Identification of Novel Direct Stat3 Target Genes for Control of Growth and Differentiation

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Signal transducer and activator of transcription 3 (Stat3) is a key regulator of gene expression in response to signaling of the glycoprotein 130 (gp130) family cytokines, including interleukin 6, oncostatin M, and leukemia inhibitory factor. Many efforts have been made to identify Stat3 target genes and to understand the mechanism of how Stat3 regulates gene expression. Using the microarray technique, hundreds of genes have been documented to be potential Stat3 target genes in different cell types. However, only a small fraction of these genes have been proven to be true direct Stat3 target genes. Here we report the identification of novel direct Stat3 target genes using a genome-wide screening procedure based on the chromatin immunoprecipitation method. These novel Stat3 target genes are involved in a diverse array of biological processes such as oncogenesis, cell growth, and differentiation. We show that Stat3 can act as both a repressor and activator on its direct target genes. We further show that most of the novel Stat3 direct target genes are dependent on Stat3 for their transcriptional regulation. In addition, using a physiological cell system, we demonstrate that Stat3 is required for the transcriptional regulation of two of the newly identified direct Stat3 target genes important for muscle differentiation.

The signal transducer and activator of transcription (STAT) family of transcriptional regulators is activated in response to extracellular signaling proteins, including cytokines and growth factors (1, 2). When cytokines bind to their cell surface receptors, the receptor-associated JAK tyrosine kinases become activated and in turn phosphorylate a single tyrosine residue in the STAT molecule. The phosphorylated STATs then enter the nucleus as dimers and bind to specific DNA sequences in the promoters of their target genes to regulate transcription. Although the seven members of the STAT family have similarity in their molecular structure and function, they play diverse physiological roles in a wide variety of biological processes (3).

One member of the STAT family, Stat3, mediates the signaling of cytokines that share the gp130 receptor chain, which include interleukin-6, oncostatin M (OSM), and leukemia inhibitory factor (LIF) (4, 5). In response to gp130 ligand stimulation, Stat3 is phosphorylated on Tyr-705 and forms dimers through phosphotyrosine-Src homology 2 domain interactions (6). The dimerized Stat3 molecules enter the nucleus and bind to a consensus DNA sequence in the promoters of its target genes to regulate transcription (7). The transcriptional activity of Stat3 is mediated by its transcription activation domain located in the carboxyl-terminal end of the molecule (8). In addition to the tyrosine phosphorylation, the Stat3 transcription activation domain contains a serine residue (Ser-724) that is also phosphorylated to achieve maximum transcription activity (9, 10). Analyses of Stat3-dependent enhancersomes demonstrate that Stat3 interacts and recruits other transcription factors and co-activators to the promoters of its target genes (10–14). Furthermore, non-tyrosine-phosphorylated Stat3 has been shown to be able to activate transcription in cooperation with other transcription factors such as NFκB (15, 16).

Stat3 plays essential roles in a diverse array of cellular processes. For example, Stat3 activation is associated with oncogenesis and tumor metastasis (17, 18). Stat3 is also necessary for the normal development of multiple cellular systems, including early embryogenesis, lymphocyte growth, wound healing, and postnatal survival, as demonstrated by the various Stat3 knockout mouse models (19, 20). LIF-mediated self-renewal of murine embryonic stem cells also requires Stat3 activation (21–23). Because of these wide ranging physiological functions of Stat3, it is critical to identify Stat3 target genes to fully understand how Stat3 mediates its effect on these various cellular processes. Several microarray analyses have been carried out, and numerous potential downstream targets of Stat3 have been identified (24–28). Particularly among them are genes that regulate cell cycle progression, cell survival/growth, and migration, correlating with the concept that Stat3 is a critical factor in oncogenesis and making it a suitable drug target for cancer treatment (29, 30). Therefore, it is essential to identify direct Stat3 target genes to develop specific therapeutic treatments as well as to understand how Stat3 regulates gene transcription to achieve diverse physiological impacts. However, the large numbers of potential Stat3 target genes from the microarray studies...
Novel Direct Stat3 Target Genes

make it difficult to systematically distinguish the direct Stat3 targets for further analyses.

In this study, we used a genome-wide screening method utilizing the chromatin immunoprecipitation (ChIP) assay to identify Stat3 target genes. Because this method is based on the binding of Stat3 to DNA, the target genes identified are direct Stat3 targets. We show here that we have identified direct Stat3 target genes in NIH3T3 cells that are regulated by OSM. These direct Stat3 target genes include some of the genes that have been identified by previous microarray screening. However, a significant number of novel Stat3 target genes were also identified by this method. These novel genes are diverse in their function, including involvement in oncogenesis, neuronal development, and muscle differentiation. We further demonstrate that Stat3 functions as both a transcriptional activator and a repressor for its direct target genes. Using the Stat3-deficient MEF cells, we show that most of the novel Stat3 direct targets are dependent on Stat3 for their transcriptional regulation. In addition, to test the physiological relevance of these novel direct Stat3 target genes, we showed that Stat3 represses two of its direct target genes in a myoblast cell line for muscle differentiation, and knock down of Stat3 in this myoblast cell line prevents their differentiation. Therefore, we have identified a set of novel direct Stat3 target genes important for a wide range of biological processes, including the control of cellular growth and differentiation.

MATERIALS AND METHODS

Cell Culture and Reagents—NIH3T3 cells were maintained in DMEM supplemented with 10% fetal bovine serum (HyClone Laboratories Inc.). Wild-type and Stat3-deficient MEFs (provided by David Levy, New York University) were grown in DMEM supplemented with 10% fetal bovine serum in growth conditions. C2C12 cells (from Chisa Hidaka, Hospital for Special Surgery) were maintained in DMEM supplemented with 10% fetal bovine serum for growth conditions. C2C12 cells were maintained in DMEM supplemented with 2% horse serum (Sigma) for differentiation conditions. The antibodies used were anti-phosphotyrosine-Stat3 (Cell Signaling Technology) and anti-Stat3 (BD Transduction Laboratories). Recombinant mouse OSM was from R&D Systems. Recombinant mouse LIF was from Chemicon International.

Genome-wide Screening of Direct Target Genes of Stat3 by ChIP—NIH3T3 cells were grown in 15-cm dishes to 80–90% confluency. Cells were then treated with 25 ng/ml mouse OSM for 30 min. ChIP assays were performed as described previously (10) with 2.5 μg of Stat3 antibody. The precipitated genomic DNA was further purified three different ways to determine whether different techniques used for the DNA isolation yielded the same target genes. DNA was ethanol-precipitated with yeast tRNA twice, isolated with QIAquick PCR purification kit once, or agarose gel purified once, respectively. For agarose gel purification, DNA fragments ranging in size from 200 bp to 2 kb were excised. DNA was resuspended in 30 μl of sterile deionized water and blunted with the DNA terminator end kit (Lucigen) according to the manufacturer’s instructions. There was no significant difference in the final numbers of clones obtained with these various DNA purification methods, and some genes were repetitively pulled out with the different methods.

The blunted genomic DNA was amplified by ligation-mediated PCR with linkers containing EcoRi sites (LMPCR.1, 5'-GCCGTGAACCCGGAGATCTGAATTC-3', and LMPCR.2, 5'-GAATTCAGATC-3') as follows. For kinase reaction of LMPCR.2, 9 μl of linker (350 ng/μl), 6 μl of sterile deionized water, 2 μl of kinase buffer, 2 μl of 10 mM ATP, and 1 μl of T4 polynucleotide kinase (New England Biolabs) were incubated at 37 °C for 30 min. The kinase was then heat-inactivated at 65 °C for 10 min. The entire 20-μl LMPCR.2 reaction was annealed to 9 μl (350 ng/μl) of LMPCR.1. The reaction was heated to 95 °C for 2 min, 65 °C for 10 min, 37 °C for 10 min, and 25 °C for 20 min. A total of 3 μl of the annealed linkers were ligated to 10 μl of blunted ChIP DNA with 1 μl (2000 units) of T4 DNA ligase (New England Biolabs) at 16 °C for 16 h. Ligation mixtures were purified with QIAquick PCR purification kit to obtain the ChIP DNA/linker mixture.

The ChIP DNA-linker complex was then PCR-amplified in a reaction containing 5 μl of the ChIP DNA-linker complex, 1 μl of Platinum Taq DNA polymerase (Invitrogen), 200 pmol of LMPCR.1, 2 mM MgCl2, and 0.75 mM dNTPs in a final volume of 50 μl. PCR was performed with the following conditions: 1 cycle 95 °C/50 s, 25 cycles 94 °C/15 s, 55 °C/30 s, 68 °C/2 min, followed by 1 cycle at 68 °C for 7 min. The PCR-amplified DNA was purified with QIAquick PCR purification kit and then digested with 60 units of EcoRI in a 200-μl reaction at 37 °C for 2 h. The EcoRI-digested DNA was purified with the QIAquick PCR purification kit again and ligated into the EcoRI site of pBluescript followed by transformation into DH5α (Invitrogen) and plated onto LB agar plates containing ampicillin and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal).

Plasmid DNAs were purified from white colonies and sequenced with T7 and T3 primers. Most of the plasmids contained inserts of concaternes of 10–20 bp in length from different genes. Sequences were identified with NCBI BLAST (www.ncbi.nlm.nih.gov) against the mouse genome using a combination of the data bases nr, refseq_genomic, and refseq_rna. All sequences were considered potential targets regardless of the location of the sequence within the gene, even if the sequence was not located in the promoter region.

GeneSpring GX 7.3.1 software (Agilent Technologies) was used to find genes that are common to a microarray list and our ChIP list. Three publications of microarray studies of mouse cell lines were utilized (24–26) to generate three initial lists, which were then combined into one final list by manual editing (removing duplicates) to generate supplemental Table S1.

Gene-specific ChIP Analysis—NIH3T3 cells were grown in DMEM supplemented with 10% FBS in 15-cm dishes to 80–90% confluency. Cells were then either treated with 25 ng/ml mouse OSM or left untreated. ChIP analysis was performed as described previously (10) with 2.5 μg of Stat3 antibody (Santa Cruz Biotechnology). DNA from one 15-cm dish was used for a total of six separate PCRs.

If promoter sequences for a potential Stat3 target gene could be obtained from NCBI, putative Stat3 sites were identified within these sequences. Some genes do not have promoter sequences available from NCBI, and putative Stat3 sites for
these genes were identified by obtaining complete genomic sequences from NCBI followed by searching for potential Stat3 sites 1–3 kb upstream of the translational start site. All subsequent gene-specific ChIP primers were designed to flank the putative Stat3 sites and could amplify PCR fragments that were about 200 bp in size. Primers for the ChIP are as follows: Boc, 5′-GTCTTCAGTGTCAGTACATA-3′ and 5′-ACACACCCACTGCTTCACTCT-3′; Cln6, 5′-GAAATGAGGAGGAGGAGGAG-3′ and 5′-CCGGAGAGGAGGAGGAGGAGGAG-3′; Perq1, 5′-CCCTTGTGGCTCTACCCTGAA-3′ and 5′-CCTTGTGGCTCTACCCTGAA-3′; Tnf, 5′-ACCTTCTCTCTCCCTCAGC-3′ and 5′-ACCTTCTCTCTCCCTCAGC-3′; Fgl2, 5′-GCTGCTAGGAAGGAGA-3′ and 5′-GCTGCTAGGAAGGAGA-3′; Cdo, 5′-GCCAGACCTTCTCAGTACATA-3′ and 5′-GCCAGACCTTCTCAGTACATA-3′; Smad9, 5′-GGTCTCTCGCTGGTGTCAGCTC-3′ and 5′-GGTCTCTCGCTGGTGTCAGCTC-3′; Gbp1, 5′-TCCTAGTCCCCAGAAGAAGGA-3′ and 5′-TCCTAGTCCCCAGAAGAAGGA-3′; TNF-R, 5′-GTGCTCAGTTCCGGGTTC-3′ and 5′-GTGCTCAGTTCCGGGTTC-3′; FasL, 5′-GGGATTGGCTAGCGTCACT-3′ and 5′-GGGATTGGCTAGCGTCACT-3′; Pcnt, 5′-GGAAGAAATCCTA-3′ and 5′-GGAAGAAATCCTA-3′; mMyog, 5′-GGCTGCGAGTACATTTTAAGGATGTT-3′ and 5′-GGCTGCGAGTACATTTTAAGGATGTT-3′.

Quantitative Real Time RT-PCR (qRT-PCR)—NIH3T3 cells were grown in 6-well dishes in DMEM supplemented with 10% FBS until cells were 80% confluent. Cells were then serum-starved for 24 h. The cells were then either left untreated or treated with mouse OSM at 25 ng/ml for lengths of time as indicated. C2C12 cells were grown to about 80% confluence in growth medium for 3 days and then cultured in DMEM supplemented with 2% horse serum (differential medium) for 24 h. A portion of the transfected cells was analyzed by Western blotting assay to determine the knockdown of Stat3. The transfected cells were further treated with OSM for 4 h or left untreated followed by real time qRT-PCR analyses.

RESULTS

Identification of Direct Stat3 Target Genes by Genome-wide ChIP Screening—To identify direct Stat3 target genes, we used a genome-wide screening method based on ChIP followed by ligation-mediated PCR and subcloning. A similar approach has been utilized to identify direct target genes for other transcription factors such as cAMP-response element-binding (CREB) protein (31). For our screening, we used NIH3T3 cells that have been extensively used as a cell line for studying Stat3 function (17). OSM activates Stat3 robustly in NIH3T3 cells (10) and was chosen as a ligand to activate Stat3 at maximum level for genome wide screening of Stat3 target genes. Because ligand-activated STATs become de-phosphorylated and inactivated rapidly (32, 33), a 30-min OSM treatment time point was chosen to capture as many direct Stat3 target genes as possible, as well as to make it feasible for a scaled up ChIP assay. NIH3T3 cells were treated with OSM for 30 min, and ChIP assays were performed with a Stat3-specific antibody. The precipitated genomic DNA fragments from the ChIP were then amplified by ligation-mediated PCR and further subcloned into pBluescript followed by sequencing of the cloned inserts (see “Materials and Methods” for details). The DNA sequences obtained were used to BLAST search against the mouse genome data base. Four separate independent ChIP-subcloning experiments were performed, and ~400 genes were identified. These potential Stat3 target genes function in a wide range of biological processes, including immune response, oncogenesis, cell cycle control, development, cell adhesion, and differentiation. Out of these potential direct Stat3 target genes, about one-fourth have been identified previously by microarray analyses of mouse cell lines (24–26) (supplemental Table S1). Most of these microarray-
Novel Direct Stat3 Target Genes

A.

<table>
<thead>
<tr>
<th>OSM</th>
<th>c-fos</th>
<th>TNF-R</th>
<th>Pcnt</th>
<th>Bcl3</th>
<th>Gbp1</th>
<th>Pax4</th>
<th>Fgl2</th>
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Fold Induction: 4 4.2 2 5 * * * 3.7

B.

<table>
<thead>
<tr>
<th>OSM</th>
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<th>Perq1</th>
<th>Smad9</th>
<th>Boc</th>
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<th>Ect2</th>
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Fold Induction: 7.5 5 3.5 2.5 10 11.5 4.1 4.1 3.3

FIGURE 1. Induction of Stat3 binding to the promoters of target genes. NIH3T3 cells were treated with mouse OSM for 30 min or left untreated followed by ChIP analyses with Stat3 antibodies and PCR primers designed to include potential Stat3-binding sites in the promoters of the indicated genes. A, Stat3 target genes identified by both microarray analyses and the ChIP method as listed in supplemental Table S1. B, novel Stat3 target genes as listed in supplemental Table S2. c-fos is shown as a positive control. Quantitation was performed with a PhosphorImager, and the ChIP signals were normalized by the input signals. Fold induction is presented as a ratio of values of OSM-treated samples to untreated samples. For samples with an asterisk, fold induction could not be obtained because of undetectable signal in the untreated samples. IP, immunoprecipitation.

identified genes have not been confirmed by ChIP to demonstrate that they are direct Stat3 target genes. Therefore, using the genome-wide screen method based on ChIP, we have identified the direct Stat3 target genes from these microarray studies.

Identification of Novel Direct Stat3 Target Genes—The majority of the Stat3 target genes isolated by ChIP using NIH3T3 cells with a 30-min OSM stimulation have not been identified previously by microarray analyses. To confirm that we have identified novel direct Stat3 target genes, gene-specific ChIP analyses were performed on these potential Stat3 target genes. Some of the novel Stat3 target genes (34 genes) were cloned out multiple times and therefore were more likely to be true Stat3 target genes (supplemental Table S2). We focused on this group of genes to perform the gene-specific ChIP analyses, as well as including several genes from supplemental Table S1 and novel genes that have only been identified once by the ChIP screening method.

Putative Stat3-binding sites (TTC/GN2–4GAA) (7, 34) were searched for in either known promoter sequences of target genes or genomic sequences upstream of transcription or translation start sites (see supplemental Table S3). ChIP PCR primers were designed to flank the potential Stat3 sites to generate ~200-bp PCR fragments. We performed gene-specific ChIP on 22 genes from supplemental Table S2, 9 genes from supplemental Table S1, and 1 novel gene that had been isolated once (FasL). 7 of the genes from supplemental Table S1 (TNF-R, Pcnt, Bcl3, Gbp1, Pax4, Fgl2, and Cdo) showed an increase in the amount of Stat3 bound to their promoters in response to OSM treatment compared with untreated cells (Fig. 1A), whereas two genes, Tek and Ltbp3, had constitutive binding of Stat3 (data not shown and see supplemental Table S4). The c-fos promoter is shown as a positive control (Fig. 1A).

Of the 22 novel Stat3 target genes tested with gene-specific ChIP assay, 11 of them showed an increase in Stat3 binding to the promoter in response to OSM treatment. Results for Cln6, Perq1, Smad9, Boc, CBP, Ect2, FasL, Angpt1, and Peg10 are shown in Fig. 1B. II28ra and Dzip1 also showed an increase in Stat3 bound to their promoters in response to OSM treatment (data not shown and see supplemental Table S4). 7 of the novel Stat3 target genes (Notch4, Bag4, V2r4, Sema3g, Itga11, Paxip1, and Pax1) showed constitutive Stat3 binding to their promoters that were not affected by OSM treatment (data not shown and see supplemental Table S4). Only 4 of the potential direct Stat3 targets (B3bp, Nav1, Ighmbp2, and Dde2) had no detectable Stat3 binding (data not shown) in either treated or untreated conditions. Because the ChIP primers usually flank only one of several putative Stat3 sites in these genes, it is possible that we did not choose the right Stat3 sites for these four genes.

However, these results demonstrate that with preliminary gene-specific ChIP analyses most of the potential novel Stat3 target genes (~82%) identified by the ChIP assay are true direct Stat3 target genes.

Stat3 Can Both Activate and Repress Its Direct Target Genes in Response to OSM—To further understand how Stat3 regulates its direct target genes, RNA expression of the target genes was analyzed by quantitative real time RT-PCR. NIH3T3 cells were either untreated or treated with OSM for 2 and 4 h. cDNA-specific RT-PCR primers were designed for all the target genes that showed inducible Stat3 binding to their promoters. The expression of these direct Stat3 target genes could either be induced or repressed by the activation of Stat3 in NIH3T3 cells in response to OSM treatment (Fig. 2 and supplemental Table S4). Of the 18 direct target genes tested, 6 (Bcl3, TNF-R, Peg10, Smad9, Gbp1, and FasL) were induced to varying degrees by OSM treatment (Fig. 2A) and 10 (Perq1, Boc, Cdo, Pcnt, Fgl2, Angpt1, Cln6, Pax4, Dzip1, and Ect2) were reduced by 2–3-fold (7 of them shown in Fig. 2B, supplemental Table S4). The expression of CBP was not affected by OSM treatment (data not shown). These results are consistent with previously published data of Stat3 acting both as an activator and a repressor on its target genes.

Requirement of Stat3 for Transcription Regulation of Novel Direct Stat3 Target Genes—To test whether Stat3 is essential for the transcriptional control of its direct target genes, we utilized the Stat3-deficient MEFs to analyze the expression of these genes. Wild-type and Stat3−/− MEFs were serum-starved for 24 h and then either treated with OSM for 4 h or left untreated. Expression of 10 representative direct Stat3 target genes (five from supplemental Table S1 and five from supplemental Table S2 and including both induced and repressed genes) were analyzed by real time qRT-PCR. For the five genes from supplemental Table S1, the OSM-induced repression of one gene (Cdo) was dependent on Stat3 (Fig. 3E); for TNF-R and Bcl3, there was an increase in their basal level of expression, and the OSM induced fold increase was lower in Stat3−/− cells (Fig. 3, A and B and supplemental Table S4). Two genes (Pcnt...
and Gbp1) were not dependent on Stat3. For the five novel direct Stat3 target genes from supplemental Table S2, 4 of them (Smad9, Peg10, Perq1, and Boc) are dependent on Stat3 for either the increase or decrease in their expression in response to OSM (Fig. 3, F–J). The basal level expression of Perq1 is also lower in Stat3 cells (Fig. 3). A summary of these results is presented in supplemental Table S4. These results demonstrate that Stat3 plays a critical role for the transcriptional regulation for most of its direct target genes, particularly the novel target genes identified by the ChIP method in this report.

**Stat3 Represses the Expression of Cdo and Boc in Myocytes**—Two of the Stat3 direct target genes, Cdo and Boc, function together as a receptor complex and are critical for muscle differentiation and axonal guidance (35–38, 40). Specifically for muscle differentiation, the expression of these two genes is required. It has been suggested that Stat3 activation could inhibit muscle cell differentiation through direct binding and inhibition of MyoD activity (39). The direct repression of Cdo and Boc expression by Stat3 observed in NIH3T3 cells provides a novel mechanistic explanation for the above observations.

To see if Stat3 activation could repress Cdo/Boc expression in muscle cells to inhibit differentiation, we further analyzed expression of Stat3 target genes in the myoblast C2C12 cell line, which has been used extensively for muscle differentiation (38–40). C2C12 cells were treated with OSM for 2 and 4 h followed by real time RT-PCR analyses of Cdo/Boc expression. In response to OSM, Stat3 was activated in these cells through tyrosine phosphorylation after 30 min of treatment (Fig. 4A). When these cells were grown in growth medium, low levels of Boc and Cdo expression are detected, whereas their levels increased significantly when the cells are in differentiation media (Fig. 4B). After treatment with OSM for 2 and 4 h in differentiation media, expression of Cdo and Boc was repressed (Fig. 4B). As a marker for muscle differentiation, the expression of myogenin was increased in differentiation media, and OSM treatment significantly inhibited myogenin expression (Fig. 4B). In growth media, OSM treatment decreased the basal level of Cdo and Boc slightly and had no effect on myogenin expression (data not shown). To further confirm that it is Stat3, not other factors activated by OSM, that represses the expression of Cdo and Boc, the C2C12 cells were treated with another gp130 ligand, LIF, which plays important physiological roles for muscle regeneration after injury (41, 42). LIF induced activation of Stat3 in C2C12 cells and inhibited the expression of Cdo, Boc, and myogenin (Fig. 4, C and D). All together, these results suggest that Stat3 activation causes a repression of Boc, Cdo, and myogenin transcription in C2C12 myoblasts, which are necessary for myocyte differentiation.

**Stat3 Is Necessary for Inhibition of Muscle Differentiation**—To further demonstrate that Stat3 plays a direct physiological role in controlling muscle growth and differentiation, we used the siRNA technique to knock down the level of Stat3 in C2C12 myocytes. C2C12 cells were
Novel Direct Stat3 Target Genes

A. OSM  
-  +  PY-Stat3  
Stat3

B. Cdo  
-  +  12.5  10  7.5  5  2.5  0  Growth  Differentiation
Boc  
-  +  8  6  4  2  0  Growth  Differentiation
Myogenin  
-  +  12.5  10  7.5  5  2.5  0  Growth  Differentiation

C. LIF  
-  +  PY-Stat3  
Stat3

D. Cdo  
-  +  5  4  3  2  1  0  Growth  Differentiation
Boc  
-  +  8  6  4  2  0  Growth  Differentiation
Myogenin  
-  +  12.5  10  7.5  5  2.5  0  Growth  Differentiation

FIGURE 4. Activation of Stat3 inhibits the expression of two direct target genes Cdo and Boc in myocytes. A and C, C2C12 myoblasts were cultured in DMEM containing 10% FBS and treated with OSM (A) or LIF (C) for 30 min or left untreated. Whole cell extracts were prepared, and Western blot analysis was performed with antibodies against phosphorylated Stat3 (PY-Stat3) or total Stat3. B and D, C2C12 cells were grown either in growth-inducing media (Growth) or differentiation-inducing media (Differentiation). The cells were then treated with OSM for 0, 2, or 4 h (B) or LIF for 4 h (D) followed by RNA extraction and real time RT-PCR analyses for the expression of Boc, Cdo, myogenin, and GAPDH. Relative expression is standardized to GAPDH, and the means ± S.D. of three independent experiments are shown.

FIGURE 5. Stat3 is required for OSM-induced repression of Cdo and Boc and myocyte differentiation. C2C12 cells were grown in growth media and transfected with either control siRNA or Stat3 siRNAs for 3 days. The transfected cells were then cultured in differentiation medium for 24 h. A portion of the transfected cells was analyzed by Western blotting assays to determine the effect of knockdown of Stat3 (A). The cells were either further treated with OSM for 4 h or left untreated followed by real time RT-PCR analyses for the expression of Boc, Cdo, myogenin and GAPDH (B–D). Relative expression is standardized to GAPDH with the values from the untreated sample of cells transfected with control siRNA set as one. The means ± S.D. of three independent experiments are shown.

grown in growth media and transfected with Stat3 siRNA or control siRNA. Three days after transfection, the cells were changed to differentiation media for 24 h. A portion of the transfected cells were analyzed by Western blotting assay to determine the knockdown of Stat3. Stat3 level was significantly reduced in cells transfected with Stat3 siRNA compared with

cellular processes. Furthermore, these Stat3 target genes are directly regulated by Stat3 through the binding of Stat3 to their promoters. About 25% of these direct target genes have been previously identified by microarray analyses of mouse cell lines. There are several reasons for the relative small overlap of genes identified by these two methods. First, because the microarray

DISCUSSION

Using the ChIP method with an antibody specific for Stat3 followed by ligation-mediated PCR and sub-cloning, we have identified a whole new set of Stat3 target genes involved in a wide ranging array of

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experiments often use much longer ligand treatment (hours versus the 30-min time point in this report), many of the Stat3 target genes identified in the microarray experiments are not direct Stat3 target genes, whereas the ChIP cloning method used here searches for the direct target genes that bind Stat3. Second, it has been shown that the kinetics of STAT binding to their target genes vary. Some targets have STAT binding quickly after ligand stimulation but only for a short time period, whereas others may have slower but sustained STAT binding (43, 44). Therefore, the 30-min time point of treatment we chose may render it difficult to clone the immediate-early genes such as c-fos (even though by radioisotope labeling we are able to detect it at the 30-min time point) and the genes that have slower Stat3-binding kinetics.

The gene-specific ChIP analyses of a subset of these direct target genes showed that some of them have inducible binding of Stat3 on their promoters after OSM treatment, whereas others had constitutive Stat3 binding. For these constitutive Stat3-bound genes, although there is no detectable phosphorylation of Tyr-705 on Stat3 in NIH3T3 cells without OSM treatment, we cannot rule out the possibility of low level Stat3 activation in NIH3T3 cells grown in serum-containing medium. Another explanation for the constitutive binding of Stat3 on these promoter DNA could be that Stat3 is present on these promoters through interaction with other DNA-bound transcription factors such as NFκB as reported recently (15). About 18% of the target genes analyzed by gene-specific ChIP showed no detectable Stat3 binding. Because most of the promoters are not defined and there are often multiple putative Stat3 sites in the promoter, it is possible that we did not choose the correct Stat3-binding sites when we designed the gene-specific ChIP primers. It is also possible that these are false positive sequences because of contamination of sticky genomic fragments in the immunoprecipitates during ChIP.

The regulation of these target genes by Stat3 was further analyzed by real time RT-PCR in response to OSM treatment. The results indicate that Stat3 can both activate and repress its direct target genes when activated by OSM in NIH3T3 cells. These results are consistent with findings from the microarray analyses, further demonstrating the complex nature of Stat3 activity not only in its diverse involvement in many different biological processes but also in the way it controls transcription of its target genes. Analyses of the expression of these direct Stat3 target genes in Stat3-deficient MEFs also showed that a majority of them are dependent on Stat3 for their transcriptional regulation (both induction and repression). Therefore, for mammalian gene promoters that require multiple transcription factors and co-activators to assemble an enhancerosome for precise transcriptional regulation, Stat3, as one component of the enhancerosome, often plays a critical role for its direct target genes, although in a few cases its function may be dispensable (at least in MEFs in response to OSM), perhaps replaced by other STATs. Furthermore, although we identified these direct Stat3 target genes in NIH3T3 cells, some of these target genes have tissue-specific functions. This could be the reason that the expressions of some genes are only moderately affected by OSM treatment in NIH3T3 cells because not all the components of a tissue-specific enhancerosome are assembled on the promoters in NIH3T3 cells in response to OSM even if the activated Stat3 can bind there. Therefore, to demonstrate the physiological relevance of these Stat3 target genes, we further analyzed the expression of two of the Stat3 target genes in a tissue-specific cell line. The two related Stat3 target genes, Cdo and Boc, have specific functions in myoblast differentiation and axonal guidance (35–38, 40). Using a myoblastic cell line, we showed that Stat3 represses their transcription that is necessary for muscle differentiation. Furthermore, the expression of these genes can no longer be suppressed in myocytes with Stat3 knockdown. The identification of these genes as direct targets for Stat3 uncovers a link in the mechanism of muscle differentiation regulated by Stat3. All together these results demonstrate that Stat3 plays a critical role in controlling muscle growth and differentiation.

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