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Leptin stimulates *IGF-1* transcription by activating AP-1 in human breast cancer cells

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Leptin, an adipokine regulating energy metabolism, appears to be associated with breast cancer progression. Insulin-like growth factor-1 (IGF-1) mediates the pathogenesis of breast cancer. The regulation of IGF-1 expression by leptin in breast cancer cells is unclear. Here, we found that leptin upregulates IGF-1 expression at the transcriptional level in breast cancer cells. Activating protein-1 (AP-1)-binding element within the proximal region of *IGF-1* was necessary for leptin-induced IGF-1 promoter activation. Forced expression of AP-1 components, c-FOS or c-JUN, enhanced leptin-induced IGF-1 expression, while knockdown of c-FOS or c-JUN abrogated leptin responsiveness. All three MAPKs (ERK1/2, JNK1/2, and p38 MAPK) mediated leptin-induced IGF-1 expression. These results suggest that leptin contributes to breast cancer progression through the transcriptional upregulation of leptin via the MAPK pathway. [BMB Reports 2019; 52(6): 385-390]

INTRODUCTION

Leptin is a peptide hormone encoded by the leptin (*LEP*) gene, a homolog of the mouse obese (*ob*) gene (1). It is mainly produced by adipocytes and mainly affects the hypothalamus to decrease food intake and modulate glucose and fat metabolism (2, 3). Mice with a homozygous mutation in *ob* (*ob*/*ob*) become massively obese (3). Leptin is also produced by various peripheral tissues, including placenta, ovaries, skeletal muscle, and mammary epithelial cells (4). In addition to energy metabolism, leptin also has numerous biological activities, including in immune function, cell proliferation, angiogenesis, metastasis, and apoptosis (2). In breast cancer cells, leptin stimulates proliferation (5-8). Additionally, overexpression of leptin and leptin receptor (Ob-R) is correlated with a high risk of tumor recurrence (9) and closely associated with the poor prognosis of breast cancer (10, 11). These studies suggest that the leptin signal contributes to promoting breast cancer progression (12).

Ob-R belongs to the type I cytokine receptor family and transmits signals through the JAK-STAT pathway (13). Leptin-regulated genes have been identified using microarray systems in MCF-7 breast cancer cells (14). They found that cell cycle regulators (e.g., cyclin D1) and anti-apoptotic genes (e.g., BCL2 and survivin) are involved in leptin-induced cell proliferation. Additionally, leptin may induce growth factor secretion. A possible candidate causing these effects is insulin-like growth factor-1 (IGF-1). IGF-1 is a potent mitogen in nearly every cell in the body (15). Numerous studies have demonstrated that IGF-1 stimulates cell proliferation, migration, and metastasis and has critical roles in the development of human breast cancer (16, 17). High levels of circulating IGF-1 are closely associated with high risks of prostate and colorectal cancer (18, 19). A recent meta-analysis revealed a positive association between high concentrations of IGF-1 and increased breast cancer risk (20), indicating that IGF-1 is an important growth factor in the development of breast cancer.

Several studies have shown the effect of leptin on IGF-1 expression in hepatocytes (21, 22) and that a positive relationship between leptin and IGF-1 expression; for example, IGF-I can induce the transcriptional activation of the leptin gene in MCF7 cells (11). However, the mechanism underlying leptin regulation of IGF-1 gene expression is unclear in breast cancer cells.

This study aimed to investigate how leptin affects IGF-1 expression in breast cancer cells. We found that leptin stimulates IGF-1 gene expression by activating AP-1 *cis*-acting elements spanning nucleotides −39 to −27 upstream of the transcription initiation site of *IGF-1* in human breast cancer cells.

RESULTS AND DISCUSSION

Upregulation of *IGF-1* transcription by leptin in breast cancer cells

The *IGF-1* expression is stimulated by various signals, including growth hormone (23, 24), thyroid hormone (24),

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epidermal growth factor (25), and parathyroid hormone (26). However, it is unclear whether leptin stimulates *IGF-1*. To investigate whether leptin regulates *IGF-1* expression in breast cancer cells, two breast cancer cell line, estrogen receptor (ER)-positive MCF-7 cells and ER-negative MDA-MB-231 cells, were treated with leptin for various times and *IGF-1* mRNA expression was measured. Real-time PCR analysis showed that *IGF-1* mRNA expression peaked at 6 h and then slowly decreased after leptin stimulation in MCF-7 cells (Fig. 1A). Similar results were obtained in MDA-MB-231 cells (Fig. 1B). Leptin-induced elevation of IGF-1 protein levels was confirmed by immunoblotting in MCF-7 cells (Fig. 1C) and MDA-MB-231 cells (Fig. 1D). Upon leptin stimulation, increased IGF-1 protein levels were easily detectable at 24 h, after which IGF-1 gradually accumulated until 48 h. Immunofluorescence microscopy also revealed strong intensities of IGF-1 staining compared to the vehicle-treated control in MCF-7 cells (Fig. 1E). These data demonstrate that leptin upregulates IGF-1 expression in both ER-negative and ER-positive breast cancer cells.

Leptin stimulates *IGF-1* promoter activity through AP-1 *cis*-acting elements within the 5'-regulatory region of *IGF-1*

The 5′-regulatory region of human *IGF-1* contains several putative *cis*-acting regulatory elements (27). To determine whether leptin upregulates *IGF-1* expression at the transcriptional level and to identify the leptin-responsive *cis*-acting element in the *IGF-1* promoter, we generated a series of deletion constructs of the 5′-flanking region of human *IGF-1* linked to the luciferase reporter gene and transfected them into MCF-7 cells. As shown in Fig. 2A, leptin triggered a significant increase in *IGF-1* promoter reporter activity. The promoter-reporter harboring the sequence from nucleotides −95 to −3 was still capable of inducing reporter activity. These data suggest that leptin response elements are located within this region.

To identify the *cis*-acting element responsible for leptin-induced stimulation of the *IGF-1* promoter, we analyzed genomic sequences between nucleotide −95 and −3 using the web-based transcription factor search tool MatInspector (http://www.genomatix.de/). The result shows that a putative AP-1-binding motif (5′-TCCTTACTCAATA-3′) spanning from nucleotide −39 to −27 (Fig. 2A). AP-1 is a well-known transcription factor complex consisting of homo- or hetero-dimers of Jun (c-Jun, JunB, JunD) and Fos (c-Fos, FosB, Fra-1, Fra-2) (28). AP-1 contributes to the proliferation and transformation of breast cells (29) and is involved in leptin-induced aromatase expression, which catalyzes estrogen biosynthesis, in MCF-7 cells (30). However, the effects of AP-1 on *IGF-1* expression have not been characterized.

To evaluate the role of this putative AP-1-binding site in leptin-induced *IGF-1* expression, we introduced a site-directed mutation in the AP-1-binding sequence using the pIGF1-Luc

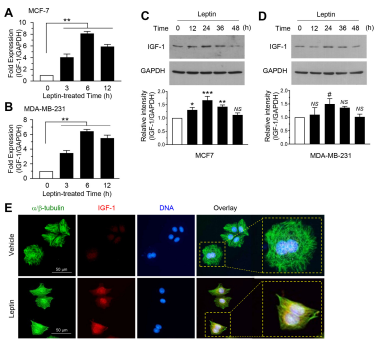

Fig. 1. Effect of leptin on *IGF-1* expression in breast cancer cells. (A, B) MCF-7 (A) and MDA-MB-231 (B) cells were treated with 100 ng/ml leptin for various times (0-12 h). Total RNA was isolated, and *IGF-1* mRNA was measured by real-time PCR. GAPDH mRNA level was used as an internal control. Bars represent mean ± S.D. (n = 3). **P < 0.001; by Sidak's multiple comparison test. (C, D) MCF-7 (C) and MDA-MB-231 (D) cells were treated with 100 ng/ml leptin for various times (0-48 h). Whole cell lysates were measured by immunoblotting using an antibody against IGF-1. GAPDH level was examined as an internal control. The band intensities of IGF-1 relative to GAPDH were measured using ImageJ software. Bars represent mean ± S.D. (n = 3). *P = 0.0069; **P = 0.0006; ***P < 0.0001; $^{\#}$P = 0.0098; *NS*, not significant; by Dunnett's multiple comparisons test. (E) MCF-7 cells were treated with PBS or 100 ng/ml leptin, and then incubated with antibodies against α/β-tubulin and IGF-1 for 2 h, followed by incubation with Alexa Fluor 488-conjugated (green signal for α/β-tubulin) or Alexa Fluor 555-conjugated (red signal for IGF-1) secondary antibody for 30 min. Nuclear DNA was stained with 1 μg/ml Hoechst 33258 for 10 min (blue signal). Bar indicates 50 μm.

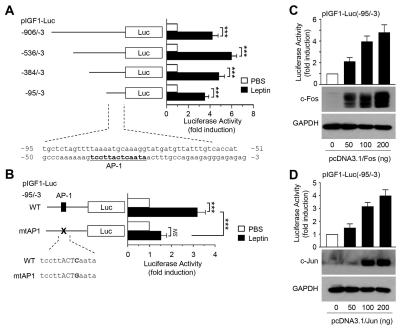

Fig. 2. Role of AP-1-binding element in leptin-induced *IGF-1* promoter activation. (A) MCF-7 cells were transfected with 0.2 μg of a series of 5′-deletion constructs of *IGF-1* promoter reporter plasmids. Putative AP-1-binding sequence is located at −39 to −27 nt. Bars represent mean ± S.D. (n = 3). ***P < 0.0001; by Sidak's multiple comparison test. (B) MCF-7 cells were transfected with 0.2 μg of wild-type (WT) pIGF1-Luc (−95/−3) or AP-1 site mutant construct (mtAP1). After 48 h, the cells were treated with either PBS or 100 ng/ml leptin for an additional 8 h, and luciferase activities were measured. Bars represent the mean ± SD (n = 3). ***P < 0.0001; *NS*, not significant; by Sidak's multiple comparison test. (C, D) MCF-7 cells were co-transfected with pIGF1-Luc (−95/−3) construct and increasing concentrations of expression plasmid (0-200 ng) for c-FOS (C) or c-JUN (D). After 48 h, the cells were collected, and luciferase activities were measured. Bars represent the mean ± SD (n = 3). Exogenous expression of c-FOS (C) or c-JUN (D) was confirmed by immunoblotting (*bottom panels*).

(−95/−3) construct. Disruption of the AP-1-binding core sequence (mtAP1; ACTC to ACTG) resulted in a significant loss of leptin-stimulated promoter activity compared to the wild-type (WT) construct (Fig. 2B). These data suggest that the putative AP-1-binding site at −39/−27 is involved in leptin-induced *IGF-1* promoter activation.

To assess the role of AP-1 in regulating *IGF-1* promoter activity, we transfected the −95/−3 construct into MCF-7 cells, along with an expression plasmid for AP-1 components. Forced expression of c-Fos (Fig. 2C) or c-Jun (Fig. 2D) stimulated promoter reporter activity in a plasmid concentration-dependent manner. These results suggest that AP-1 can *trans*-activate the *IGF-1* promoter in MCF-7 cells.

Knockdown of c-Fos or c-Jun by shRNA abrogates leptin-induced *IGF-1* mRNA expression

To further verify the role of AP-1 in leptin-induced *IGF-1* promoter activity, we established MCF-7 variant cell lines expressing lentiviral shRNA against scrambled control (MCF7/shCT), c-Fos (MCF7/shFos), and c-Jun (MCF7/shJun). Stable knockdown of basal and leptin-induced expression of c-Fos (Fig. 3A) or c-Jun (Fig. 3B) was confirmed after leptin treatment in serum-starved cells. The ability of leptin to induce accumulation of IGF-1 protein was substantially attenuated when either c-Fos or c-Jun expression was reduced compared to in MCF7/shCT cells (Fig. 3C). Real-time PCR analysis shows that leptin elevated *IGF-1* mRNA expression by 6.33 ± 1.04-fold in MCF7/shCT cells, which was reduced to 2.4 ± 0.6- and 1.7 ± 0.43-fold in MCF7/shFos and MCF7/shJun cells, respectively (Fig. 3D). These results demonstrate that AP-1 contributes to leptin-induced *IGF-1* mRNA expression.

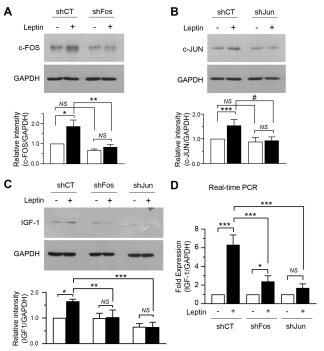

Fig. 3. Effect of c-Fos or c-Jun knockdown on leptin-induced IGF-1 expression. (A, B) MCF-7 variant cells expressing scrambled (shCT), c-Fos shRNA (shFos), or c-Jun shRNA (shJun) were treated with 100 ng/ml leptin for 1 h. Cell lysates were immunoblotted with an antibody against c-FOS (A) or c-JUN (B). GAPDH level was examined as an internal control. The band intensities of c-FOS or c-JUN relative to GAPDH were measured using ImageJ software. Bars represent mean ± S.D. (n = 3). *P = 0.001; **P = 0.0003; ***P = 0.0158; $^{\#}$P = 0.0079; *NS*, not significant; by Sidak's multiple comparison test. (C) MCF7/shCT, MCF7shFos, and MCF7/shJun cells were treated with 100 ng/ml leptin for 12 h. Whole cell lysates were measured by immunoblotting using an antibody against IGF-1. GAPDH level was examined as an internal control. The band intensities of IGF-1 relative to GAPDH were measured using ImageJ software. Bars represent mean ± S.D. (n = 3). *P = 0.0043; **P = 0.0060; ***P < 0.0001; *NS*, not significant; by Sidak's multiple comparison test. (D) MCF7/shCT, MCF7shFos, and MCF7/shJun cells were treated with 100 ng/ml leptin for 6 h. Total RNA was isolated, and *IGF-1* mRNA was measured by real-time PCR. GAPDH mRNA level was examined as an internal control. Bars represent mean ± S.D. (n = 3). *P = 0.0318; ***P < 0.0001; *NS*, not significant; by Sidak's multiple comparison test.

Leptin induces AP-1 expression through multiple MAP kinase pathways

It has been demonstrated that leptin induces AP-1 expression by activating the extracellular signal-related kinase (ERK) mitogen-activated protein kinase (MAPK) pathway to induce aromatase mRNA expression in MCF-7 cells (30). We confirmed that leptin increased the levels of both c-Fos and c-Jun proteins within 30 min after leptin stimulation in MCF-7 cells (Fig. 4A). To delineate the signal pathway mediating leptin-induced AP-1 expression, we treated serum-starved MCF-7 cells with leptin at various times, and the phosphorylation status of MAPKs was assessed using phospho-specific antibodies. The increases in phosphorylated levels of ERK1/2 at Thr202/Tyr204 and p38 MAPK at Thr180/Tyr182 were maximal within 30 min, while JNK1 at Thr183/Tyr185 was evident within 30 min and then progresively increased to 120 min after leptin treatment (Fig. 4B). To determine which MAPK signaling pathway is necessary for leptin-induced AP-1 expression, we used chemical inhibitors against MAPKs. Pretreatment of MCF-7 cells with the MAPK kinase (MEK) inhibitor U0126 led to a substantial reduction in leptin-induced accumulation of both c-Fos and c-Jun proteins (Fig. 4C). In contrast, pre-treatment with the p38 MAPK inhibitor SB203580 had a minimal effect, while the JNK inhibitor SP600125 increased c-Fos but decreased c-Jun protein levels, suggesting that multiple MAPKs differentially regulate AP-1 expression. We next examined whether inhibition of each MAPK affects leptin-induced IGF-1 protein accumulation. Treatment with all three MAPK inhibitors substantially decreased the accumulation of IGF-1 protein (Fig. 4D). To precisely quantify the effect of MAPK inhibitors, we performed real-time PCR analysis. Upon leptin stimulation, the *IGF-1* mRNA level was enhanced by 6.8 ± 0.7-fold, which was significantly reduced to 1.1 ± 0.31-, 2.7 ± 0.27-, and 3.6 ± 0.2-46-fold (n = 3, all P < 0.0001) in the presence of U0126, SB203580, and SP600125, respectively (Fig. 4E). These data suggest that multiple MAPK pathways are important in leptin-induced *IGF-1* expression dependently or independently of AP-1.

The present study shows that leptin stimulates AP-1-mediated *IGF-1* expression in breast cancer cells. We found that (i) disruption of the AP-1-binding site spanning from nucleotides −39 to −27 within the *IGF-1* promoter region attenuated leptin-induced *IGF-1* promoter activity, (ii) exogenous expression of either c-Fos or c-Jun stimulated the *IGF-1* promoter, and (iii) knockdown of either c-Fos or c-Jun abrogated

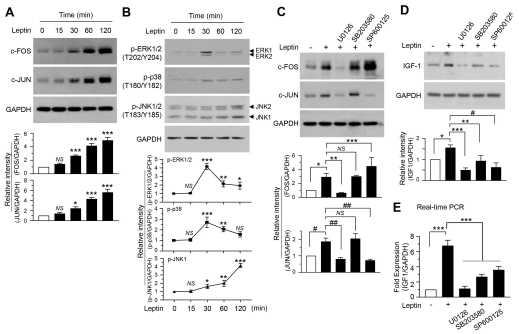

Fig. 4. Differential role of MAPK pathways in leptin-induced AP-1 expression. (A) MCF-7 cells were treated with 100 ng/ml leptin for various times (0-120 min). Cell lysates were immunoblotted with an antibody against c-Fos or c-Jun. GAPDH level was examined as an internal control. The band intensities of c-FOS or c-JUN relative to GAPDH were measured using ImageJ software. Bars represent mean ± S.D. (n = 3). *P = 0.0016; ***P < 0.0001; *NS*, not significant; compared with control (0 min) by Dunnett's multiple comparisons test. (B) MCF-7 cells were treated with 100 ng/ml leptin for various times (0-120 min). Cell lysates were immunoblotted using a phospho-specific antibody against ERK1/2 (Thr202/Tyr204), p38 (Thr180/Tyr182), or JNK1/2 (Thr183/Tyr185) MAPK. GAPDH level was examined as an internal control. The band intensities of phosphorylated MAPKs relative to GAPDH were measured using ImageJ software. Bars represent mean ± S.D. (n = 3). *P = 0.0095; **P = 0.0026; ***P < 0.0001; *NS*, not significant; compared with control (0 min) by Sidak's multiple comparison test. (C) MCF-7 cells were pre-treated with U0126 (10 μM), SB203580 (20 μM), or SP600125 (20 μM) for 30 min before stimulation with 100 ng/ml of leptin. After 1 h, cell lysates were prepared and immunoblotted using an antibody against c-Fos or c-Jun. GAPDH level was examined as an internal control. The band intensities of c-FOS or c-JUN relative to GAPDH were measured using ImageJ software. Bars represent mean ± S.D. (n = 3). *P = 0.0123; **P = 0.0038; ***P = 0.0430; $^{\#}$P = 0.0005; $^{\#\#}$P < 0.0001; *NS*, not significant; by Sidak's multiple comparison test. (D) MCF-7 cells were pre-treated with U0126 (10 μM), SB203580 (20 μM), or SP600125 (20 μM) for 30 min before stimulation with 100 ng/ml of leptin. After 12 h, whole lysates were prepared, and IGF-1 protein level was measured by immunoblot analysis. The band intensities of IGF-1 relative to GAPDH were measured using ImageJ software. Bars represent mean ± S.D. (n = 3). *P = 0.0074; **0.0034; ***P < 0.0001; $^{\#}$P = 0.0002; *NS*, not significant; by Sidak's multiple comparison test. (E) Cells were treated as in (D). Total RNA was isolated and real-time PCR analysis was carried out. GAPDH mRNA level was examined as an internal control. Bars represent mean ± S.D. (n = 3). *** P < 0.0001 by Sidak's multiple comparison test.

leptin-induced *IGF-1* expression. Additionally, we found that all three major MAPKs, ERK1/2, JNK1/2, and p38 MAPK, which differentially regulate AP-1 expression, are necessary for leptin-induced *IGF-1* expression in MCF-7 breast cancer cells. The previous study has demonstrated that combined treatment with leptin and IGF-1 increases proliferation as well as migration and invasion of MCF-7 and MDA-MB-231 breast cancer cells (31). We also observed that the combination of leptin and IGF-1 led to a synergistic increase in cell proliferation when compared with either treatment alone (Supplemental Fig. S1). As downstream signaling of leptin and IGF-1 involves PI3K-AKT and MAPK signalings, leptin might share a common mechanism of action with IGF-1 (32, 33). Moreover, leptin and IGF-1 synergistically transactivate epidermal growth factor receptor (EGFR) in breast cancer cells (31). Thus, it seems likely that leptin and leptin-induced IGF-1 are thought to promote the progression of breast cancer synergistically. Our results further support the role of leptin in crostalk with IGF-1 to contribute to the progression of breast cancer.

MATERIALS AND METHODS

Cells and cell culture

MCF-7 and MDA-MB-231 human breast cancer cells were from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Reagents

Human leptin was obtained from Peprotech (Rocky Hill, NJ, USA). U0126, SB203580, and SP600125 were purchased from Calbiochem (San Diego, CA, USA). The firefly and Renilla Dual-Glo™ Luciferase Assay System was purchased from Promega (Madison, WI, USA). The pRL-null plasmid, which encodes Renilla luciferase, was purchased from Promega. Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Reverse transcription-PCR and quantitative real-time PCR

Total RNA was extracted using a TRIzol RNA extraction kit (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Quantitative real time-PCR was performed on an iCycler iQ™ system (Bio-Rad) according to the manufacturer's recommendations. The reaction mixture (20 μl) contained 10 μl of iQ™ SYBR® Green Supermix (Bio-Rad). Gene-specific IGF-1 PCR primers were as follows: forward, 5′ GTCTGATAA TCTTGTTAGTCTATA-3′; reverse, 5′-CACAG ATGGAATCTTGTG-3′. GAPDH PCR primers were: forward, 5′-CCAAGGAGTAAGAAACCCTGGAC-3′; reverse, 5′-GGGCC GAGTTGGGATAGGG-3′. The specificity of real-time PCR was verified by melting curve analysis. GAPDH was used to normalize the RNA levels in the tested samples.

Immunoblot analysis

Antibodies against GAPDH, c-Fos, and c-Jun were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Phospho-ERK1/2 (Thr202/Tyr204), phospho-p38 (Thr180/Tyr182), and phospho-JNK1/2 (Thr183/Tyr185) were obtained from Cell Signaling Technology (Danvers, MA, USA). IGF-1 antibody was obtained from Gene Tex (Irvine, CA, USA). Cells were lysed and separated as described previously (34). The blots were incubated with the primary and secondary antibodies

and developed using an enhanced chemiluminescence detection system. Relative band intensity was quantified using ImageJ version 1.52a software (National Institute of Health, Bethesda, MD, USA) and was expressed as a ratio to GAPDH.

Immunofluorescence microscopy

MCF-7 cells cultured on coverslips were either treated with phosphate-buffered saline (PBS) or 100 ng/ml leptin for 30 min, followed by fixation, permeabilization, and incubation of primary antibodies specific to αβ-tubulin and IGF-1. After 2 h, the cells were incubated with Alexa Fluor 488-conjugated (green signal) or Alexa Fluor 555-conjugated (red signal) secondary antibody for 30 min as described previously (35). Nuclear DNA was stained with 1 μg/ml Hoechst 33258 for 10 min (*blue signal*). Fluorescent staining of cells was examined under an EVOS FL fluorescence microscope (Advanced Microscopy Group; Bothell, WA, USA).

Generation and mutagenesis of human *IGF-1* promoter-reporter constructs

An *IGF-1* promoter fragment spanning nucleotides −906 to −3 upstream of the transcription start site was synthesized from human genomic DNA (Promega) by PCR using the primers 5′-CAA AACAGC TGGCTTGGA CC-3′ (forward primer; −906F) and 5′-TCTCTCTCCCTCTTCTGGCA-3′ (reverse primer; −3R). The amplified PCR products were ligated into a T&A vector (RBC Bioscience, Taipei County, Taiwan) and then digested with *Kpn*I and *Bgl*II. The products were ligated into the *Kpn*I and *Bgl*II sites of the pGL4-basic vector (Promega), yielding pIGF1-Luc (−906/−3). A series of deletion constructs of human *IGF-1* promoter fragments was synthesized by PCR using the pIGF1-Luc (−906/−3) construct as a template plasmid. Forward primer sequences were 5′-CCTCATCGCA GAGAAAAAG-3′ (−536 to −3), 5′-CCCCAGTCACTTCA GGGTTA-3′ (−384 to −3), and 5′-TGCTCTAGTTTTAAAA TGCAAAGG-3′ (−95 to −3). One reverse primer, −3R, was used to generate all deletion constructs. The PCR products were ligated into the T&A vector, followed by digestion with *Kpn*I and *Bgl*II. The products were ligated into the same sites of the pGL4-basic vector. Site-specific mutation of the AP-1-binding site (mtAP1) was performed using the QuickChange site-directed mutagenesis system (Stratagene, La Jolla, CA, USA) with the −95/−3 construct used as a template plasmid. Primer sequences used to generate the point mutation were as follows: mtAP1 forward, 5′-AATAACTTTGCCAGAAGAGGG AGAGA-3′; mtAP1 reverse 5′-CAGTAAGGACTTTTTTGGGC ATGGTG-3′. PCR conditions were as follows: hold for 4 min at 95°C, followed by 30 cycles consisting of denaturation at 95°C (30 s), annealing at 55°C (1 min), and elongation at 60.3°C (3 min). The point mutation site was verified by DNA sequencing (Macrogen, Seoul, Republic of Korea).

IGF-1 promoter reporter assay

MCF-7 cells were seeded into 12-well plates and transfected with 0.1 μg of the *IGF-1* promoter construct using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. Where indicated, co-transfected concentrations of mammalian expression vectors for c-Fos (pCDNA3.1/Fos) or c-Jun (pCDNA3.1/Jun) were included. At 24 h post-transfection, firefly luciferase activity was measured using the Dual-Glo™ Luciferase Assay System (Promega). The relative level of luciferase activity in the untreated cells was designated as 1. Luminescence was measured with a dual luminometer (Centro LB960; Berthold Tech, Bad Wildbad, Germany).

Stable knockdown of c-Fos and c-Jun by using shRNA

MISSION shRNA Lentiviral Transduction Particles targeting c-Fos (SHCLNV-NM_005252) or c-Jun (SHCLNV-NM_002228) were introduced into MCF-7 cells according to the manufacturer's instruction (Sigma-Aldrich). After 2 weeks, silencing of STAT3, c-Fos or c-Jun expression was determined by immunoblotting.

Statistical analysis

Statistical significance was analyzed by one-way analysis of variance followed by Sidak's multiple comparisons test or by Dunnett's multiple comparisons test using GraphPad Prism version 7.04 software (GraphPad Software, Inc., La Jolla, CA, USA). A value of $P < 0.05$ was considered statistically significant.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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