of C/EBPβ began to increase at 4 h into differentiation and was maintained for up to 48 h, after which the expression level was noticeably reduced. The expression of C/EBPα and PPARγ increased at 38 h into differentiation and was followed by expression of the terminal marker gene FABP4 at approximately 72 h (Fig. 2A). During the adipogenesis of 3T3-L1 preadipocytes, STAT3 expression was maintained throughout the entire period, whereas STAT 2, STAT4, and STAT6 were not expressed at all. The only other STAT genes with any significant expression pattern were STAT1 and STAT5, which showed gradual increase in expression during adipogenesis (Fig. 2B). Differentiated cells were stained with Oil Red O on day 6 after the induction of differentiation (Fig. 2C). Using western blot and RT-PCR, STAT5A and STAT5B were found to be weakly expressed prior to the onset of adipogenesis, followed by an initial decrease in expression at early stages of differentiation and a gradual increase after 38 h (Fig. 2D, E).

### *C/EBPα and C/EBPβ control the expression of STAT5B, and PPARγ and RXRα control the expression STAT5A*

In a previous study, it was shown that STAT5A and STAT5B have alternative promoters in different cells types [29,30]. To determine whether STAT5A and STAT5B have at least 2 promoters in mouse preadipocytes, we analyzed STAT5A and STAT5B mRNA transcripts using RT-PCR. Figure 3 shows a map of the STAT5A and STAT5B loci, which identifies STAT5A promoters 1 (P1) and 2 (P2) and STAT5B promoters 1 (P1) and 2 (P2), as well as the locations of primer sets for RT-PCRs to distinguish between the transcripts produced by these promoters. RT-PCR of STAT5A was analyzed using primer set 1F-3R and 2F-3R, and the analysis showed that promoter 1 was used to drive the ex-

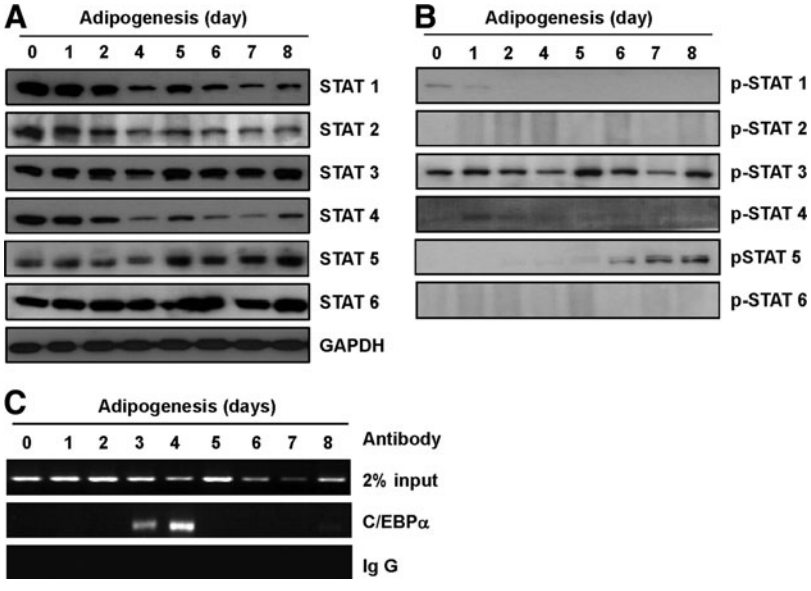


**FIG. 1.** The expression of STATs and phosphorylated STATs during hBMSCs adipogenesis. hBMSCs were induced with 0.5 mM isobutyl-methylxanthine, 1 μM dexamethasone, 200 μM indomethacin, and 5 μM insulin in DMEM-LG medium containing 10% FBS for 14 days. The expression levels of STATs **(A)** and phosphorylated STATs **(B)** were confirmed by western blot. GAPDH was used as an internal control. **(C)**: At different times after adipogenesis induction, cells were cross-linked with formaldehyde, the DNA was fragmented, and the chromatin-associated DNA was immunoprecipitated with IgG or antibodies against C/EBPα. PCR amplification of the DNA fragments was performed with specific primers flanking the C/EBP regulatory element in the STAT5B promoter. STAT, signal transducers and activators of transcription; hBMSC, human bone marrow-derived stromal cell; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; IgG, immunoglobulin; C/EBP, CCAAT/enhancer-binding protein; PCR, polymerase chain reaction.

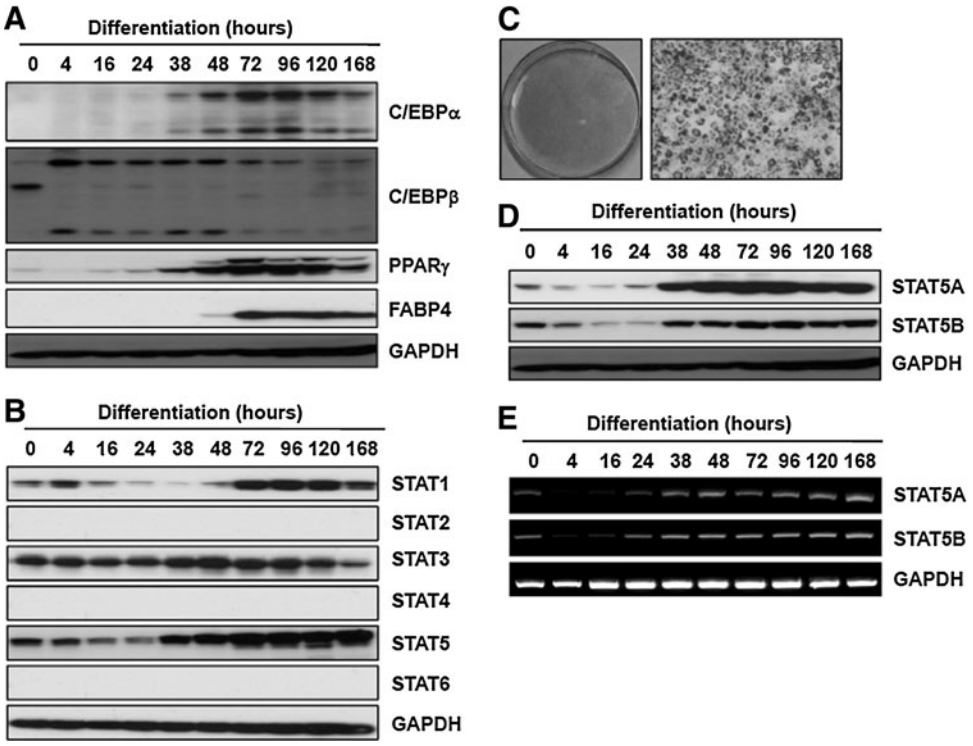


**FIG. 2.** The expression of adipocyte-specific marker genes during the differentiation of 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were induced with DMEM-HG containing 10% FBS, 1 μg/mL insulin, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine until day 2. The cells were treated with DMEM supplemented with 10% FBS and 1 μg/mL insulin for 2 days, and they were then treated every other day with DMEM containing 10% FBS. **(A)** Analysis of marker protein expression levels by western blot. 3T3-L1 cells were differentiated into adipocytes and lysed at each time point, and total protein was prepared for immunoblotting with the indicated antibodies. **(B)** The expression of STATs during adipogenesis of 3T3-L1 preadipocyte. 3T3-L1 preadipocytes were differentiated into adipocytes using hormonal stimuli. STAT expression levels were confirmed by western blot. GAPDH was used as an internal control. **(C)** Differentiated cells were stained with Oil Red O on day 6 after differentiation induction. **(D, E)** The expression of STAT5A and STAT5B during the differentiation of 3T3-L1 preadipocytes. The expression of STAT5A, STAT5B, and adipocyte marker genes was analyzed at different time points after the induction of differentiation. 3T3-L1 preadipocytes were differentiated into adipocytes. The analysis of STAT5A and STAT5B expression levels was confirmed by western blot **(D)** and RT-PCR **(E)**. GAPDH was used as an internal control. PPARγ, peroxisome proliferator-activated receptor γ; FABP4, fatty acid binding protein 4; RT-PRC, reverse transcription-polymerase chain reaction.

pression of STAT5A in 3T3-L1 preadipocytes. RT-PCR of STAT5B in 3T3-L1 preadipocytes using primer sets 4F-6R and 5F-6R showed that promoter 1 was used to drive the expression of STAT5B gene. RT-PCR of STAT5A and STAT5B in 3T3-L1 preadipocytes during 3T3-L1 adipogenesis showed that promoter 1 of STAT5A and STAT5B, respectively, were used to drive gene expression (Fig. 3).

Following the observation that C/EBPα binds to the STAT5B promoter during adipogenesis of hBMSCs (Fig. 1C), we examined luciferase assays to verify whether other transcription factors such as C/EBPα, C/EBPβ, PPARγ, or RXRα also affect the transcriptional activity of STAT5A and STAT5B through DNA binding. As shown in Fig. 4A, we observed that the transcriptional activity of STAT5A was not changed by the presence of either C/EBPα, C/EBPβ, or both C/EBPα and C/EBPβ. However, the transcriptional activity of STAT5B showed a 2–6 fold increase in the presence of either C/EBPα, C/EBPβ, or both C/EBPα and C/EBPβ. Additionally, we tested the effect of PPARγ and RXRα on the transcriptional activity of STAT5A and STAT5B. As shown in Fig. 4B, PPARγ and RXRα induced a 12-fold increase in STAT5A transcriptional activity. However, they did not induce significant changes in STAT5B transcriptional activity. Collectively, these data demonstrate that C/EBPα and C/EBPβ induce the transcriptional activity of STAT5B, and PPARγ and RXRα induce the transcriptional activity of STAT5A.

### *Identification of the element responsible for the transcriptional activation induced by C/EBPα, CEBPβ, PPARγ, and RXRα at the STAT5A and STAT5B promoters*

To precisely localize the regions in the STAT5A and STAT5B promoters targeted by C/EBPα, C/EBPβ, PPARγ, and RXRα, several truncated STAT5A and STAT5B promoter-luciferase constructs were produced (Fig. 5A, D). We

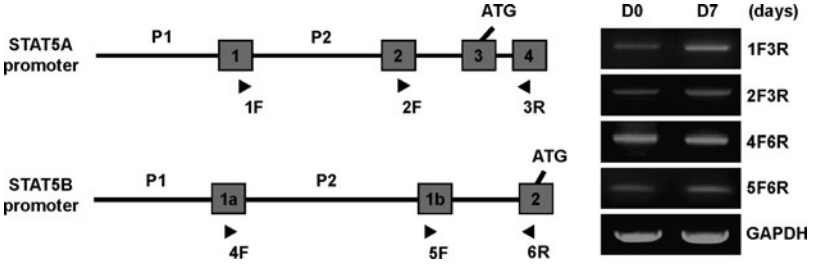


**FIG. 3.** Analysis of STAT5A and STAT5B mRNA transcripts by RT-PCR. Map of STAT5A and STAT5B showing the locations of primers 1F and 4F (specific for promoter P1), 2F and 5F (specific for promoter P2), as well as the reverse primers (3R and 6R) prior to induction and after differentiation of 3T3-L1 cells transcripts.

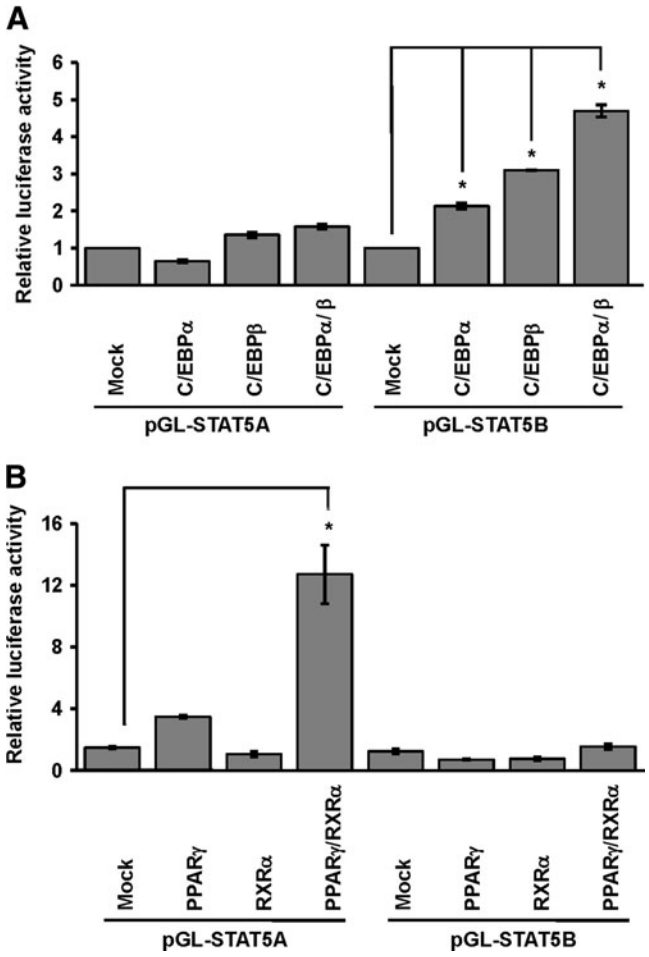


**FIG. 4.** The transcriptional activity of STAT5A and STAT5B by C/EBPα, C/EBPβ, PPARγ, and RXRα expression. NIH3T3 cells were cotransfected with each of the indicated luciferase constructs, pGL-STAT5A and pGL-STAT5B, and an overexpression vector, pCMV-C/EBPα, pCMV-C/EBPβ, pCMV-PPARγ, or pCMV-RXRα. **(A)** Transcriptional activity was measured from the STAT5A or STAT5B promoter by C/EBPα and C/EBPβ overexpression vectors using luciferase assays. **(B)** Transcriptional activity was measured from STAT5A or STAT5B promoters by PPARγ and RXRα. Results are shown as relative luciferase activities. The assays were repeated in at least 3 times. Each sample was normalized to the level of β-galactosidase activity, and *asterisk* (*) indicates $P < 0.05$ versus the mock vector group. RXRα, retinoid X receptor α.

then examined the elements responsible for C/EBPα, C/EBPβ, PPARγ, and RXRα binding to STAT5A and STAT5B promoters by performing transient transfection assays in 3T3-L1 preadipocytes and NIH3T3 fibroblasts using the respective reporter constructs. The constructs pGL-STAT5A-1500/+57-luc, −766/+57-luc, and −346/+57-luc did not show any changes in transcriptional activities, whereas the pGL-STAT5A-101/+57-luc construct lost its transcriptional response to PPARγ and RXRα (Fig. 5B, C). pGL-STAT5B-1433/+45-luc, −600/+45-luc, and −196/+45-luc did not show any changes in the transcriptional response to C/EBPα and C/EBPβ, but pGL-STAT5B-118/+43-luc lost its transcriptional response to C/EBPα and C/EBPβ (Fig. 5E, F). Additionally, PPARγ, RXRα, C/EBPα, and C/EBPβ did not affect transcriptional activity of the pGL3-basic construct.

As shown in Figs. 4 and 5, C/EBPα and C/EBPβ increased the transcriptional activity of STAT5B, whereas PPARγ increased the transcriptional activity of STAT5A. To confirm the transcriptional relevance of PPARγ in regulating STAT5A expression and the transcriptional relevance of C/EBPα and C/EBPβ in regulating STAT5B expression, 3T3-L1 preadipocytes were induced to undergo adipogenesis and ChIP analyses were performed. PPARγ was bound to the −346/−101 region of the STAT5A promoter on day 3 following the induction of differentiation (Fig. 6A). C/EBPα bound the STAT5B promoter in the −196/−118 region on days 1 and 2, and C/EBPβ bound the STAT5B promoter in the −196/−118 region on days 1, 2, and 3 of differentiation (Fig. 6B). These results indicate that C/EBPα and C/EBPβ directly interact with the STAT5B promoter and increase the transcriptional activity of STAT5B, whereas PPARγ directly interacts with the STAT5A promoter and increases the transcriptional activity of STAT5A.

To confirm the exact sequence of the PPRE binding region within the STAT5A promoter, we performed a preliminary test using 3 putative PPRE binding sequences and the previously identified PPRE binding element of the FABP4 promoter. The PPARγ and RXRα $T_NT$ mixture combined with an individual probe showed a noticeable shift in the CNTL group, and we observed the disappearance of the shifted band in the presence of competitor. Individual binding of the probes to PPARγ and RXRα occurred only with probes 1 and 2, and PPARγ and RXRα showed stronger binding to the probe 1 element sequence. No significant binding of PPARγ and RXRα was observed for probe 3 (Fig. 6C).

To locate the C/EBP binding site within the STAT5B promoter, we constructed 2 putative C/EBP sequences. Following a similar preliminary test, we identified the sequence with higher binding efficiency, which was then used for EMSA. Nuclear extracts from cells that had undergone adipocyte differentiation for 16 h were used to confirm probe binding, and the shift assay using both C/EBPα and C/EBPβ antibodies yielded a supershift in nuclear extracts from days 2 and 3. Additionally, when a known competitor, C/EBP binding sequence, was added, we observed the disappearance of the shifted band in the cold probe group (Fig. 6D). Based on the results presented in Figs. 5 and 6, the C/EBP element located at position −132 is likely to be involved in C/EBPα- and C/EBPβ-mediated activation of the STAT5A promoter, whereas the PPRE element located at positions −194 and −105 likely functions in PPARγ- and RXRα-mediated activation of the STAT5A promoter.

### *C/EBPβ is a key regulator of 3T3-L1 differentiation during adipogenesis*

We used luciferase assays and ChIP analysis to determine that STAT5A is regulated by PPARγ and RXRα; however, STAT5B is regulated by C/EBPα and C/EBPβ during the differentiation of 3T3-L1 preadipocytes. To more specifically determine the function of C/EBPβ and PPARγ, 3T3-L1 cells were transfected with C/EBPβ siRNA or PPARγ siRNA to block the expression of C/EBPβ or PPARγ and the cells were then induced to differentiate into adipocytes. The cells transfected with C/EBPβ or PPARγ siRNA had significantly lower expression levels of C/EBPβ and PPARγ compared with the cells transfected with negative control siRNA.

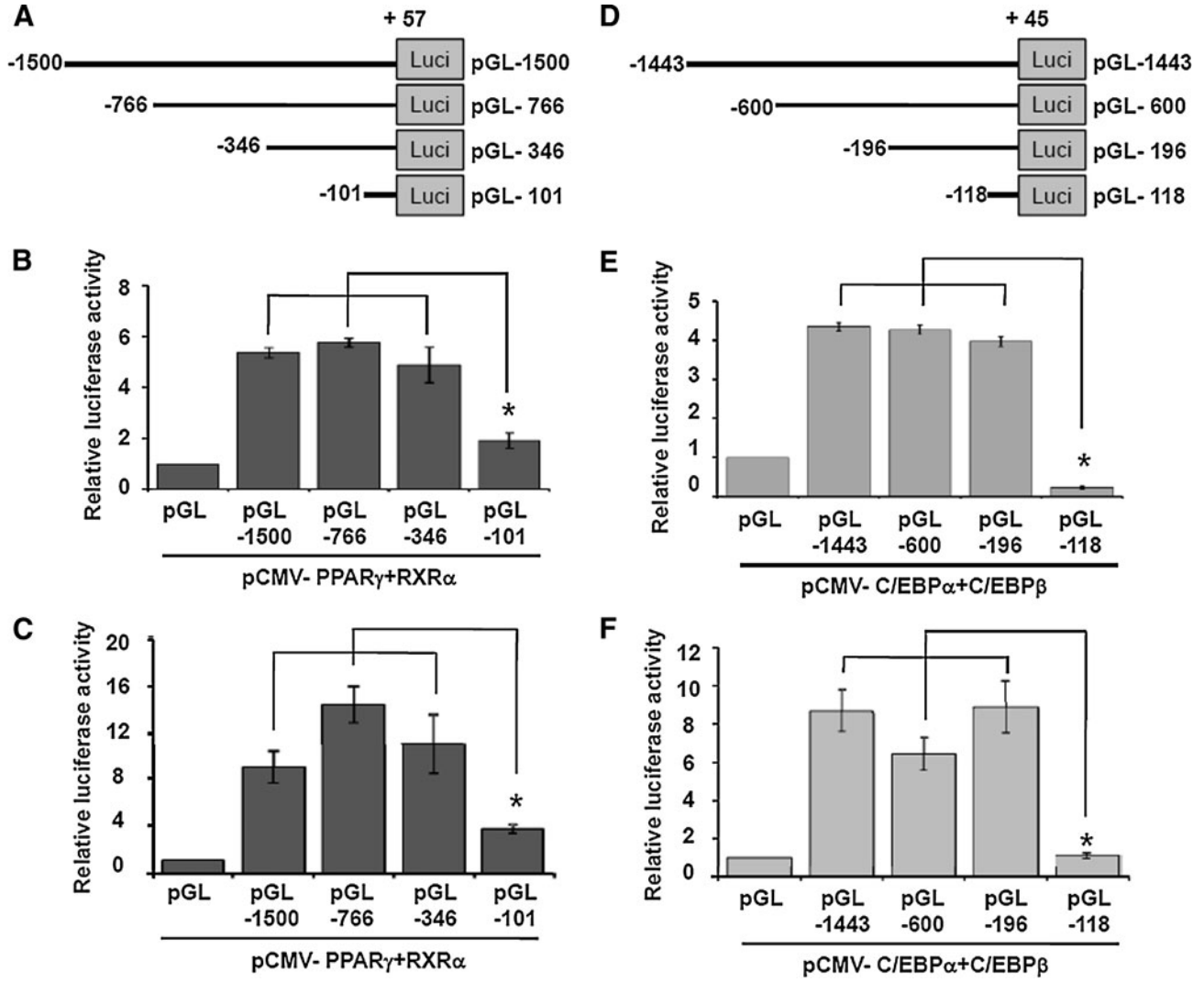


**FIG. 5.** Localization of the element in the STAT5A and STAT5B promoters responsible for transcriptional activation by C/EBPα, C/EBPβ, PPARγ, and RXRα. Schematic representations showing STAT5A promoter-luciferase constructs **(A)** and STAT5B promoter-luciferase constructs **(D)**. A luciferase reporter construct, pGL, pGL-1500, pGL-766, pGL-346, or pGL-101 (STAT5A promoter luciferase constructs), was cotransfected with PPARγ and RXRα overexpression vectors in 3T3-L1 preadipocytes **(B)** and NIH3T3 fibroblasts **(C)**. A luciferase reporter construct, pGL, pGL-1443, pGL-600, pGL-196, or pGL-118 (STAT5B promoter luciferase constructs), was cotransfected with C/EBPα and C/EBPβ overexpression vectors in 3T3-L1 preadipocytes **(E)** and NIH3T3 fibroblasts **(F)**. The assays were repeated in at least 3 times. Each sample was normalized to the level of β-galactosidase activity, and *asterisk* (*) indicates $P < 0.05$.

Additionally, C/EBPβ siRNA completely blocked differentiation, whereas the inhibition of PPARγ only partially blocked differentiation (Fig. 7A). The cells transfected with C/EBPβ siRNA showed reduced C/EBPβ expression as well as C/EBPα, PPARγ, STAT5A, STAT5B, and FABP4 expression. However, PPARγ siRNA inhibited only PPARγ, C/EBPα, and FABP4 expression and did not affect the expression of C/EBPβ or STAT5B (Fig. 7B). PPARγ siRNA partially inhibited the expression of STAT5A.

### *STAT5 is necessary for 3T3-L1 and hBMSC adipogenesis*

To assess the effects of STAT5A and STAT5B on adipogenesis, the cells were transfected with STAT5A and STAT5B overexpression plasmids. When 3T3-L1 preadipocytes were transfected with either mock, STAT5A, STAT5B, or STAT5A/STAT5B overexpression plasmids, all 3 overexpression vectors displayed an increased accumulation of lipids compared with the group treated with mock vector (Fig. 7C). To more specifically determine the function of STAT5A and STAT5B, 3T3-L1 cells were transfected with STAT5A siRNA and STAT5B siRNA to block STAT5A and STAT5B expression, followed by adipogenesis induction. To assess the progress of adipogenesis, Oil Red O staining was performed on day 6 after transfection with each siRNA. In the scramble group in which negative siRNA was transfected as a control, we were able to detect lipid droplet formation, whereas the STAT5A siRNA-treated group showed a complete lack of lipid droplets. The STAT5B siRNA-treated group displayed less lipid droplet formation compared with the control. In the STAT5A and STAT5B siRNA cotransfected group, lipid

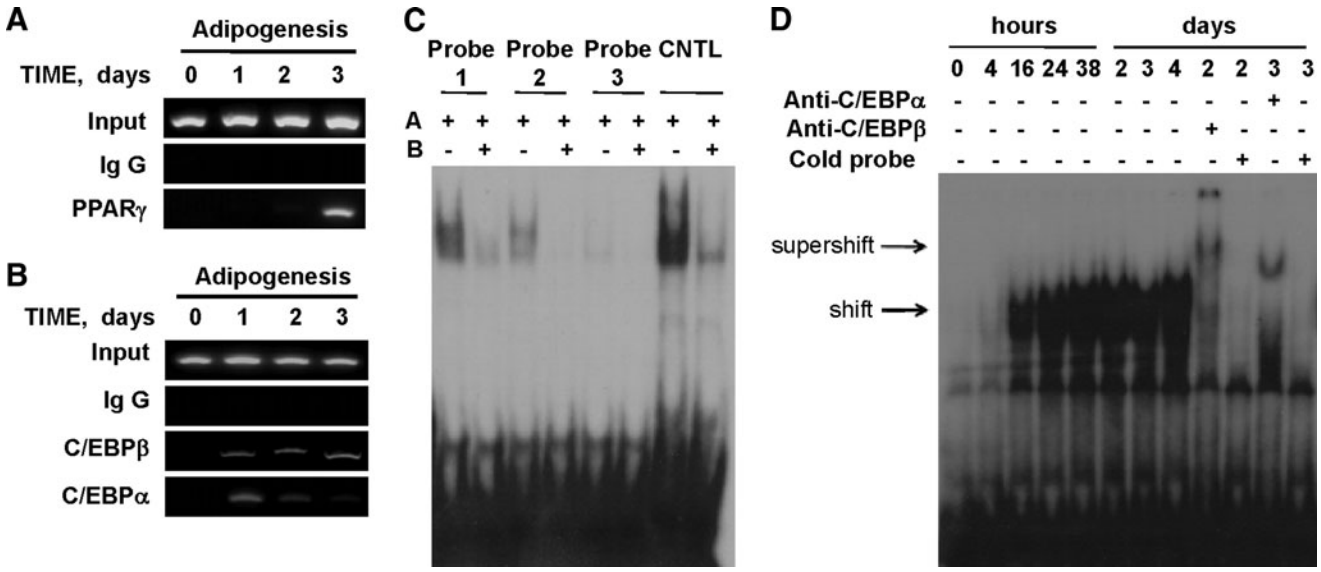


**FIG. 6.** CEBPα and C/EBPβ bind to the STAT5B promoter, and PPARγ binds to the STAT5A promoter. At different times after the induction of differentiation, cells were cross-linked with formaldehyde, the DNA was fragmented, and the chromatin-associated DNA was immunoprecipitated with IgG or antibodies against C/EBPα, C/EBPβ, or PPARγ. PCR amplification of the DNA fragments was performed with specific primers flanking the PPRE regulatory element in the STAT5A promoter **(A)** and the C/EBP regulatory element in the STAT5B promoter **(B)**. **(C)** In vitro translated PPARγ and RXRα proteins were tested by electrophoretic mobility shift analysis with the putative PPRE binding sequence on the STAT5A promoter (probe 1, probe 2, and probe 3). The PPRE regulatory element in the FABP4 promoter was used as a positive control. CNTL sequence was used to construct the cold probe. **(D)** Postconfluent 3T3-L1 preadipocytes were induced to differentiate using MDI on day 0. Nuclear extracts were prepared at various time points after induction. For the supershift assay, 2 μg of anti-C/EBPα or anti-C/EBPβ antibody was added into the reaction mixture. C/EBP regulatory element in the C/EBPα promoter was used to make the cold probe. CNTL, positive control.

droplet formation was completely inhibited similar to the group transfected with STAT5A siRNA alone (Fig. 7D). Following the confirmation that STAT5A and STAT5B play crucial roles in adipogenesis, we next asked whether STAT5A and STAT5B had similar functions in hBMSCs. In hBMSC adipogenesis, the inhibition of STAT5A and STAT5B expression by siRNA also resulted in reduced lipid droplet formation similar to what was seen for 3T3-L1 preadipocytes (Fig. 7E).

These data confirm that the importance of STAT5A and STAT5B during adipogenesis is conserved between 3T3-L1 cells and hBMSCs. Following the inhibition of STAT5A and STAT5B, we also tested the expression of various marker genes. In the scrambled group, all of the marker genes, including STAT5A, STAT5B, PPARγ, C/EBPα, C/EBPβ, and FABP4, were detected, whereas the STAT5A siRNA-transfected group showed a reduction in PPARγ, C/EBPα, and FABP4 expression. In the STAT5B siRNA-transfected group, STAT5B expression was inhibited, and FABP4 expression was reduced compared with the scramble group. When both STAT5A and STAT5B siRNAs were transfected, the expression of all marker genes except C/EBPβ was reduced (Fig. 7F). Collectively, these data indicate that the presence of STAT5A and STAT5B is crucial for differentiation; however, STAT5A may act as a particularly important factor in adipogenesis.

## Discussion

Previous studies have shown that STAT expression increases during adipogenesis. In particular, STAT5 expression increases in both mouse and human adipogenesis, and overexpression of STAT5A promotes the accumulation of triglycerides within cells [23,31,32]. However, the exact mechanism of STAT5A and STAT5B regulation remains poorly understood. Previous studies have established C/EBPα, C/EBPβ, and PPARγ as key regulators of the differentiation of 3T3-L1 preadipocytes during adipogenesis [10,13,15]. Here, we investigated the transcriptional regulation of STAT5A and STAT5B by C/EBPα, C/EBPβ, and PPARγ and examined its roles in adipogenesis. Our studies demonstrate that (i) C/EBPα and C/EBPβ transactivator activity regulates the expression of STAT5B; (ii) PPARγ and RXRα transactivator activity regulates the expression of STAT5A; (iii) inhibition of STAT5A blocks adipogenesis; and (iv) inhibition of STAT5B partially blocks adipogenesis.

In reporter assays, we found that C/EBPα and C/EBPβ activated STAT5B but not STAT5A, whereas PPARγ and RXRα controlled only STAT5A activity. We discovered a PPRE responsive region spanning bases −346 to −101 in the STAT5A promoter and a C/EBP responsive region spanning bases −196 to −118 in the STAT5B promoter. We identified 3 sites that are similar to the PPRE consensus sequence (RGGTCANRGGTCA or RGGTCANACTGGR) in the STAT5A promoter, but our EMSA analysis indicated that 2 sites at positions −301 to −277 and −206 to −183 were bound by a PPARγ and RXRα complex. Also, we identified 2 sites that were similar to the C/EBP consensus sequence (RTTGCGYAAY) in the STAT5B promoter, but only 1 site, at position −153 to −132, was bound by the C/EBPα and C/EBPβ nuclear protein complex.

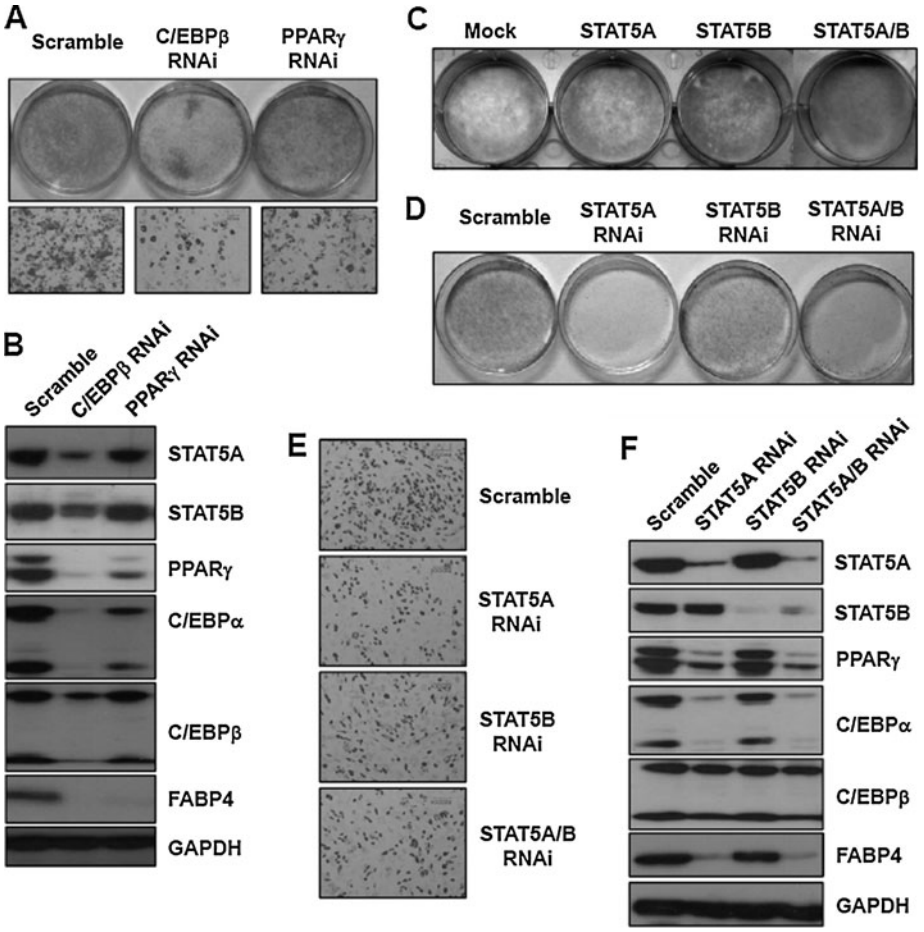


**FIG. 7.** C/EBPβ, PPARγ, STAT5A, and STAT5B expression is important during adipogenesis. **(A)** C/EBPβ RNAi, PPARγ RNAi, or scramble siRNA transfected cells were stained with Oil Red O solution on day 6 after differentiation induction. **(B)** 3T3-L1 cells were transfected with C/EBPβ RNAi (50 nM), PPARγ RNAi (50 nM), or scramble siRNA (50 nM) using Lipofectamine 2000. After transfection, the cells were induced to differentiate into adipocytes, and C/EBPβ and PPARγ knockdown was confirmed by western blot analysis on day 3. **(C)** 3T3-L1 preadipocytes were transfected with pcDNA3 empty vector (mock vector), pcDNA3-STAT5A, or pcDNA3-STAT5B and induced to differentiate without postconfluent incubation for 2 days. Transfected cells were stained with Oil Red O on day 6 after differentiation induction. **(D)** 3T3-L1 preadipocytes were transfected with STAT5A RNAi, STAT5B RNAi, STAT5A RNAi and STAT5B RNAi, or scramble siRNA and induced to differentiate without postconfluent incubation for 2 days. Transfected cells were stained with Oil Red O solution on day 6 after differentiation induction. **(E)** STAT5A RNAi (50 nM), STAT5B RNAi (50 nM), STAT5A RNAi (50 nM) and STAT5B RNAi (50 nM), or scramble siRNA (50 nM)-transfected cells were stained with Oil Red O solution on day 14 after hBMSC adipogenesis. **(F)** 3T3-L1 cells were transfected with STAT5A RNAi (50 nM), STAT5B RNAi (50 nM), or scramble siRNA (50 nM) using Lipofectamine 2000. After transfection, the cells were induced into adipocytes, and STAT5A and STAT5B knockdown was confirmed by western blot analysis on day 3. Additionally, the expression of adipogenic marker genes was confirmed by western blot analysis. GAPDH was used as an internal control.

C/EBPβ is a well-known initiation factor of adipogenesis [17], and consequently, C/EBPβ siRNA completely inhibited adipogenesis and the expression of C/EBPα, PPARγ, STAT5A, STAT5B, and FABP4. PPARγ siRNA also inhibited adipogenesis and the expression of C/EBPα and PPARγ, but it did not have any effect on the expression of C/EBPβ. Because C/EBPβ is an initiator and upstream molecule involved in differentiation, the inhibition of PPARγ did not affect the expression of C/EBPβ. In the feedback regulation between PPARγ and C/EBPα [33,34], the inhibition of PPARγ reduced C/EBPα expression.

Inhibition of STAT5A expression completely blocked adipocyte differentiation, whereas the inhibition of STAT5B only partially blocked adipocyte differentiation. Therefore, our results indicate that the expression of STAT5A is necessary, whereas the expression of STAT5B is supplementary in adipogenesis. These results support the findings of a previous study that demonstrated that the overexpression of STAT5A induces an increase in adipocyte differentiation; however, overexpression of STAT5B alone does not yield the same effects in NIH3T3 cells [22]. Additionally, STAT5A and STAT5B double knock-out mice showed somewhat reduced epidermal fat pads [35]. In the present study, the inhibition of STAT5A expression decreased C/EBPα and PPARγ expression, but not C/EBPβ expression, and the inhibition of STAT5B expression did not affect C/EBPα, C/EBPβ, or PPARγ expression. The expression of FABP4, a known target gene of PPARγ, was also reduced in the STAT5A siRNA-treated group; however, it was unaffected by STAT5B siRNA treatment. Based on this result, one can speculate that PPARγ regulates the transcriptional activity of STAT5A, which in turn regulates C/EBPα or PPARγ expression. This hypothesis is supported by other studies indicating that dominant STAT5 partially blocks the expression of C/EBPα, PPARγ, and FABP4 during adipogenesis, as shown via northern blots [36]. It is also supported by the finding that PPARγ promoters can be regulated by STAT5 [37,38]. During angiogenesis, STAT5 transactivator activity controls PPARγ expression. Additionally, heterodimer formation of the STAT5/PPARγ transcriptional complex binds to cyclin D1 and regulates its transcriptional activity [39]. In summary, STAT5A is regulated by PPARγ; however, it also directly regulates the expression of PPARγ via a feedback loop.

The RNA interference study showed the potential for STAT5 autoregulation, and we performed luciferase assays

to verify this possibility. The results indicated that STAT5B cannot be autoregulated by STAT5A or STAT5B; however, STAT5A may be autoregulated by either STAT5A or STAT5B (data not shown). These results suggest that STAT5A is initially activated by PPARγ during the early stages of adipogenesis. However, its expression may switch to autoregulation afterward. Consequently, the inhibition of PPARγ caused only a slight reduction of STAT5A expression, which further supports the idea that STAT5A expression was maintained and enforced by autoregulation, despite the initial absence of PPARγ.

## Conclusion

C/EBPα and C/EBPβ bound to the STAT5B promoter, whereas PPARγ bound to the STAT5A promoter. We showed that C/EBPα and C/EBPβ directly regulate the transcriptional activity of STAT5B, and PPARγ regulates the transcriptional activity of STAT5A. In RNA interference experiments, the expression of STAT5A was required, whereas the expression of STAT5B was supplementary to adipogenesis. These data indicate that STAT5A plays a major role in adipogenesis and STAT5B has only a supportive function.

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## Author Disclosure Statement

No competing financial interests exist.

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