Mechanistic Roles of Leptin in Osteogenic Stimulation in Thoracic Ligament Flavum Cells*

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Obesity is a risk factor for thoracic ossification of ligament flavum (TOLF) that is characterized by ectopic bone formation in the spinal ligaments. Hyperleptinemia is a common feature of obese people, and leptin, an adipocyte-derived cytokine with proliferative and osteogenic effects in several cell types, is believed to be an important factor in the pathogenesis of TOLF. However, how leptin might stimulate cell osteogenic differentiation in TOLF is not totally understood. We reported here that leptin-induced osteogenic effect in TOLF cells is associated with activation of signaling molecules STAT3, JNK, and ERK1/2 but not p38. Blocking STAT3 phosphorylation with a selective inhibitor, AG490, significantly abolished leptin-induced osteogenic differentiation of TOLF cells, whereas blocking ERK1/2 and JNK phosphorylation with their selective inhibitors PD98059 and SP600125, respectively, had only marginal effects. In addition, we showed that STAT3 interacted with Runx-related transcription factor 2 (Runx2) in the nucleus, and STAT3, Runx2, and steroid receptor coactivator steroid receptor coactivator-1 were components of the transcription complex recruited on Runx2 target gene promoters in response to leptin treatment. Our experiments identified STAT3, Runx2, and steroid receptor coactivator-1 as critical molecules in mediating leptin-stimulated cell osteogenesis in TOLF.

Ossification of ligament flavum (OLF) of the spine is characterized by a heterotopic bone formation in the ligament flavum that is normally composed of fibrous tissues (1). Ossification could enlarge the spinal canal and compresses the spinal cord, resulting in serious neurological damages. Epidemiology has shown that high incidence rate of OLF occurs in thoracic spine (2). It has been documented that obesity represents a risk factor for thoracic ossification of ligament flavum (TOLF), particularly in Asian people (3). Indeed, hereditary obese rats, Zucker fatty (fa/fa) rats, are prone to OLF (4). A common feature of obese people is hyperleptinemia (5). Leptin, an adipocyte-derived cytokine, can stimulate the proliferation and osteogenic differentiation of various cell lines, such as the embryonic cell line C3H10T1/2, human NHOst cells, and human osteoblastic cells (6, 7). However, the molecular mechanism underlying the osteogenic effect of leptin in TOLF is not totally understood.

Leptin exerts its biological activity through binding to its receptors, which belong to cytokine receptor superfamily. Different leptin receptor isoforms exist, including a long form (ObRb) and a short form (ObRa) (8). In vitro and in vivo studies have shown that leptin activates cytokine-like signal transduction via the long form receptor. Upon leptin stimulation, intracellular Janus tyrosine kinases are activated via transphosphorylation and phosphorylate tyrosine residues on the long form leptin receptor and on signal transducers and activators of transcription (STAT) proteins (9). Phosphorylated STAT proteins dimerize and translocate to the nucleus to activate gene transcription.

We report here that STAT3 signaling pathway was involved in the osteogenic differentiation of TOLF cells in response to leptin. The p160 family of nuclear receptor coactivators SRC-1, but not GRIP1 and AIB1, interacted with STAT3. We showed that STAT3, SRC-1, and Runx2 are components of the transcription complex recruited to the Runx2 target gene promoters to regulate the target gene transcription.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Polyclonal anti-ob-receptor, polyclonal anti-ERK1/2, anti-p38 MAPK, anti-JNK, anti-phospho-ERK1/2, anti-phospho-p38 MAPK, anti-phospho-JNK, anti-osteocalcin, and anti-Runx2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-STAT3 and anti-phospho-STAT3 Tyr705 were from Upstate Biotechnology (Lake Placid, NY) and New England Biolabs (Beverly, MA), respectively. Recombinant human leptin was from R&D Systems (Minneapolis, MN). PD98059, SP600125, and AG490 were obtained from Promega and Calbiochem and were dissolved in dimethyl sulfoxide (Me2SO) before use.

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3 The abbreviations used are: OLF, ossification of ligament flavum; TOLF, thoracic OLF; SRC, steroid receptor coactivator; Runx2, Runx-related transcription factor 2; STAT3, signal transducer and activator of transcription 3; MAPK, mitogen-activated protein kinase; ALP, alkaline phosphatase activity; ChIP, chromatin Immunoprecipitation; Re-ChIP, ChIP re-immunoprecipitation; JNK, c-Jun NH2-terminal kinase; ERK, extracellular signal-regulated kinase; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT, reverse transcription.
Clinical Diagnosis and Spinal Ligament Samples—The diagnosis of TOLF or non-TOLF (i.e., other thoracic diseases) was confirmed by x-ray, computed tomography, and magnetic resonance imaging of the whole spine preoperatively to avoid any other non-thoracic OLF patients. The clinical diagnoses and the spinal ligament tissues used in this study are shown in Table 1. Ligaments were aseptically harvested from patients during surgery and rinsed with phosphate-buffered saline. Surrounding tissue was carefully removed under a dissecting microscope. In all cases the ligaments were extirpated carefully from nonossified sites to avoid any possible contamination of osteogenic cells. This study was approved by the Ethics Committee of Peking University Health Science Center.

Cell Cultures—Collected ligaments were minced into about 0.5-mm³ pieces and washed twice with phosphate-buffered saline then plated in 6-cm culture dishes and maintained in DMEM medium (10% FBS, 1% l-glutamine, 100 units/ml of penicillin G sodium, 100 μg/ml of streptomycin sulfate) in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. The cells derived from explants were removed from the dish with 0.02% EDTA, 0.05% trypsin for passage. The first and third passages were used in the following studies (10).

Colorimetric 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay—Cell proliferation was measured by MTT dye reduction assay. Briefly, cells were seeded into 96-well plates overnight and exposed to the leptin at different concentrations for different times. The cells then were incubated with MTT (0.5 mg/ml) for 4 h at 37 °C. After removal of MTT, 150 μl of Me₃SO were added to the cells, and the plates were shaken at room temperature for 10 min. The absorbance was measured at 540 nm using a microplate reader (7).

Mineralization Assay—Cells from TOLF patients (TOLF cells) and non-TOLF patients (non-TOLF cells) were plated at 40,000 cells/well in 6-well dishes and maintained in DMEM supplemented with 10% FBS. On confluence, designated day 0, cells were exposed to leptin medium containing DMEM supplemented with 10% FBS, 10 mm β-glycerophosphate, 100 ng/ml leptin. Medium was replaced for every 3 to 4 days. Samples were processed at 4 weeks, and alizarin red assay (Sigma) was performed to determine mineralization. Briefly, cells were washed with phosphate-buffered saline and water for several times. Extracellular matrix mineral-bound stain was photographed under microscopy (11).

Tissue samples used in the study, showing clinical diagnosis, patient’s sex and age, and tissue derived

| Code   | Diagnosis | Sex/age | Tissue | BMI  |
|--------|-----------|---------|--------|------|
| O-1    | TOLF      | M/51    | TLF    | 25.6 |
| O-2    | TOLF      | M/52    | TLF    | 23.1 |
| O-3    | TOLF      | M/60    | TLF    | 25.3 |
| O-4    | TOLF      | M/68    | TLF    | 25.7 |
| O-5    | TOLF      | M/69    | TLF    | 23.3 |
| O-6    | TOLF      | F/48    | TLF    | 26.2 |
| O-7    | TOLF      | F/53    | TLF    | 25.5 |
| O-8    | TOLF      | F/64    | TLF    | 26.1 |
| O-9    | TOLF      | F/65    | TLF    | 25.8 |
| O-10   | TOLF      | F/66    | TLF    | 25.1 |

| Code   | Diagnosis | Sex/age | Tissue | BMI  |
|--------|-----------|---------|--------|------|
| N-1    | TT        | M/46    | TLF    | 23.7 |
| N-2    | ST        | M/48    | TLF    | 23.2 |
| N-3    | TT        | M/47    | TLF    | 23.4 |
| N-4    | TT        | M/56    | TLF    | 25.3 |
| N-5    | TSS       | M/57    | TLF    | 22.8 |
| N-6    | TT        | F/43    | TLF    | 23.5 |
| N-7    | TT        | F/57    | TLF    | 25.2 |
| N-8    | TT        | F/66    | TLF    | 25.1 |
| N-9    | TF        | F/66    | TLF    | 23.4 |
| N-10   | TT        | F/73    | TLF    | 23.1 |

* Mean obesity.
Results are expressed as nmol of p-nitrophenol/µg of cellular protein/min (12).

Luciferase Assays—Cells were transfected with an osteocalcin promoter-driven luciferase construct (pOC-LUC) (13) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instruction. Forty-eight hours after transfection, the cells were harvested, and luciferase and renilla activities were measured using dual luciferase kit (Promega). The firefly luciferase data for each sample were normalized based on transfection efficiency measured by renilla luciferase activity. Each assay was performed in triplicate and repeated at least three times.

Immunoprecipitation and Western Blotting—Nuclear extracts were precleared with 50 µl of protein A-agarose beads for 30 min followed by pelleting the beads. Anti-RUNX2 antibody was then added and incubated for 2 h at 4 °C with gentle rocking. The immune complexes were collected after the addition of 30 µl of protein A-agarose beads and incubation for 1 h at 4 °C followed by centrifugation. Precipitates were suspended in washing buffer (20 mM Tris-HCl (pH 8.3), 0.5% sodium deoxycholate, 0.5% Nonidet P-40, 50 mM NaCl, 2 mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride), centrifuged, and resuspended in 0.5 M Tris-buffered saline (pH 6.8), containing 2% SDS, 1 mM dithiothreitol, 10% glycerol, and 0.01% phenol blue. The protein samples were then denatured at 95 °C for 4 min, resolved by 10% SDS-PAGE, and transblotted to Hybond/ECL nitrocellulose membranes. The membranes were blocked overnight in Tris-buffered saline containing 1% bovine serum albumin, 1% polyvinylpyrrolidone, and 0.01% Tween 20 and incubated with an appropriately diluted primary antibody followed by incubation with horseradish peroxidase-conjugated secondary IgG. Signals were visualized with diaminobenzidine on LAS3000 Lumi-Imager (Fuji Photo Film Co., Ltd.).

Chromatin Immunoprecipitation (ChIP) and ChIP Re-immunoprecipitation (Re-ChIP) Assay—ChIP experiments were performed according to the method described elsewhere (14–18). The presence of the target gene promoter sequences in both the input DNA and the recovered DNA immunocomplexes were detected by real-time PCR using DNA as template for real-time PCR with SYBR Green PCR Master Mix (Applied Biosystems).

FIGURE 1. Effects of leptin on ALP and osteocalcin mRNA expressions in TOLF and non-TOLF cells. A, the effect of leptin on the proliferation of TOLF (top panel), non-TOLF (middle panel), and MCF-7 (bottom panel) cells. MCF-7 were incubated with 0–100 ng/ml leptin for 24, 48, and 72 h, and colorimetric MTT assays was performed as described under “Experimental Procedures.” Each bar represents the mean ± S.D. from sextuplet experiments, and the asterisks indicate statistical significance (p < 0.05). OD, optical density. B and C show the level of mRNA expression of genes measured by real-time RT-PCR in cells treated with leptin at various concentrations (0–100 ng/ml) for 24 h. D and E show the level of mRNA expression of genes measured by real-time RT-PCR in cells treated 10 ng/ml leptin for different periods of time (0–7 days). Each bar represents mean ± S.D. from 8 samples, and the asterisks indicate statistical significance (p < 0.05).
The primer sequences for specific promoter regions (within −500 to +100 regions, corresponding to the transcription start sites of genes) are shown below. The data obtained were normalized to the corresponding DNA input control. The sequences of the primers used were as follows: for osteocalcin: forward, 5′-CGGGCAGCTGATTGTGGC-3′ (position −261), and reverse, 5′-CTCATGGTGTCTCGGTGGC-3′ (position +23); control sequence, forward, 5′-AAAGTGTTGGTGACAAAGGC-3′ (position −3368), and reverse, 5′-TGAGTGCCAAGTGAGGC-3′ (position −3234). The Re-ChIP assays were performed as described earlier (15). The immunoprecipitated complexes obtained by ChIP were eluted by incubation for 30 min at 37 °C in 25 μl of 10 mM dithiothreitol. After centrifugation, the supernatant was diluted 20 times with sonication buffer and subjected to the ChIP procedure.

Statistical Analysis—The data were analyzed by analysis of variance, and the Student-Newman-Kleuss method was used to estimate the level of significance. p < 0.05 was considered significant.

**RESULTS**

Effect of Leptin on Proliferation and Osteogenic Differentiation of TOLF and Non-TOLF Cells—As stated above, hyperleptinemia is a risk factor for TOLF; to investigate the molecular mechanism underlying leptin-stimulated TOLF, we first examined the effect of leptin on the proliferation and osteogenic differentiation of TOLF and non-TOLF cells. In these experiments TOLF and non-TOLF cells were treated with various concentrations of leptin for different periods of time. The effect of leptin treatment on cell proliferation was evaluated by MTT methodologies, and cell osteogenic differentiation was assessed by real-time RT-PCR, Western blotting, ALP assay, and mineralization assay. As shown in Fig. 1, real-time RT-PCR analysis showed that whereas leptin stimulated the proliferation of a breast carcinoma cell line, MCF-7, it had no significant effect on the proliferation of either TOLF cells or non-TOLF cells (Fig. 1A). However, leptin treatment resulted in a significant increase in mRNA expression of ALP and osteocalcin in TOLF cells but not in non-TOLF cells, and the effect was both dose- and time-dependent (Fig. 1B–E). In agreement with the real-time RT-PCR experiments, ALP activity assay and Western blotting demonstrated that both the activity of ALP and the protein expression of osteocalcin were elevated in response to leptin stimulation in TOLF cells but not in non-TOLF cells (Fig. 2, A and B). In addition, mineralization assays showed that whereas in the absence of leptin treatment, both TOLF cells and non-TOLF cells exhibited a fibroblast-like, spindle-shaped appearance and none of these cells manifested mineralization, under leptin stimulation the cell matrix began to mineralize, and crystals appeared at 4 weeks in 8 of 10 TOLF cell cultures, whereas no mineralization was observed in all 10 non-TOLF cell cultures (Fig. 2C). Collectively, all these experiments indicated that whereas leptin had no appreciable effect on the proliferation of both TOLF cells and non-TOLF cells, it stimulated osteogenic differentiation of TOLF cells but not non-TOLF cells.

Molecular Events Involved in Leptin-stimulated Osteogenesis in TOLF Cells—To gain insight into the mechanism underlying the osteogenic effect of leptin, we first examined the expression of leptin receptors in TOLF cells and non-TOLF cells. These cells were grown in normal media for 72 h and harvested for

**FIGURE 2. Effects of leptin on osteocalcin proteins expression and ALP activity in TOLF and non-TOLF cells.** Cells were treated with 10 ng/ml leptin for different periods of time (0–7 days), and cellular proteins were extracted for osteocalcin protein detection (A) and ALP activity measurement (B). Each bar represents the mean ± S.D. from 8 samples, and the asterisks indicate statistical significance (p < 0.05). Controls were cells maintained under resting state (0 days). C, comparison of TOLF and non-TOLF cells for the ability to form mineralized nodules. Cells were incubated in DMEM medium containing 10% FBS, 10 mM glycerophosphate, and 100 μm β-glycerophosphate, and 100 ng/ml leptin for the indicated periods of time (0–4 weeks) and then stained with alizarin red.
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**FIGURE 3. Activation of STAT3 and mitogen-activated protein kinase (MAPK) signaling pathways in TOLF cells.** A, the expression of ObRb mRNA and protein in TOLF cells and non-TOLF cells. Total RNA was extracted from TOLF cells and non-TOLF cells and analyzed by RT-PCR. Total proteins of TOLF cells and non-TOLF cells were examined by immunoblot analysis using a rabbit polyclonal antibody against ObRb. p-, phosphorylated. B, activation of STAT3 and mitogen-activated protein kinase signaling by leptin. TOLF cells were treated with different concentrations of leptin for the time indicated, and then lysates were immunoblotted with specific antibodies against total or phosphorylated form of STAT3, ERK, JNK, and p38.

As stated before, leptin is believed to exert its biological function through binding to its receptors, which in turn transduce the signal through the activation of STAT3, ERK, JNK, and p38 pathways (20, 21). Thus, we next examined the status of STAT3, ERK, JNK, and p38 phosphorylation in response to leptin treatment in TOLF cells. Cellular proteins were extracted from TOLF cells that were exposed to leptin for various periods of time and were then immunoblotted with antibodies against phospho-STAT3, phospho-ERK1/2, phospho-JNK, and phospho-p38. These experiments showed that, whereas the total expression levels of STAT3, ERK1/2, and JNK were unchanged over the time of leptin treatment, the phosphorylation of STAT3, ERK1/2, and JNK was stimulated by leptin (Fig. 3B).

However, leptin treatment did not affect both the expression and the phosphorylation of p38 in TOLF cells, although leptin was capable of stimulating p38 phosphorylation in breast cancer cells MCF-7 and T47-D in our experimental system (Fig. 3B). Collectively, these experiments indicated the osteogenic effect of leptin in TOLF cells could be mediated via STAT3, ERK1/2, and/or JNK signaling pathways.

**STAT3 Signaling Pathway Is Critically Involved in Leptin-induced Osteogenic Differentiation of TOLF Cells**—To investigate whether activation of STAT3, ERK1/2, and JNK signaling pathways was indeed linked to the osteogenic effect of leptin in TOLF cells, we used selective inhibitors to block STAT3, ERK1/2, or JNK phosphorylation, respectively, and detected the expression of osteocalcin, a marker for cellular osteogenic differentiation, by real-time RT-PCR under the treatment of leptin. As shown in Fig. 4A, treatment of TOLF cells with Janus tyrosine kinase/STAT3 phosphorylation-selective inhibitor AG490 selectively inhibited the phosphorylation of STAT3 protein. Analogously, treatment of TOLF cells with ERK1/2 phosphorylation-selective inhibitor PD98059 and JNK phosphorylation-selective inhibitor SP600125 selectively inhibited the phosphorylation of ERK1/2 and JNK, respectively. Measurement of osteocalcin expression indicated that blocking phosphorylation of ERK1/2 and JNK had only marginal effects on osteocalcin expression, whereas blocking STAT3 phosphorylation resulted in a significant reduction of leptin-induced osteocalcin expression (Fig. 4B), suggesting that STAT3 signaling pathway is the major mediator for the osteogenic effect of leptin in TOLF cells.

**Transactivation of Osteocalcin by STAT3 in TOLF Cells under Leptin Treatment**—STAT3 is a cytoplasmic protein, which once phosphorylated is translocated into the nucleus where it regulates gene expression through interacting with cofactors (22). Runx2 is a key transcriptional factor in cell osteogenic differentiation. Activated Runx2 transactivates the transcription of its target gene such as osteocalcin (23), which in turn promotes cellular osteogenic differentiation (24). The observation that osteocalcin expression was increased by leptin treatment in TOLF cells (Fig. 1, C and E) suggests that leptin-activated STAT3 could be functionally linked to Runx2 in these cells. To investigate a functional interaction between STAT3 and Runx2 in transactivating Runx2 target genes, thus in mediating the osteogenic effect of leptin, we first examined if STAT3 could interact with Runx2 in the nucleus of TOLF cells under leptin stimulation. Co-immunoprecipitation experiments were performed with nuclear extractions of TOLF cells treated with leptin using STAT3 antibodies, and the immunoprecipitates were then immunoblotted with antibodies against Runx2. As shown in Fig. 5A, a physical interaction between STAT3 and Runx2 was detected in the nucleus of TOLF cells upon the stimulation of leptin treatment, providing a basis for a functional interaction between STAT3 and Runx2.

STAT3 activates gene transcription through recruitment of coactivators that modify the chromatin architecture (25–28).
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Previous studies have shown that the p160 family coactivators, SRC-1, GRIP1, and AIB1, although originally identified as cofactors involved in nuclear receptor-mediated gene transcription, also participate in gene transcriptional activation mediated by other transcription factors, including STATs (29–33). To investigate whether the p160 family coactivators are also implicated in the osteogenic activity of leptin, transient transfection experiments were performed to examine the enhancement of Runx2 target gene transcription by p160 coactivators. In these experiments osteocalcin gene promoter-driven luciferase (pOC-LUC) (13) was cotransfected with mammalian expression vectors for p160 coactivators SRC-1, GRIP1, or AIB1 in TOLF cells, and the luciferase activity was measured in the presence or absence of leptin stimulation. As shown in Fig. 5B, the experiments indicated that SRC-1, but not GRIP1 or AIB1, was able to enhance the expression of the luciferase gene. This discrepancy was not a result of the coactivator protein abundance because the expression levels of SRC-1, GRIP1, and AIB1 in these cells were similar (Fig. 5C) and the enhanced expression of endogenous osteocalcin gene by SRC-1 was also evident as measured by Western blot analysis (Fig. 5D).

These observations suggested that there was a differential involvement of p160 coactivators in transcription of genes that are important in osteogenesis.

Transactivation of Osteocalcin Gene by STAT3, Runx2, and SRC-1—To further dissect the leptin-activated STAT3 interaction with Runx2 and to understand the involvement of SRC-1 as a coactivator in Runx2 target gene coactivation, we examined the recruitment of STAT3, Runx2, and SRC-1 on the promoter of osteocalcin gene using ChIP assays (14). In these experiments soluble chromatin were prepared using a formaldehyde cross-linking protocol, and the occupancy of Runx2 target gene promoter by SRC-1, STAT3, and Runx2 was analyzed using specific antibodies against SRC-1, STAT3, or Runx2 and using a pair of primers spanning the Runx2-responsive region in the osteocalcin promoter (Fig. 5E, upper panel). Measurement of GRIP1 and AIB1 also was included in the experiment. As shown in Fig. 5E (lower panel), ChIP experiments detected the occupancy of SRC-1, but not GRIP1 or AIB1, along with STAT3 and Runx2 on the promoter of osteocalcin gene under the treatment of leptin. The increased presence of SRC-1 on osteocalcin promoter was not resulted from leptin-induced accumulation of SRC-1 in the cell nucleus as Western blotting did not detect any increase of nuclear SRC-1 with leptin treatment (Fig. 5F).

We next performed Re-ChIP of the cross-linked and sonicated chromatin from TOLF cells with antibodies against STAT3 and then with anti-SRC-1, antibodies against SRC-1, and then with anti-STAT3 or antibodies against Runx2 and then with anti-SRC-1. This was followed by quantitative PCR analyses for determining whether osteocalcin promoter sequences were detected in the final precipitate (Fig. 5G). When chromatin were first immunoprecipitated with anti-STAT3 (Fig. 5G, column 3) and the collected samples were subsequently re-precipitated with anti-SRC-1 antibodies (Fig. 5G, column 7), the presence of osteocalcin promoter was detected in the precipitate. Similarly, chromatin first immunoprecipitated with anti-SRC-1 (Fig. 5G, column 4) and then re-precipitated with anti-STAT3 antibodies (Fig. 5G, column 8) revealed the presence of osteocalcin promoter. These results indicated that both STAT3 and SRC-1 were recruited to the promoter of osteocalcin gene. In addition, when chromatin extracts were sequentially immunoprecipitated with anti-Runx2 and then anti-SRC-1 antibodies (Fig. 5G, column 6), the osteocalcin promoter was also detected in the collected materials. Collectively, these results suggested that all STAT3, Runx2, and SRC-1 occupied the promoter and, thus, participated in transactivation of osteocalcin gene. The binding of Runx2 and STAT3 on osteo-
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A

B

C

D

E

F

G

FIGURE 5. Leptin-induced transactivation of osteocalcin gene by STAT3, Runx2, and SRC-1 in TOLF cells. A, Runx2 interacts with STAT3. TOLF cells were serum-starved 8 h and then stimulated with 100 ng/ml leptin or left untreated for 30 min. Cell extracts were directly analyzed by Western blotting (lanes 1 and 2) or first immunoprecipitated with antibodies against STAT3 (lanes 4 and 6) or a control serum (lanes 3 and 5) and then immunoblotted with Runx2 antibodies. B, SRC-1, but not GRIP1 or AIB1, enhanced the leptin-induced osteocalcin transactivation in TOLF cells. TOLF cells were cotransfected with an osteocalcin-luciferase reporter construct and plasmids for SRC-1, GRIP1, or AIB1 expression for 48 h. Transfected cells were treated with 100 ng/ml leptin for 6 h or left untreated (control). Luciferase activities were determined. Each bar represents the mean ± S.D. from triplicate experiments. C, overexpression of SRC-1, GRIP1, and AIB1 in TOLF cells. TOLF cells were transfected with an expression construct of SRC-1, GRIP1, or AIB1. Forty-eight hours after transfection, the total proteins were extracted and examined by immunoblotting using the antibodies against SRC-1, GRIP1, or AIB1. β-gal, β-galactosidase. D, overexpression of SRC-1 enhanced leptin-induced osteocalcin expression. TOLF cells were transfected with the SRC-1 expression plasmid and then left untreated or treated with 100 ng/ml leptin for 8 h. The protein levels were examined by Western blotting using specific antibodies against osteocalcin. E, schematic representation of the Runx2 consensus binding sites in the osteocalcin promoter and the primers for ChIP assays (upper panel). Soluble chromatin was prepared from serum-starved TOLF cells treated with 100 ng/ml leptin for 30 min or left untreated followed by immunoprecipitation with antibodies against STAT3, Runx2, SRC-1, GRIP1, or AIB1 (lower panel). The final DNA extractions were amplified using pairs of primers that cover the Runx2 binding sites of the osteocalcin promoter by real-time PCR with normal IgG as a control. F, the protein level of SRC-1 in the TOLF nuclear extracts before and after leptin treatment. TOLF cells were serum-starved for 8 h and stimulated with 100 ng/ml leptin for 30 min or left untreated. Nuclear extracts were then analyzed by Western blotting with antibodies against SRC-1. G, re-ChIP experiments were carried out as described previously (15). Column 1, ChIP with nonspecific IgG; column 2, ChIP with anti-Runx2; column 3, ChIP with anti-STAT3; column 4, ChIP with anti-SRC-1; column 5, ChIP and re-ChIP with a nonspecific IgG; column 6, ChIP with anti-Runx2 and re-ChIP with anti-STAT3; column 7, ChIP with anti-SRC-1 and re-ChIP with anti-STAT3; column 8, ChIP with anti-STAT3 and re-ChIP with anti-SRC-1. The final DNA extractions were amplified using pairs of primers that cover the Runx2 binding sites of the osteocalcin promoter by real-time PCR with normal IgG as a control. The data obtained were normalized to the corresponding DNA input.

calcin promoter is also supported by electrophoretic mobility shift assays (Fig. 6A).

Because SRC-1 was the only p160 coactivator involved in the leptin-induced osteocalcin activation, we sought to further substantiate the role of SRC-1 in leptin-induced osteogenesis of TOLF cells. We used RNA-mediated interference (RNAi) approach to silence the expression of SRC-1, and the effect of RNAi on the stimulation of osteocalcin transcription by leptin in TOLF cells was measured by real-time PCR. As shown in Fig. 6B, the expression of SRC-1 could be effectively silenced by a vector carrying specific sequence against the mRNA of SRC-1, and leptin-stimulated osteocalcin transcription was severely affected with silenced SRC-1 expression in TOLF cells. These results further supported that SRC-1 plays an important role in leptin signaling pathway that leads to the osteogenesis of TOLF cells.

DISCUSSION

It is believed that obesity is a risk factor for TOLF. Although other endocrine abnormalities such as hyperinsulinemia, a feature of obese people, might account for TOLF, a recent study shows that hyperleptinemia is closely correlated with TOLF (5, 34, 35), suggesting that leptin, an adipocyte-derived cytokine, is critical in connecting at molecular levels the phenotypical manifestation of obesity and the pathological development of TOLF. In supporting this notion, leptin has been reported to stimulate the osteogenic differentiation of various cell types (36–38).

We reported here the molecular events involved in leptin-stimulated osteogenesis in TOLF cells. We showed that leptin treatment induced the expression of osteocalcin and ALP as well as in vitro mineralization in TOLF cell cultures. In addition, we demonstrated that the STAT3 signaling pathway is activated in response to leptin treatment in TOLF cells and STAT3, along with steroid receptor coacti-
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The p160 coactivator family is thought to contribute to transcriptional activation by being recruited to the gene promoter and forming a platform for the binding of other coactivators. Initially identified as coactivators for nuclear receptor-mediated gene transcription, recent studies have shown that the p160 coactivators also are involved in transcriptional activation by various other transcription factors, such as activator protein, p160 coactivators also are involved in transcriptional activation.

FIGURE 6. The roles of STAT3, Runx2, and SRC-1 on leptin-stimulated TOLF cells osteogenesis. A, the binding of STAT3 and Runx2 on osteocalcin promoter. Electrophoretic mobility shift assays were performed with nuclear extracts (NE) from TOLF cells treated with leptin (100 ng/ml) for 30 min to determine the binding of STAT3 and Runx2 on osteocalcin promoter sequence. B, RNA interference was carried out by introducing pSUPER vector carrying a specific small interfering RNA against the mRNA of SRC-1 into TOLF cells for 48 h. Total proteins were extracted, and Western blotting was performed to monitor protein expression. Transfection efficiency was monitored by cotransfection with an Escherichia coli lacZ construct, and the protein expression of β-galactosidase (β-gal) was detected by Western blotting. B, leptin-induced osteogenesis of TOLF cells after RNA-mediated interference. TOLF cells were transfected with pSUPER vectors carrying specific small interfering RNAs against the mRNAs of SRC-1, GRIP1, or AIB1. Transfected cells were serum-starved, and stimulated with 100 ng/ml leptin for 1, 3, or 7 days followed by osteocalcin expression detection with real-time PCR. Each bar represents the mean ± S.D. from sextuplet experiments, and the asterisks indicate statistical significance (p < 0.05).

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Vigor SRC-1 and osteogenic transcription factor Runx2, transactivates osteocalcin, one of the Runx2 downstream target genes that may in turn mediate the osteogenic activity of leptin. STAT3 plays an essential role during embryonic development, cell survival, and differentiation (39). At the molecular level, STAT3 acts as a transcription activator and interacts with other nuclear factors to regulate a number of genes that are critically involved in cell proliferation and differentiation (40, 41). The biological function of STAT3 in osteotropic activity has been well documented. The gp130 knock-in mice gp130F759/F759, in which interleukin-6 family cytokine-mediated STAT3 activation is enhanced, showed an osteosclerotic phenotype, whereas mice with osteoblast-specific disruption of the STAT3 gene exhibited an osteoporotic phenotype (42). Runx2, also referred to as Cbfa1, Osf2, AML3, and PEBP2αA, is an osteoblast-related transcription factor that is essential for bone formation (43). Homozygous deletion of Runx2 arrests osteoblast maturation, resulting in the absence of endochondral and intramembranous ossification (44). Runx2 can directly stimulate osteogenic differentiation by binding to an osteoblast-specific cis-acting element, termed OSE2, in the promoter region of skeletal target genes and regulating their expression (45). Our experiments identifying a physical and functional interaction between STAT3 and Runx2 in TOLF cells add another line of evidence to support the important roles of STAT3 and Runx2 in osteogenesis.

The p160 coactivator family is thought to contribute to transcriptional activation by being recruited to the gene promoter and forming a platform for the binding of other coactivators. Initially identified as coactivators for nuclear receptor-mediated gene transcription, recent studies have shown that the p160 coactivators also are involved in transcriptional activation by various other transcription factors, such as activator protein, serum response factor, NFκB, and STATs (46, 47), implying a functional complexity for these proteins. We showed that one member of the p160 family, SRC-1, but not GRIP1 and AIB1, was recruited to Runx2 target gene promoter and coactivated the gene transcription, indicating that SRC-1 plays an important role in leptin signaling pathway that leads to the osteogenesis in TOLF cells.

Leptin receptors are expressed in both TOLF cells and non-TOLF cells. Nonetheless, the osteogenic effect of leptin is different in TOLF cells versus non-TOLF cells. Moreover, even different TOLF cells exhibited different levels of mineralization in response to leptin treatment in our experiments. It is conceivable that osteogenic commitment of the cells in the ossification process of TOLF and the metaplasia of TOLF cells into osteogenic cells have already occurred in TOLF, which is consistent with other studies of OLF pathogenesis (19, 48). Further studies are warranted to investigate the molecular mechanism underlying osteogenic commitment of TOLF cells and molecular events involved in the initiation of TOLF.
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