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JAK2/STAT3 Pathway Is Involved in the Early Stage of Adipogenesis Through Regulating *C/EBPβ* Transcription

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ABSTRACT

Signal transducer and activator of transcription 3 (STAT3) was reported to be involved in adipogenesis. However, the regulating mechanism of STAT3 remains unclear. The present results showed that STAT3 was activated within 2-h adipogenic induction, in which the phosphorylated STAT3 translocated from cytoplasm to the nucleus. In addition, we detected Janus kinase2 (JAK2) acted upstream of the STAT3 activation at the early stage of adipogenesis. Accordingly, the JAK2 inhibitor AG490 and siRNAs led to the partial inhibition of the STAT3 activation, and the inhibition of 3T3-L1 adipocyte differentiation. Furthermore, the results based on luciferase, chromatin immunoprecipitation, and gel shift approaches indicated that STAT3 could regulate the transcription of *C/EBPβ* by binding the distal region of *C/EBPβ* promoter at the early stage of adipogenesis. Collectively, our findings reveal that JAK2/STAT3 pathway is involved in the early stage of 3T3-L1 adipocyte differentiation though regulating the *C/EBPβ* transcription. J. Cell. Biochem. 112: 488–497, 2011.

KEY WORDS: JAK2; STAT3; C/EBPβ; ADIPOGENESIS

Adipose tissue plays an important role in the regulation of metabolic homeostasis, and its abnormal growth such as obesity is associated with various metabolic diseases [Friedman, 2004; Bays, 2009]. To better understand the molecular mechanisms governing adipogenesis, 3T3-L1 cell line has been widely used as an in vitro adipogenic differentiation model [Green and Meuth, 1974; Green and Kehinde, 1975; MacDougald and Lane, 1995; Gregoire et al., 1998]. The standard differentiation process of 3T3-L1 cells involves three distinct stages: contact inhibition, mitotic clonal expansion, and terminal differentiation [MacDougald and Lane, 1995; Gregoire et al., 1998]. Proliferating 3T3-L1 preadipocytes usually first undergo 48-h contact inhibition before the adipogenic induction. Upon stimulation with a cocktail of hormones including isobutylmethylxanthine, dexamethasone and insulin (MDI), the growth-arrested 3T3-L1 cells re-enter a period of cell cycle called mitotic clonal expansion. About 2 days later, the cells enter the terminal differentiation stage. Approximately 4 days after induction, most of the cells display the lipid-visualized adipocyte phenotype.

During the past decades, a number of reports have revealed that adipogenesis is controlled by a complicated network of transcription factors in which CCAAT-enhancer binding proteins (C/EBPs) and peroxisome proliferator-activated receptor-γ (PPARγ) play critical roles [Cao et al., 1991; MacDougald and Lane, 1995; Gregoire et al., 1998; Cowherd et al., 1999; Lane et al., 1999; Farmer, 2006]. Among the transcription factors, C/EBPβ and C/EBPδ are induced at the early stage of adipogenesis, which subsequently stimulate the transcription of C/EBPα and PPARγ. However, little is known about what kind of transcription factors controls these early regulators such as C/EBPβ, whereas some reports showed the early involvements of cAMP regulatory element-binding proteins (CREB), Krüppel-like factor 4 (KLF4) and Krox20 [Zhang et al., 2004; Chen

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et al., 2005; Birsoy et al., 2008]. Therefore, the identification of factors that regulate the early differentiation-related genes will provide insights into the mechanisms regulating the initiation of adipogenesis.

JAK/STAT signaling pathway has been studied extensively in cytokine signaling during immune responses [Leonard and O'Shea, 1998; Kisseleva et al., 2002; Levy and Darnell, 2002]. The Janus kinase (JAK) family comprises four members: JAK1, JAK2, JAK3, and Tyk2, whereas the STAT family has seven members: STAT1-4, STAT5a/b, and STAT6. In 1996, Stephens et al. first explored the expression and regulation of STATs during adipogenesis. They found that signal transducer and activator of transcription 3 (STAT3) expression was markedly elevated in adipogenesis, whereas its up-regulation was unaffected when adipogenesis was inhibited by TNFα, suggesting that STAT3 expression might be not tightly regulated during adipogenesis [Stephens et al., 1996]. Later, Deng et al. [2000] showed that STAT3 was activated and bound to DNA during proliferative phases of 3T3-L1 adipogenesis, and a protein inhibitor of activated STAT3 (PIAS3) could inhibit adipogenic gene expression [Deng et al., 2006], suggesting that STAT3 might function in 3T3-L1 adipocyte differentiation. This conclusion was confirmed by a recent study, in which STAT3 regulated adipocyte differentiation via PPARγ [Wang et al., 2009]. However, the precise molecular mechanism of this transcription factor during adipogenesis remains unclear.

In the present study, the regulating mechanism of STAT3 was analyzed in depth during 3T3-L1 adipogenesis. STAT3 was proved to directly regulate the transcription of *C/EBPβ* gene by binding to the distal region of its promoter. Furthermore, our results indicated that JAK2 acted upstream of STAT3. Collectively, the findings indicate that JAK2/STAT3 pathway is involved in the early stage of 3T3-L1 adipocyte differentiation through regulating the expression of *C/EBPβ*.

MATERIALS AND METHODS

PLASMIDS AND REAGENTS

STAT3 expression plasmid was a gift from Dr. Zhenguo Wu (Hong Kong University of Science and Technology). LAP/C/EBPβ (NC_000068.6) promoter (nucleotides −2,912 to +33) construct was made by PCR and cloned into pGL3 basic luciferase reporter expression vector (Promega). Cell-Permeable STAT3 inhibitor peptide (STAT3 inhibitor) was purchased from Calbiochem. Tyrphostin AG 490 (AG 490) was purchased from Sigma. The following antibodies were used: anti-STAT3 (Cell Signaling), anti-Tyr $(P)^{705}$-STAT3 (Santa Cruz), anti-actin (Santa Cruz), anti-aP2 (R&D Systems), anti-STAT6 (Cell Signaling), anti-α-tubulin (Sigma), anti-LAMIN B (Santa Cruz), anti-JAK2 (Santa Cruz), anti-Tyr$(P)^{1007}$-JAK2 (SAB), anti-Glut4 (Cell Signaling).

CELL CULTURE AND DIFFERENTIATION

3T3-L1 cells were maintained and differentiated in a 37°C incubator with 10% CO_2 as previously described [Qiu et al., 2001; Guo et al., 2009]. Briefly, the cells were grown up to the contact inhibition stage and remained in the post-confluent stage for 2 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf bovine serum (Gibco), 32.8 μM biotin (Sigma), 16.8 μM Ca-pantothenate (Sigma), and penicillin–streptomycin (Gibco). Differentiation was then induced by changing the medium to DMEM supplemented with 10% fetal bovine serum (FBS; Gibco) plus a cocktail of hormones (MDI) that includes 0.5 mM 3-isobutyl-1-methylaxanthine (Sigma), 1 μM dexamethasone (Sigma), and 1.7 μM insulin (Sigma). After 48-h (day 2) MDI-induction, the differentiation medium was replaced with DMEM supplemented with 10% FBS and 1.7 μM insulin. The cells were fed every other day with DMEM containing 10% FBS until day 8. NIH 3T3 cells were cultured in DMEM supplemented with 10% calf bovine serum in a 37°C incubator with 5% CO_2.

OIL-RED-O STAINING AND QUANTITATIVE MEASUREMENT

Differentiated 3T3-L1 adipocyte monolayers were washed three times with phosphate-buffered saline (PBS) and then fixed with 3.7% formaldehyde in PBS. 0.5% Oil-Red-O (Sigma) in isopropanol was diluted with 2/3 volumes of water, filtered and added to the fixed cell monolayers for 1 h at room temperature. The cell monolayers were washed and visualized directly or by microscopy. Quantification of Oil-Red-O staining was carried out by extracting Oil-Red-O-stained triglyceride droplets with 100% isopropanol and then the extracts were analyzed by a spectrophotometer at OD 510.

TRANSFECTION AND LUCIFERASE REPORTER GENE ASSAYS

Transient transfections were carried out using Lipofectamine™ 2000 reagent (Invitrogen, 11668-019). For luciferase reporter gene assays, NIH 3T3 cells were plated into the wells of 24-well plates, and the cells were co-transfected with either pcDNA3 or STAT3-pcDNA3 expression plasmid and pC/EBPβ-luc. pRL-SV40 that carries renilla luciferase was also transfected as an internal control for transfection efficiency. After 24-h transfection, the cells were lysed and subjected to analysis of firefly and renilla luciferases with the Promega dual luciferase reporter assay system (Promega). A promoter fragment of *C/EBPβ* (from −2,912 to +33 bp according to the transcription start-site) and a series of 5′-truncated promoter fragments were cloned into the pGL3 basic luciferase reporter vector. The promoter–luciferase constructs were co-transfected with STAT3 expression vector and pRL-SV40 plasmid into NIH 3T3 cells when cells reached about 80% confluence.

CYTOPLASMIC AND NUCLEAR EXTRACTS PREPARATION

For preparation of whole cell extracts, the cells were rinsed with PBS and harvested with 1× SDS Lysis Buffer. Protein concentration of the cell lysates was measured by Lowry assay. Proteins were separated by SDS–PAGE and transferred to PVDF membranes. Following transferring, the membrane was blocked in 5% milk for 1 h at room temperature and probed with individual antibody. Cytoplasmic and nuclear extracts were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo). The procedure was carried out according to the manufacturer's instructions. After separation, the components were prepared with 5× SDS Lysis Buffer followed by Western-blotting analysis.

Tubulin and actin are widely used as a loading control for Western-blotting analysis. However, it has been reported that the expression of tubulin and actin decreased during adipogenesis

[Spiegelman and Farmer, 1982]. Therefore, STAT6, as previously reported [Stephens et al., 1996], was used as a loading control in this study.

CONFOCAL MICROSCOPY

3T3-L1 cells were first fixed with 3.7% formaldehyde in PBS for 15 min at room temperature and then permeabilized with ice-cold 100% methanol for 10 min at −20°C. Next, the cells were blocked with 3% BSA in PBS for 30 min at room temperature followed by incubation with the primary antibody against STAT3 (1:50) overnight at 4°C. Appropriate secondary antibody was added to the cells together with 4′,6′-diamino-2-phenylindole (DAPI) for 1 h at room temperature. The images were taken by a Leica TCS SP2 + ABOS confocal fluorescence microscope system using an HC × PL Apo × 63 oil immersion objective (NA = 1.4).

SILENCING BY siRNA

For small interfering RNA (siRNA) duplexes transfection, the 2-day post-confluent 3T3-L1 cells were transfected with 100 nM chemically synthesized siRNA using Lipofectamine^{TM} 2000. We designed three pairs of siRNAs for STAT3 and JAK2, respectively. The following siRNAs (top strand sequence is shown) were used: STAT3, 3-829 (5′-CCGCCAACAAAUUAAGAAATT-3′), 3-1067 (5′-GAGUUGAAUUAUCAGCUUATT-3′), 3-1134 (5′-GAGGGUCUCGGAAAUUUAATT-3′); JAK2, JAK2-1 (5′-GCAAACCAGGAAUGCUCAA-3′), JAK2-2 (5′-GGAAUGGCCUGCCUUACAA-3′), JAK2-3 (5′-GGACAAAGAAUACUACAAATT-3′). A scramble-sequence (5′-UUCUCCGAACGUGUCACGUTT-3′) was synthesized as a internal control. The efficiency of siRNA knockdown was confirmed by Western-blotting assay. Chemically synthesized siRNA was made by Gene Pharma.

CHROMATIN IMMUNOPRECIPITATION (ChIP)

ChIP experiment was performed in 3T3-L1 cells using a kit (Upstate Biotechnology) according to the manufacturer's instructions. Briefly, the 2-day post-confluent cells were treated with MDI for 2 h followed by being cross-linked with 1% formaldehyde for 10 min at 37°C. The cell pellets were resuspended in SDS lysis buffer. After incubation for 10 min in ice, the cell lysates were sonicated. The supernatant was diluted in ChIP dilution buffer and precleared with 80 μl of salmon sperm DNA/protein A agarose–50% slurry for 30 min at 4°C, and then incubated overnight at 4°C with STAT3 antibody or IgG. The immune complexes were recovered by the addition of 60 μl of salmon sperm DNA/protein A agrose–50% slurry and incubation for 1 h at 4°C. Agarose beads were pelleted and sequentially washed with low salt buffer, high salt buffer, LiCl buffer, and TE buffer (Tris, 10 mM, pH 8.0; EDTA, 1 mM), respectively. Then, the immune complexes were eluted by incubation with 250 μl of fresh elution buffer (1% SDS, 0.1 M $NaHCO_3$) for 15 min at room temperature. To reverse the cross-linking of DNA, 5 M NaCl were added and incubated for 4 h at 65°C. After treatment with proteinase K for 1 h at 45°C, the DNA fragments were recovered and resuspended in 50 μl of TE buffer. The following primers based on C/EBPβ promoter sequences were designed for PCR: P1 forward (beginning at −2,564 bp), 5′-CACACCAGGCACACCAAGCACAC-3′, P1 reverse (ending at −2,288 bp), 5′-CCACGGGGAGGCCAGAGGAT-3′; P2 forward (beginning at −2,744 bp), 5′-AAAAAGAGGGCTGGGGTGTAG-3′, P2 reverse (ending at −2,560 bp), 5′-GTGTGCCTC TTTTCTTTCCTCTTC-3′; P3 forward (beginning at −1,662 bp), 5′-GACCTGTGACAGTCAGCGAA-3′, P3 reverse (ending at −1,489 bp), 5′-ATCTCACCTGGCCTGTCATC-3′; P4 forward (beginning at −1,690 bp), 5′-CAAT ACCCAGGACCCATCTG-3′, P4 reverse (ending at −1,489 bp), 5′-ATCTCACC TGGCCTGTCATC-3′; P5 forward (beginning at −81 bp), 5′-GCCCTCTCGCGCTC-3′, P5 reverse (ending at +90 bp), 5′-GGCTCCGCTGCGTC-3′.

ELECTROPHORETIC MOBILITY-SHIFT ASSAY (EMSA)

EMSA was performed according to the manufacturer's instructions (PIERCE 20148). The post-confluent 3T3-L1 cells were induced by MDI for 2 h and then were collected for nucleocytoplasmic separation. The nuclear extracts were used for the EMSA experiment. The DNA sequences used for EMSA are as follows: Seq-1 forward (5′-GCTGGGGTCTGGTAGCACAGGCCTGTGATTCCAGCTGCTCTGGA GGTTGA-biotin-3′); Seq-2 forward (5′-GGAATGAATTCACTTTAAAGGGACAGCTGGGGTCTGG TAGCACAGGCCTG-biotin-3′); The competitor (wt) forward (5′-GGAATGAATTCACTTTAAA GGGACA-3′); competitor (mut-1) forward (5′-GGCCGGAATTCACTTTAAAGGGACA-3′); competitor (mut-2) forward (5′-GGAATGCCGTCACTTTAAAGGGACA-3′); competitor (mut-1/2) forward (5′-GGCCGGCCGTCACTTTAAAGGGACA-3′).

QUANTITATIVE REAL-TIME PCR

Total RNA of 3T3-L1 cells was isolated by TRIzol reagent (Invitrogen) according to the manufacturer's instructions. About 1.5 μg of total RNA from each sample was reversed transcribed into cDNA using the ReverTra Ace qPCR RT Kit (Toyobo), and real-time PCR was performed using the SYBR Green Realtime PCR Master Mix (Toyobo) and ABI-7500 fast detection system (Applied Biosystems). The following primers were used for detection of the gene expression: *C/EBPβ* (forward, 5′-ACCGGGTTTCGGGACTTGA-3′; reverse, 5′-GTTTCGATATCACTGGAG-3′), *GAPDH* (forward, 5′-TGGCAAAGTGGAGATTGTTGCC-3′; reverse, 5′-AAGATGGTGATGGGCTTCCCG-3′). All real-time PCRs were done with the following conditions: 10 min at 95°C and then 40 cycles of 15 s at 94°C, 30 s at 62°C, and 20 s at 72°C.

STATISTICAL ANALYSIS

All values are mean ± s.e.m. For statistical analysis, the P value was calculated using a two-tailed unpaired Student's t-test with $P < 0.05$ being considered statistically significant.

RESULTS

THE REQUIREMENT OF STAT3 FOR ADIPOGENESIS DURING THE FIRST 2 H AFTER THE DIFFENTIATIONAL INDUCTION

We examined the expression profile of STAT3 during the adipogenic progression of 3T3-L1 cells. The results showed that STAT3 proteins were constitutively expressed from days 0 to 8 but dramatically phosphorylated in the first 2 days after MDI stimulation of post-confluent 3T3-L1 preadipocytes (Fig. 1A). To detect whether STAT3 involves in adipogenesis, we analyzed the relationship between STAT3 and adipogenesis by RNA interference assay. The effect of

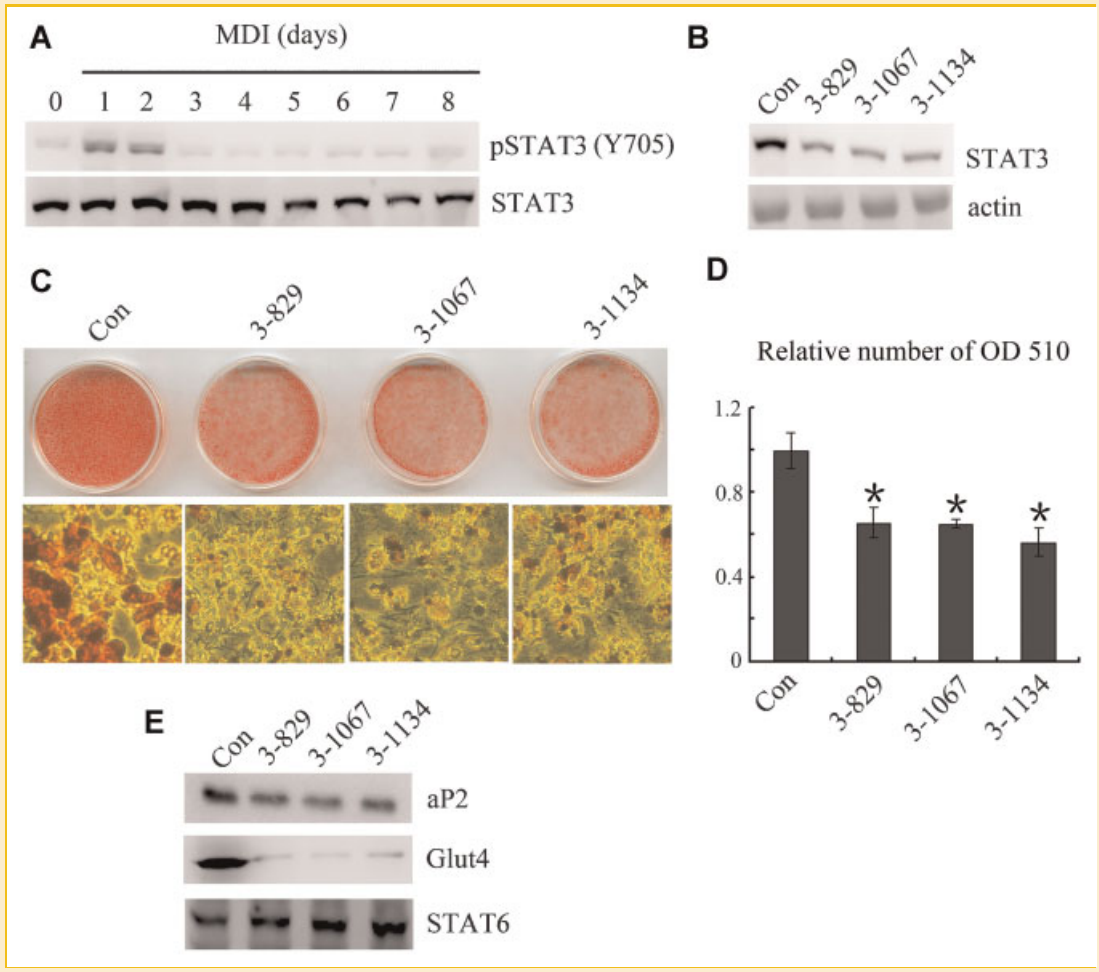

Fig. 1. STAT3 is required for 3T3-L1 adipocyte differentiation. A: The expression profiles of STAT3 and its phosphorylation statue (pSTAT3 Y705) during 3T3-L1 preadipocyte differentiation. Whole cell extracts were prepared from 3T3-L1 cells at various time points following MDI induction. B: Down-regulation of STAT3 by RNAi assay. Post-confluent cells were transfected with either a scramble-siRNA (Con) or STAT3-siRNAs as indicated. The expression profiles of STAT3 after 24-h transfection were detected by Western-blotting assay. Actin served as a loading control. C–E: STAT3 is required for 3T3-L1 adipocyte differentiation. On day 8, the differentiated cells were stained with Oil-Red-O (C). The Oil-Red-O staining cells were extracted with isopropanol and measured at OD 510 (D). The data are presented as means ± s.e.m. from three independent experiments. Significance is determined by two-tailed unpaired Student's *t*-test ($^*P < 0.01$ vs. Con). For Western blotting of aP2 and Glut4, whole cell extracts were subjected to Western-blotting assay, and STAT6 served as a loading control (E).

STAT3 knocking down was shown in Figure 1B. After being transfected with three different STAT3-specific siRNAs, respectively, 3T3-L1 cells were subjected to adipogenesis induced with the MDI cocktail, and then the differentiated cells were measured with Oil-Red-O assay. The results showed that the knockdown of STAT3 by siRNAs led to a strong reduction of 3T3-L1 adipocyte differentiation (Fig. 1C,D). In addition, adipocyte-specific markers, aP2 and Glut4 proteins, in these cells were also assessed by Western-blotting assay. As shown in Figure 1E, the expression of aP2 and Glut4 was significantly reduced in the cells treated with STAT3-siRNAs. Taken together, these results illustrate that STAT3 is required for 3T3-L1 adipocyte differentiation.

Since phosphorylated STAT3 was only elevated at the early stage of adipocyte differentiation (Fig. 1A), implying a functional role during the early stage of adipogenesis, we further examined the expression level and the phosphorylation status of STAT3 proteins in the first 2 h after MDI induction by Western-blotting assay. The result showed that phosphorylated STAT3 was gradually increased within the first 2 h after MDI induction, whereas the total protein level of STAT3 remained constant (Fig. 2A). Previous studies reported that the activation of STAT3 by tyrosine phosphorylation (Y705) conferred its translocation from the cytoplasm to the nucleus [Leonard and O'Shea, 1998; Kisseleva et al., 2002; Levy and Darnell, 2002]. So we analyzed the subcellular localization of STAT3 protein by Western-blotting and immunofluorescence assays. The results showed that amount of STAT3 protein in cytoplasmic extracts decreased at 2 h after the MDI induction, whereas the increased STAT3 was detected in the nuclear extracts (Fig. 2B). The cytoplasmic tubulin and nuclear LAMIN B were also subjected to Western-blotting analysis as a control for the subcellular localization (Fig. 2B). Furthermore, in agreement with this observation, immunofluorescence analysis displayed a similar STAT3 transloca-

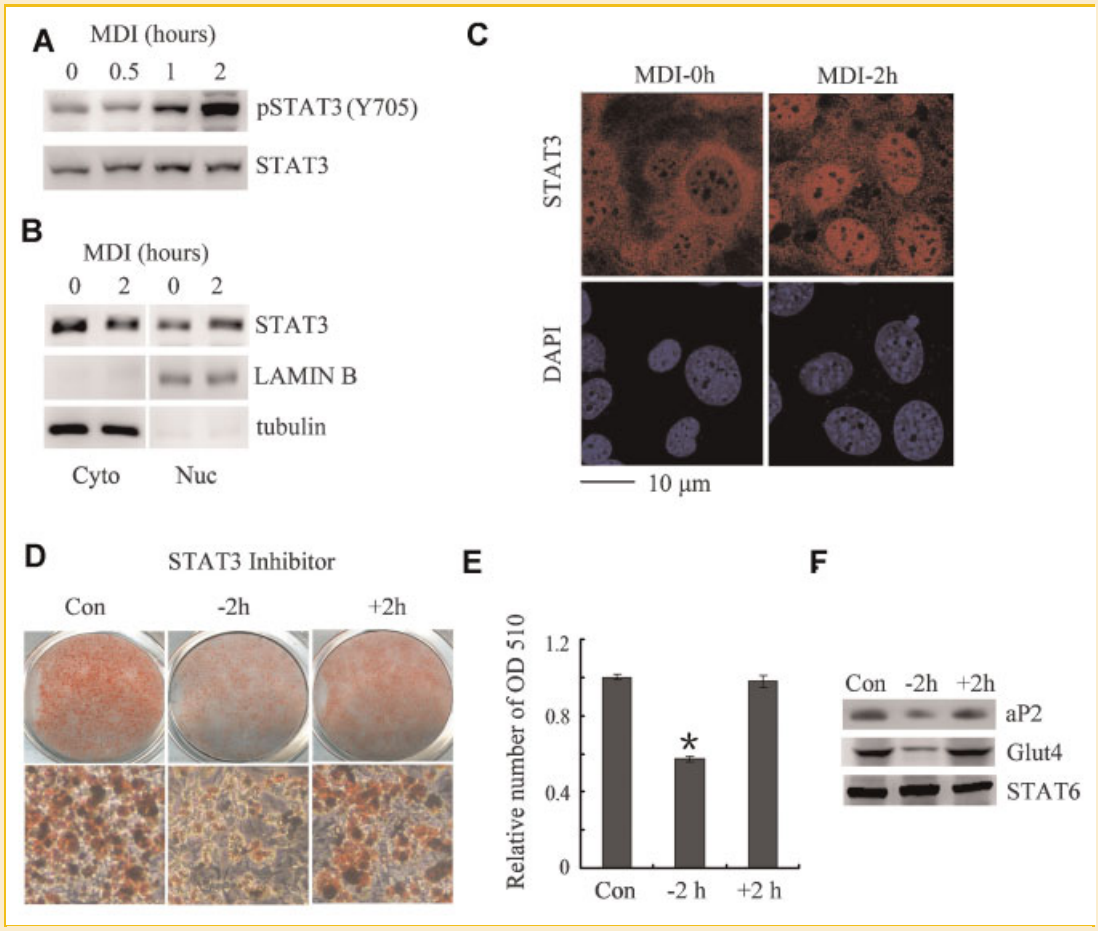

Fig. 2. STAT3 activation is required for adipogenesis during the first 2 h of MDI induction. A: Tyrosine phosphorylation of STAT3 during the first 2 h of MDI induction. The level of pSTAT3 (Y705) and STAT3 were determined by Western-blotting assay. B,C: Nuclear localization of STAT3 tyrosine phosphorylation. The level of STAT3 in cytoplasmic and nuclear extracts was determined by Western-blotting assay, and β-tubulin served as a cytoplasmic marker and LAMIN B served as a nuclear marker (B). The cells were fixed with 3.7% formaldehyde at the indicated time points following the MDI induction. Then the location of STAT3 was determined by immunofluorescence microscopy. 4′,6′-diamino-2-phenylindole (DAPI) was added to counterstain the nuclei (C). D–F: STAT3 activation is required for adipogenesis during the first 2 h of MDI induction. The cells were treated with a cell-permeable Stat3-inhibiting peptide (Calbiochem Cat. No. 573096) for 2 days, which was administered either at 2 h before the MDI induction (−2 h) or at 2 h after the post-induction (+2 h). On day 8, differentiated cells were stained with Oil-Red-O (D). The Oil-Red-O staining cells were extracted with isopropanol and measured at OD 510 (E). The data are presented as means ± s.e.m. from three independent experiments. Significance is determined by two-tailed unpaired Student's *t*-test (*$P < 0.01$ vs. Con). For Western blotting of aP2 and Glut4, whole cell extracts were subjected to Western-blotting assay, and STAT6 served as a loading control (F).

tion pattern from cytoplasm to the nucleus at 2 h after MDI induction (Fig. 2C). Collectively, STAT3 proteins in 3T3-L1 cells are significantly phosphorylated within 2 h after MDI induction, and then translocated to the nucleus.

We further explored the role of the activation of STAT3 for adipogenesis during this first 2-h stage of MDI induction. The post-confluent 3T3-L1 cells were administered with a cell-permeable STAT3-inhibiting peptide either at 2 h before MDI induction (−2 h group) or at 2 h after the post-induction (+2 h group), respectively. Then these two kinds of the drug-treated cells were continuously incubated in the culture medium containing the STAT3 inhibitor for 2 days. On day 8 of the post-induction, differentiated cells were measured by Oil-Red-O assay (Fig. 2D,E), and by Western-blotting assay of aP2 and Glut4 expression (Fig. 2F). The results showed that adipogenesis of "−2 h" group was significantly inhibited by the STAT3 inhibitor, whereas "+2 h" group had less inhibition for their differentiation (Fig. 2D–F). Taken together, these results indicate that STAT3 is activated in the first 2-h stage after MDI induction, and then mainly plays its role for adipogenesis at this early stage.

JAK2 IS THE UPSTREAM REGULATOR OF STAT3 ACTIVATION

JAK proteins have been well recognized as the upstream regulators of STATs. Of the four JAK family members, JAK2 was recently found to be involved in myogenic differentiation through regulating STAT3 activation [Wang et al., 2008], so we asked whether JAK2 was the upstream regulator of STAT3 during 3T3-L1 adipogenesis. Firstly, the activation status of JAK2 in 3T3-L1 cells at the early stage of adipocyte differentiation was analyzed by Western-blotting assay. As indicated in Figure 3A, JAK2 was phosphorylated at the first half hour after MDI induction, suggesting that JAK2 acts as an upstream factor of STAT3 since the time of JAK2 activation is prior to that of STAT3 activation (compare Figs. 3A and 2A). Secondly,

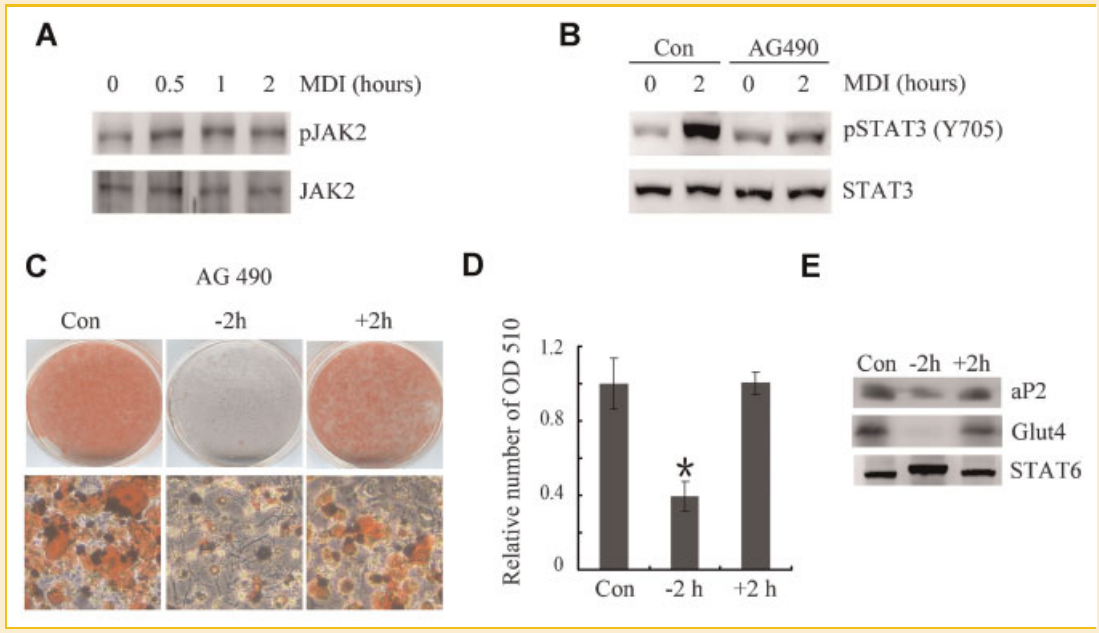

Fig. 3. JAK2 is the upstream regulator of STAT3 activation. A: Phosphorylation of JAK2 during the first 2 h of MDI induction. The level of pJAK2 and JAK2 were determined by Western-blotting assay. B: Inhibition of JAK2 activity by AG490 (20 μM) results in the decrease of STAT3 phosphorylation. C–E: JAK2 activation is required for adipogenesis during the first 2 h of MDI induction. The cells were administered either at 2 h before the MDI induction (−2 h) or at 2 h after the post-induction (+2 h). On day 8, differentiated cells were stained with Oil-Red-O (C). The Oil-Red-O staining cells were extracted with isopropanol and measured at OD 510 (D). The data are presented as means ± s.e.m. from three independent experiments. Significance is determined by two-tailed unpaired Student's *t*-test (*$P < 0.01$ vs. Con). For Western blotting of aP2 and Glut4, whole cell extracts were subjected to Western-blotting assay, and STAT6 served as a loading control (E).

STAT3 could not be activated when JAK2 of the post-confluent 3T3-L1 cells was inhibited by AG490, a known inhibitor of JAK2 [Freedman et al., 1996], before MDI induction (Fig. 3B). Collectively, these results argue that JAK2 is, at least partially, the up-activator of STAT3 by phosphorylating STAT3 at the early stage of 3T3-L1 adipocyte differentiation.

Since our results indicate that the function of STAT3 in the first 2-h stage after MDI induction is required for adipogenesis (Fig. 2D–F), JAK2 as the upstream regulator of STAT3 should be involved in adipogenesis in the same time segment. Therefore, the post-confluent 3T3-L1 cells were administrated with AG490 either at 2 h before MDI induction (−2 h group), or at 2 h after MDI induction (+2 h group), respectively. The results showed that the adipocyte differentiation was significantly inhibited when the drug was added at 2 h before MDI induction, whereas no such inhibition was detected if the drug was added at 2 h after MDI induction (Fig. 3C–E, compare "−2 h" to "+2 h"). Taken together, these findings demonstrate that the effect of JAK2/STAT3 pathway on adipogenesis largely depends on its function within the first 2 h of the post-induction.

We further confirmed the JAK2 role for adipogenesis based on the treatment of JAK2 siRNAs (Fig. 4A). The results showed that the knockdown of JAK2 gene expression by JAK2 siRNAs inhibited adipogenesis of 3T3-L1 cells (Fig. 4B–D), which was consistent with the results of AG490 treatment (Fig. 3C–E). Furthermore, we also found that the knockdown of JAK2 by siRNAs could reduce the activation of STAT3 (Fig. 4E), which is also consistent with the AG490-treated result (Fig. 3B). Collectively, these results indicate that the activation of JAK2 is required for STAT3 activation at the early stage of 3T3-L1 adipogenesis.

STAT3 DIRECTLY REGULATES THE *C/EBPβ* TRANSCRIPTION

STAT3 has been reported as both a signal transducer and a transcription factor. In the above study, we had showed that STAT3 as a signal transducer was regulated by JAK2. To verify what kind of target genes STAT3 as a transcription factor would modulate, we compared the transcription patterns of well known adipogenic factors such as C/EBPs, PPARs, aP2 with the activation pattern of STAT3 after MDI induction. The results showed that the *C/EBPβ* expression was up-regulated at the first hour of the post-induction (Fig. 5A), which was kinetically similar to STAT3 activation (Fig. 2A). To confirm this observation, we further analyzed the *C/EBPβ* expression by knockdown of STAT3 with siRNAs in 3T3-L1 cells. The results showed that the inhibition of *C/EBPβ* mRNA and protein levels by the STAT3-siRNAs was also within the first 2 h of the post-induction (Fig. 5A,B), suggesting that STAT3 participates in the regulation of the *C/EBPβ* expression during the early stage of adipogenesis.

To further understand the regulation relationship between STAT3 and C/EBPβ, we first constructed the luciferase reporters containing a promoter fragment of *C/EBPβ* and a series of 5′-truncated promoter fragments, respectively (see Materials and Methods Section). And then, NIH3T3 cells were co-transfected with the luciferase reporters and STAT3 expression plasmid, in which STAT3 was overexpressed. After 24-h transfection, the cells were lysed and

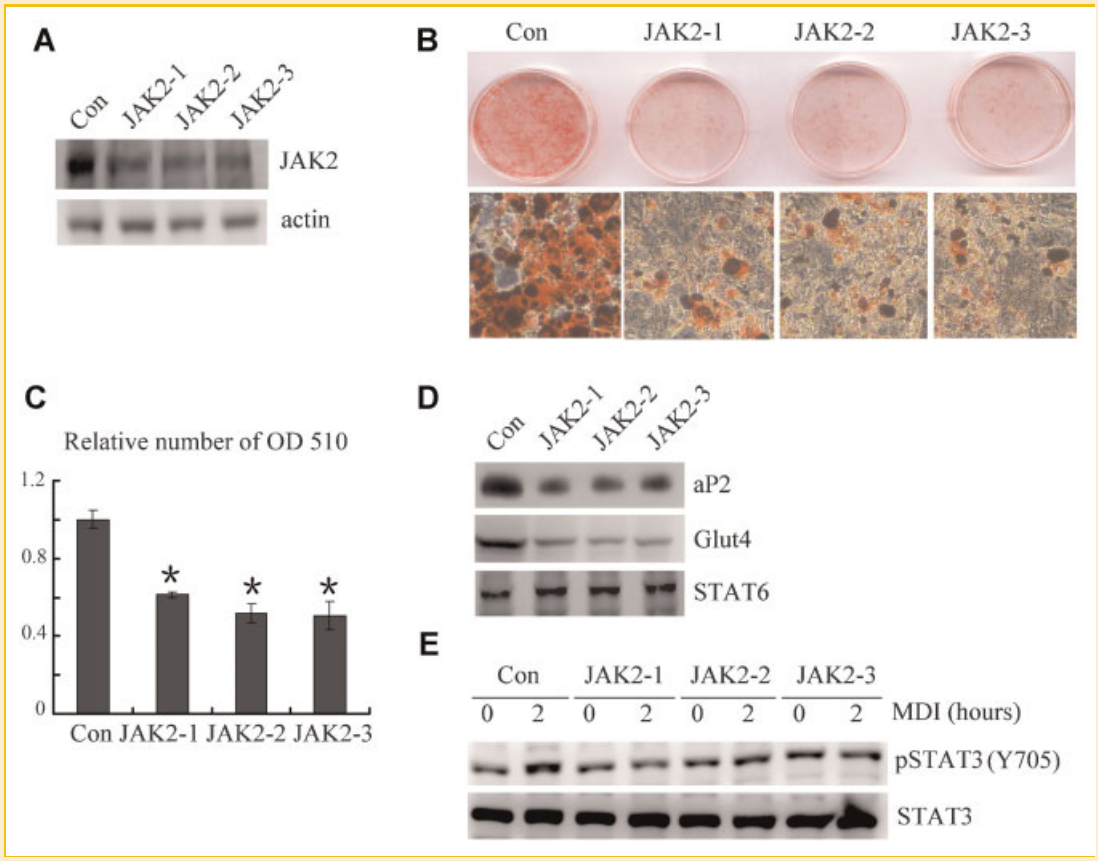

Fig. 4. Knockdown of JAK2 with RNAi assay inhibits 3T3-L1 adipocyte differentiation. Two-day post-confluent cells were transfected with either a scramble-siRNA (control) or JAK2-siRNA as indicated for 24 h followed by MDI induction. A: Post-confluent cells were transfected with either a scramble-siRNA (Con) or JAK2-siRNAs as indicated. The expression profiles of JAK2 after 24-h transfection were detected by Western-blotting assay. Actin served as a loading control. B–D: JAK2 is required for 3T3-L1 adipocyte differentiation. On day 8, the differentiated cells were stained with Oil-Red-O (B). The Oil-Red-O staining cells were extracted with isopropanol and measured at OD 510 (C). The data are presented as means ± s.e.m from three independent experiments. Significance is determined by two-tailed unpaired Student's *t*-test ($^*P < 0.01$ vs. Con). For Western blotting of aP2 and Glut4, whole cell extracts were subjected to Western-blotting assay, and STAT6 served as a loading control (D). E: Inhibition of JAK2 activity by RNAi assay results in the reduction of STAT3 phosphorylation. Whole cell extracts were prepared as indicated and subjected to immunoblotting with P-STAT3 (Y705) antibody.

subjected to luciferase assay (see Materials and Methods Section). The results showed that the strong induction of the luciferase reporter gene was observed when the whole fragment of promoter between −2,912 and +33 was used, whereas the reporter activity was abolished when the segment between −2,912 and −2,812 was deleted, indicating that the region located between −2,912 and −2,812 is involved in mediating transcription of *C/EBPβ* regulated by STAT3 (Fig. 5C).

ChIP and gel shift assays were further applied to investigate the association of endogenous STAT3 with *C/EBPβ* promoter. The post-confluent 3T3-L1 cells induced with MDI for 2 h were treated with formaldehyde to cross-link DNA–protein complexes, and then subjected to ChIP analysis (see Materials and Methods Section). The results showed that STAT3 was found to be enriched with the distal segment of *C/EBPβ* promoter, whereas there was no association of STAT3 with the promoter of *C/EBPβ* prior to MDI induction or with the proximal region of *C/EBPβ* promoter post-induction (Fig. 5D). The gel shift results shown in Figure 5E indicated that STAT3 bound to the region between −2,887 and −2,838 (lane 4) rather than the region between −2,862 and −2,813 (lane 2), whereas the signal shift observed in lane 4 could be prevented by competition from excess non-labeled DNA sequence between −2,887 and −2,863 (lane 8). Furthermore, we found that the competitor partially or greatly lost the competitive ability when two "AAT" sites were respectively or simultaneously substituted by "CCG" sites in the competitor-sequences (Fig. 5E, lanes 5–7), which is in agreement with the previous report that the "AAT" site was important for STAT3 binding [Yu et al., 1995]. Taken together, these results revealed that STAT3 as a transcription factor could regulate the expression of *C/EBPβ* by directly binding to the distal region of *C/EBPβ* promoter at the early stage of 3T3-L1 adipogenesis.

In addition, we examined the expression level of C/EBPβ protein when the cells were treated by STAT3 inhibitor or AG490 either at 2 h before MDI induction (−2 h group), or at 2 h after MDI induction (+2 h group), respectively. As shown in Figure 6, the level of C/EBPβ protein was inhibited in −2 h group, whereas no such inhibition was detected in +2 h group, which is in agreement with the observations that STAT3 is activated in the first 2-h stage after MDI induction and plays an important role for adipogenesis (Figs. 2D–F, 3C–E, and 5).

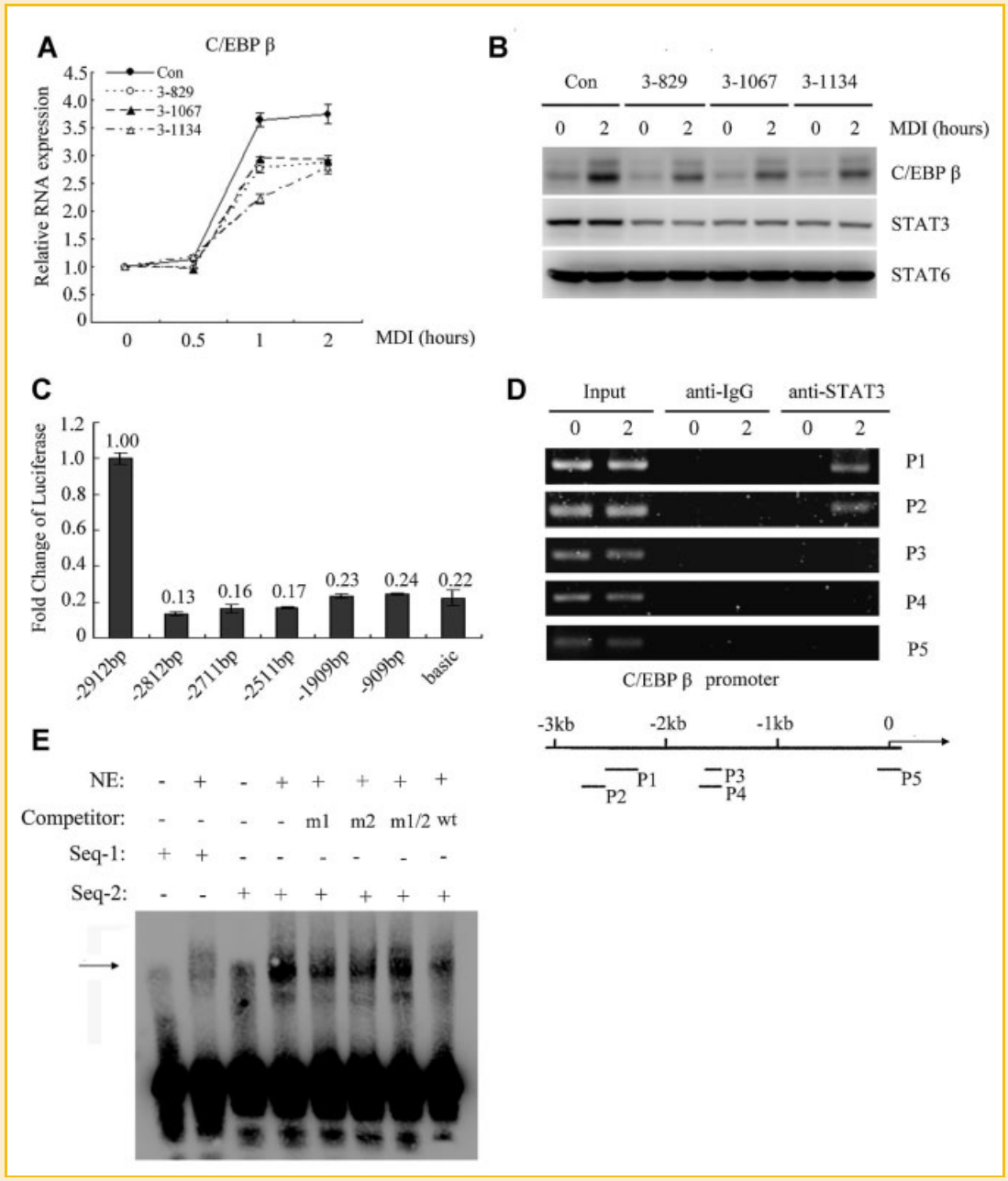

Fig. 5. STAT3 directly regulates the transcription of *C/EBPβ*. A,B: *C/EBPβ* mRNA and protein expressions were interfered by siRNA-mediated knockdown of STAT3. mRNA levels of *C/EBPβ* at the indicated time points were determined by Real-Time Quantitative PCR. The data are presented as mean ± s.e.m. from three independent experiments (A). The protein levels of *C/EBPβ* and STAT3 at the indicated time points were determined by Western-blotting assay. STAT6 was used as a loading control (B). C,D: −2,912 and −2,812 fragments of *C/EBPβ* promoter are required in STAT3 mediating transcription. NIH 3T3 cells were co-transfected with either a control (Con) or STAT3 expression plasmid (STAT3), 5′-truncated promoter of *C/EBPβ* reporter constructs and pRL-SV40. The data of luciferase experiments are shown in mean ± s.e.m. from three independent experiments (C). The post-confluent cells were induced by MDI for 2 h, and then chromatin samples were prepared as indicated and then subjected to ChIP assays using a STAT3 antibody or IgG (D, upper panel). The PCR products of different primers (P1–P5) located in the corresponding regions of *C/EBPβ* promoter were also shown (D, lower panel). E: The post-confluent cells were induced by MDI for 2 h, and then the nuclear extracts were prepared for EMSA experiment. The arrow indicates the STAT3–oligonucleotide probe complex. The seq-1 and seq-2, respectively, locate in the regions of −2,862 to −2,813 and −2,887 to −2,838 of the *C/EBPβ* promoter. NE, nuclear extract. wt, unlabeled wild-type competitor sequence. m1, m2, or m1/2, unlabeled mutated competitor sequences.

DISCUSSION

Since 1970s, the progress has been made in understanding of adipocyte differentiation and many factors, including STAT family, involved in adipogenesis have been characterized [MacDougald and Lane, 1995; Stephens et al., 1996; Gregoire et al., 1998; Farmer, 2006]. The STATs were firstly investigated in adipogenesis by Stephens et al. They found that STAT3 was up-regulated during

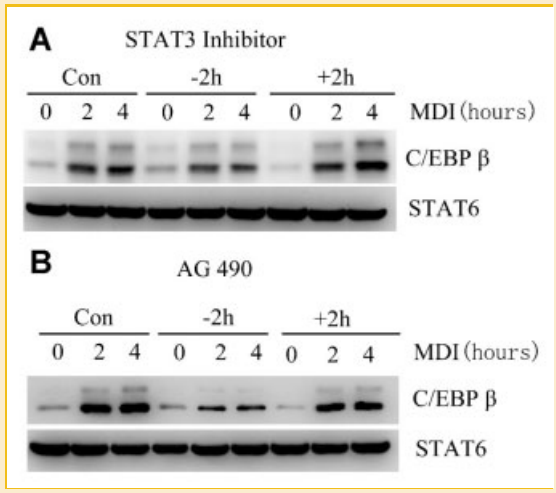

Fig. 6. STAT3 inhibitor or AG490 treatment in the first 2 h after MDI induction has more effect on the *C/EBPβ* protein expression. The cells were administered by STAT3 inhibitor (A) or AG490 (B) at 2 h before the MDI induction (−2 h) or at 2 h after the post-induction (+2 h). Whole cell extracts were collected at the indicated time points and subjected to immunoblotting with C/EBPβ antibody. STAT6 serves as a loading control.

adipocyte differentiation, however, the expression of STAT3 was unaffected when adipogenesis was inhibited by TNFα, which indicated that STAT3 expression was not highly correlated with the adipocyte phenotype. Since this work did not analyze the activation of STATs, it was possible that STAT3 activation rather than expression played the role in adipogenesis, which was proved by our present works (Figs. 1A and 2). Afterwards, Deng et al. reported that STAT3 activation might serve a regulatory role in proliferative phases of adipogenesis, while their work did not provide direct evidence to verify the prerequisite of STAT3 for adipogenesis [Deng et al., 2000].

Recently, Wang et al. [2009] have reported that STAT3 was required for adipogenesis and PPARγ was a potential downstream target of STAT3. But this report could not provide evidences about the regulation relationship between STAT3 and PPARγ. Hence, the mechanism of STAT3 in adipogenesis still remains unclear. In our present work, STAT3 was confirmed to be activated at the early stage of adipogenic induction (Figs. 1 and 2). More importantly, the findings that STAT3 could directly modulate the transcription of C/EBPβ revealed the regulating target of STAT3 in adipocyte differentiation (Fig. 5). It has been known that C/EBPβ can bind the promoter of PPARγ and modulate the transcription of PPARγ in adipogenesis [MacDougald and Lane, 1995; Gregoire et al., 1998; Cowherd et al., 1999; Farmer, 2006]. Therefore, we believe that the effect of STAT3 on PPARγ expression reported by Wang et al. indicates an indirect regulation relationship. In addition, the prerequisite of STAT3 for adipogenesis relied on its function in the first 2 h of the post-induction, which is in agreement with STAT3 transactivating C/EBPβ expression in the same time segment.

C/EBPβ has been considered to be one of the initiators of a transcriptional cascade which is critical for adipogenesis [Cao et al., 1991; Farmer, 2006]. Many works have been done to characterize the upstream factors regulating *C/EBPβ* expression. CREB was firstly verified to activate the expression of *C/EBPβ* and its binding sites located in two regions of *C/EBPβ* promoter, between −117 and −107 bp and between −107 and −50 bp [Zhang et al., 2004]. In addition, Chen and Birsoy reported that Krox20 and KLF4 also functioned as an immediate-early regulator of adipogenesis to induce *C/EBPβ* [Chen et al., 2005; Birsoy et al., 2008]. Both Krox20 and KLF4 transactivated *C/EBPβ* in −1.45 to −1.1 kb region of the promoter, and both factors could cooperate to activate *C/EBPβ* expression in adipogenesis [Chen et al., 2005; Birsoy et al., 2008]. In the present study, the luciferase experiments with a series of deletions localized the critical binding sites of STAT3 to the region between −2,912 and −2,812 bp of *C/EBPβ*. The binding site of STAT3 located in the distal region of *C/EBPβ* promoter is complementary to that of CREB, Krox20, and KLF4, which provided new insights into the transcription regulation of *C/EBPβ*. As a key factor of adipogenesis, *C/EBPβ* might be regulated by several transcription factors binding to at least four different sites (−117 to −107, −107 to −50, −1.45 to −1.1, and −2,912 to −2,812 bp) of *C/EBPβ* promoter, which appears to clarify the phenomenon that the knockdown of STAT3 could not completely abolish the expression of *C/EBPβ* (Fig. 5A). A recent work showed that STAT3 and ERK1/2 signaling pathways synergistically mediated the expression of *C/EBPβ* in lymphoma cells [Anastasov et al., 2010]. It should be interesting to explore what kind of transcription factor(s) co-regulates the expression of *C/EBPβ* with STAT3 in adipogenesis.

JAK2 was verified to be an upstream regulator of STAT3, and the activation of JAK2/STAT3 pathway at the first 2-h stage after MDI induction was essential for adipogenesis. However, we cannot exclude that JAK2/STAT3 pathway plays other roles in adipogenesis since STAT3 remained activated for about 2 days after MDI induction (Fig. 1A). In addition, what factors regulating the JAK2 activation should be explored. Midkine, a protein secreted by 3T3-L1 cells, proved to activate STAT3 [Cernkovich et al., 2007], and in our unpublished results, we found that midkine could also lead to the activation of JAK2. As the receptor of midkine [Meydan et al., 2002], PTPζ was also identified to be involved in JAK2 activation (data not shown). Can PTPζ directly interact with JAK2? How do MDI induce the expression or secretion of midkine in adipogenesis? To answer these questions will provide more insights for understanding regulation mechanisms at the early stage of adipocyte differentiation.

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