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Upregulation of survivin by leptin/STAT3 signaling in MCF-7 cells

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Abstract

Leptin and its receptors are overexpressed in breast cancer tissues and correlate with poor prognosis. Survivin, a member of the inhibitor of apoptosis protein (IAP) gene family, is generally upregulated in tumor tissues and prevents tumor cells from apoptosis. Here we showed that leptin upregulated survivin mRNA and protein expression in MCF-7 breast cancer cells. Meanwhile, leptin suppressed docetaxel-induced apoptosis by inhibiting caspase activity. Knockdown of signal transducer and activator transcription 3 (STAT3) expression by small interfering RNA (siRNA) blocked leptin-induced upregulation of survivin. TransAM ELISA showed that leptin increased nuclear translocation of active STAT3. In addition, chromatin immunoprecipitation (ChIP) assay detected an enhanced binding of STAT3 to survivin promoter in MCF-7 cells after treatment by leptin. Further studies showed that leptin enhanced the transcriptional activity of survivin promoter. Collectively, our findings identify leptin/STAT3 signaling as a novel pathway for survivin expression in breast cancer cells.
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Keywords: Leptin; Survivin; Signal transducer and activator transcription 3; MCF-7; Apoptosis

Obesity is a risk factor for postmenopausal breast cancer [1]. Multiple adipocyte-derived cytokines have been implicated in obesity-related breast carcinogenesis including leptin [2]. Initially identified as a neurohormone regulating food intake and energy expenditure, leptin is well documented as a mitogen for many cancers [3]. In breast cancer, overexpression of leptin and its receptors (OBR) correlates with poor prognosis [4,5]. Binding of leptin to OBR promotes the growth of breast cancer cells through several signaling cascades such as janus-activated kinase 2/signal transducer and activator of transcription 3 (Jak2/STAT3) [6].

STAT3 belongs to a family of latent cytoplastic transcription factors. After it is activated by cytokines or growth factors, STAT3 becomes phosphorylated, translocates to the nucleus and regulates transcription of specific target genes [7]. STAT3 is regarded as an oncogene, promoting proliferation and inhibiting apoptosis.

Very recently, survivin has been identified as a direct downstream gene of STAT3 in breast cancer cells [8]. Survivin is a member of inhibitor of apoptosis (IAP), which is generously upregulated in tumor tissues but not in normal ones [9]. It has been reported that overexpression of survivin correlates with loss of apoptosis and poor prognosis in breast cancer cells [10,11].

Since STAT3 is downstream of leptin and upstream of survivin, we hypothesized that leptin could upregulate survivin expression and exert an anti-apoptotic effect in breast cancer cells. In this study, we found that leptin increased survivin expression at the transcriptional level and reduced apoptosis induced by the chemotherapeutic drug docetaxel. Furthermore, we demonstrated that leptin-induced upregulation of survivin was strongly dependent on the activation of STAT3 in MCF-7 cells.

Materials and methods

Cell culture. Human MCF-7 breast cancer cells were purchased from the cell bank of Peking Union Medical College (Beijing, China) and

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cultured in phenol-red free DMEM (Sigma Chemical, St. Louis, MO) supplemented with 8% charcoal-stripped fetal bovine serum (FBS), L-glutamine (2 mM), streptomycin (0.1 mg/ml), and penicillin (100 U/ml) at 37 °C, under 5% CO_2 atmosphere.

Analysis of cell survival and apoptosis. Cells (5×10^4 per well) were cultured in 48-well plates, containing complete medium for 24 h and then serum-starved overnight. Subsequently, leptin (10 nM) (R&D Systems, Minneapolis, MN) and/or docetaxel (50 nM) (Aventis Pharma, Bridgewater, NJ) were added to the culture medium and further cultured for 24 h. Finally, cell apoptosis was quantified by measuring the intracellular nucleosome concentration in cell lysates using the cell-death enzyme-linked immunosorbent assay (ELISA) kit (Roche, Germany) following the manufacturer's instructions. On the other hand, after leptin and/or docetaxel treatment for 48 h, the percentage of viable cells was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay (Sigma) as previously described [6].

Caspase 9 activity. Cells were treated with leptin (10 nM) and/or docetaxel (50 nM) for 48 h. Then cells were collected and their lysates were quantified for caspase 9 activity as previously described [12].

Quantitative real-time RT-PCR. Total RNA was extracted using TRIzol reagent (Invitrogen), 2 μg RNA was reverse transcribed with superscript 2 (Invitrogen). RT product (2 μl) was amplified by real-time PCR for quantifying survivin expression as previously described [12].

Western-blot analysis. For Western-blot analysis, 30 μg of total extracted proteins from cell lysates was applied per lane before SDS–PAGE. Following transfer to nitrocellulose membranes, protein expression levels were detected using anti-STAT1, anti-STAT3, anti-survivin (Santa Cruz) or anti-actin antibodies (Sigma). Western-blot strips were examined by reflectance densitometry with Image Master Software (SYDR-1990, SYNGENE).

Small interfering RNA (siRNA) treatment. siRNA was designed and transfected into cells as described elsewhere with some modifications [13]. Briefly, cells were treated with siRNA targeting STAT3 for 48 or 72 h. siRNA transfection (600 nM) was performed with lipofectamine-2000 (Invitrogen). The sequences for siRNA against STAT3 were 5′-AAC AUC UGC CUA GAU CGG CUA dTdT-3′; 3′-dTdT GUA GAC GGA UCU AGC CGA U-5′.

TransAM ELISA. Confluent cells were serum-starved overnight before treatment by leptin (10 nM) or IL-6 (20 ng/ml) for 30 min. Subsequently, nuclear extraction was performed using a nuclear extraction kit (Active Motif, Belgium). Ten micrograms of nuclear extraction protein was used for assessing the nuclear translocation of active STAT3 by the STAT3 TransAM ELISA (Active Motif) according to the manufacturer's instructions.

Chromatin immunoprecipitation (ChIP) assay. Cells were serum-starved overnight before stimulation with leptin (10 nM) or IL-6 (20 ng/ml) for 30 min. After cross-linking chromatin with proteins by 1% of formaldehyde, the assay was performed using a ChIP Assay kit (Upstate, Charlottesville, VA) according to the manufacturer's protocol. An anti-STAT3 polyclonal antibody (Santa Cruz) was added to precipitate the protein–chromatin complexes as described previously [8]. A PCR primer pair for amplification of a survivin promoter region (−1231 to −1009) is forward primer, 5′-CAGTGAGCTGAGATCATGCC-3′; reverse primer, 5′-TATTAG CCCTCC AGCCCCAC-3′.

Expression constructs and site-directed mutagenesis. Human survivin promoter luciferase constructs include: (1) pLuc1430 that contains 1430 nt of 5′-flanking region from −39 to −1469 bp of the survivin gene; (2) pLuc-cyc1.2, which contains a 269 nt 5′-flanking region from +1 to −268 bp of the survivin gene. Generation of these plasmids was described previously [14]. pGL3-Basic plasmid and the pRL-CMV-*Renilla* plasmid were purchased from Promega. Mutant STAT3 binding sites (TTN_5AA to TTN_5TT) were introduced into the pLuc1430 construct by using a site-specific mutagenesis kit (Stratagene, La Jolla, CA).

Transfection and luciferase assay. Cells were seeded at 1×10^5 per well in 12-well plates and serum-starved overnight. The cells were then cotransfected with 1 μg each of the reporter constructs (pGL3-Basic, pLuc1430, and pLuc-cyc1.2, specific site-directed mutant constructs) and 10 ng pRL-CMV-*Renilla* internal control plasmids with lipofectamine-2000 (Invitrogen). After transfection for 48 h, cells were treated with leptin (10 nM) for 2 h. Firefly and *Renilla* Luc activities were assessed by a Dual Luciferase Assay System (Promega) according to the manufacturer's instructions.

Statistic analysis. All values were expressed as means ± SEM. Statistical analysis was performed using Student's t test. A p value above 0.05 was considered statistically significant.

Results and discussion

The present study aimed to investigate the anti-apoptotic effect of leptin on breast cancer cells and the underlying mechanism.

In order to determine the effect of leptin on the apoptotic response in breast cancer cells, we treated MCF-7 breast cancer cells with docetaxel which was shown to induce the apoptosis of MCF-7 cells at the dose of 50 nM [12]. The concentration of leptin selected for the experiment (10 nM) was in the range of those tested in normal and obese subjects [15]. The MCF-7 breast cancer cell line was chosen because it expresses the functional leptin receptor (Ob-Rb) [6]. In addition, the MCF-7 cell line has been suggested as a good model for identifying molecular events for breast carcinogenesis [16]. We quantified cell apoptosis by measuring intracellular nucleosomes as previously reported [17]. Leptin reduced docetaxel-incuced apoptosis by ~39%. Interestingly, leptin also attenuated serum starvation-induced apoptosis by ~30% (Fig. 1A). Consistent with the results from the apoptosis assay, leptin significantly increased the overall numbers of docetaxel-treated MCF-7 cells as shown by MTT assay (Fig. 1B). Previous literature confirms the proliferative effect of leptin on MCF-7 cells [6,18]. From these and our present findings, we postulate that the overall increase of MCF-7 cell numbers in response to leptin results from a combination of stimulating proliferation and inhibiting apoptosis. Bruno et al. have reported the anti-apoptotic effect of leptin through inhibition of caspase family members (caspase 3, 8) on neutrophils [19]. In agreement with these findings, we found that caspase 9 activity was significantly inhibited by leptin in MCF-7 cells (Fig. 1C). It is of note that we also detected a suppression of caspase 3 like DEVDase by leptin in MCF-7 cells despite the deficiency of caspase 3 in the cells (data not shown). We postulate that this effect may be due to the inhibition of caspase 7 which shares a common substrate with caspase 3 [20]. These findings suggest that leptin exerts an anti-apoptotic effect through targeting multiple caspase family members and this effect is not limited to tumor cells.

IAP family members, including survivin and XIAP, have been shown to inhibit caspase activities [21]. Recently, survivin has been demonstrated to decrease caspase 9 activity in breast cancer cells [22]. We next detected whether leptin could upregulate survivin, thus conferring a resistance of apoptosis. As we expected, Western-blot analysis showed that survivin protein was significantly increased after exposure to leptin for 2 h (Fig. 2A and B). Quantitative real-time PCR also showed a rapid induction of

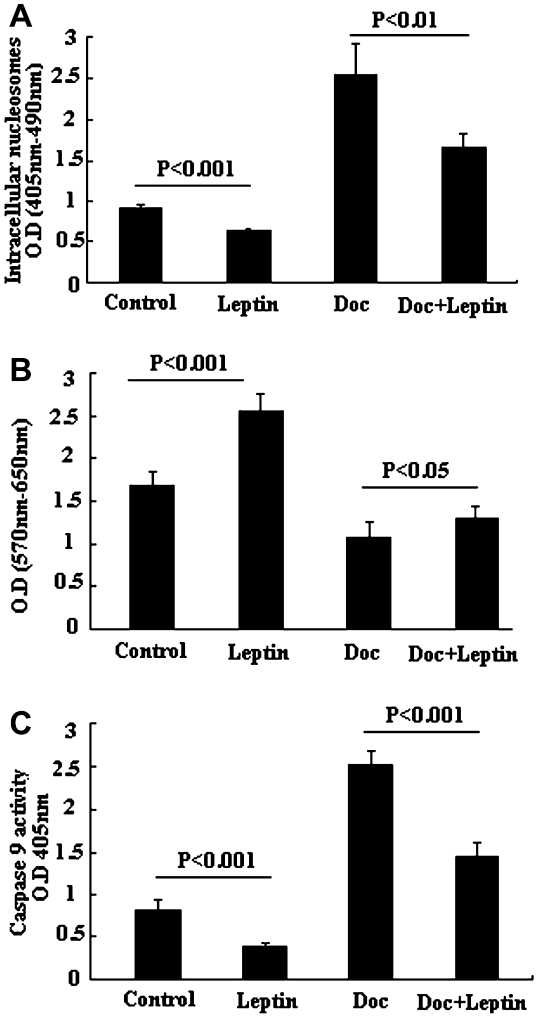

Fig. 1. Leptin reduces docetaxel-induced apoptosis in MCF-7 breast cancer cells. (A) Apoptotic analysis using ELISA for quantifying intracellular nucleosomes. Values were normalized for cell numbers. (B) MTT analysis for viable cells. (C) Twenty-five micrograms of proteins from total cell lysates was examined for caspase 9 activity using a specific substrate (Ac-LEHD-AFC). All data represent means ± SEM ($N = 4$–6).

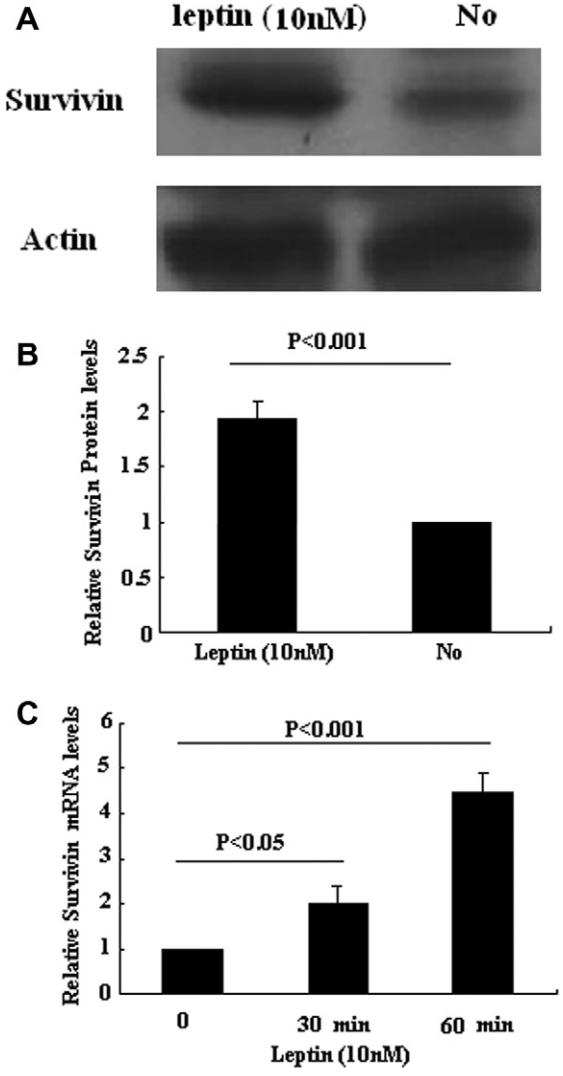

Fig. 2. Leptin promotes survivin expression in MCF-7 cells. (A) Western-blot analysis of the level of survivin protein after treatment by leptin for 2 h. Actin was used as a loading control. (B) Densitometric analysis of the level of survivin protein after normalization to actin expression. (C) Total RNA was isolated and examined by quantitative real-time RT-PCR, to determine changes in the level of survivin mRNA expression after normalization to β-actin expression. All data were presented as a fold induction relative to the control group. Mean ± SEM ($N = 3$–4).

survivin mRNA by leptin (Fig. 2C). Survivin mRNA was increased ~2.0-fold by 30 min and ~4.5-fold at 60 min after treatment by leptin. Worthy of mentioning is that we did not detect an upregulation of XIAP by leptin (data not shown), indicating that survivin plays a key role in leptin-mediated suppression of caspase activity.

To determine whether STAT3 could mediate the effect of leptin on survivin expression, we transfected siRNA against STAT3 into MCF-7 cells and detected the expression of STAT3 and survivin protein. Western blot showed that STAT3 siRNA reduced STAT3 expression compared with the mock transfection group (Fig. 3A). Meanwhile, STAT1 protein expression was not affected by STAT3 siRNA, suggesting that the siRNA is specific to inhibit STAT3. We further demonstrated that both basal levels and leptin-induced upregulation of survivin protein and mRNA were significantly inhibited after knocking down STAT3 expression (Fig. 3B and C). Our data suggest that the STAT3 signaling pathway plays a crucial role in leptin-mediated survivin expression.

Previous studies showed that leptin increased STAT3 phosphorylation, which in turn promoted cell proliferation through binding to its target gene [6]. In agreement with this result, we also found that leptin increased nuclear translocation of active STAT3 in MCF-7 cells (Fig. 4A). After treatment with leptin for 30 min, nuclear translocation of active STAT3 increased ~2.5-fold. The efficacy of leptin (10 nM) is similar to that of IL-6 (20 ng/ml), a well-known cytokine to activate STAT3. STAT3 has been shown to directly bind to survivin promoter. Meanwhile, two STAT3 binding sites have been identified in the 5′-flanking region of survivin gene [8]. To further identify whether leptin could enhance the binding of STAT3 to

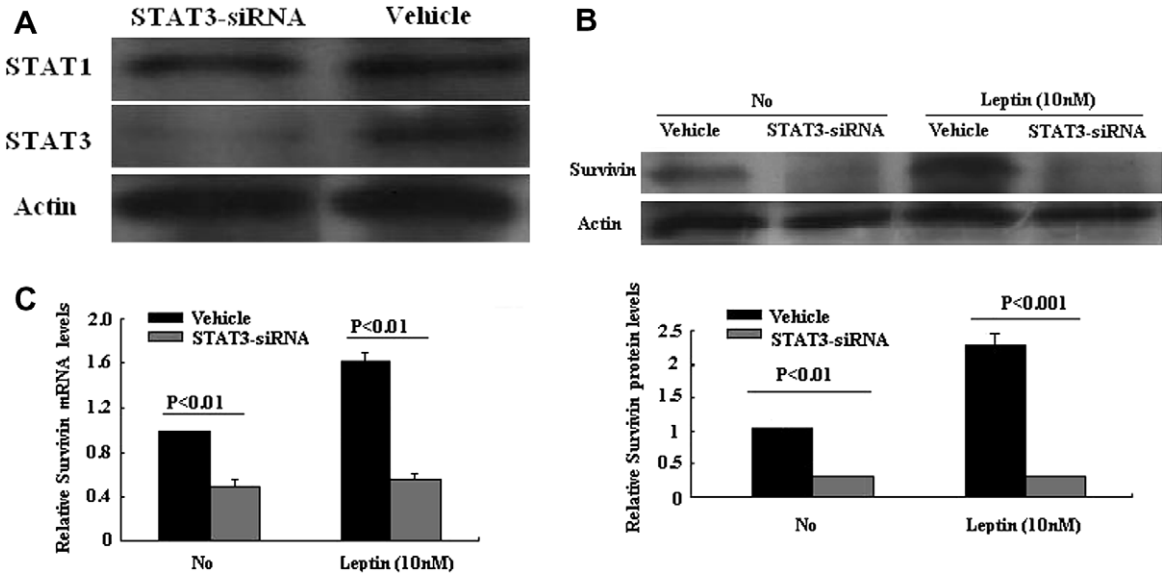

Fig. 3. Knockdown of STAT3 reverses leptin-induced upregulation of survivin. MCF-7 cells were either mock transfected with lipofectamine-2000 or transfected with STAT3 siRNA (600 nM) for 48 h. (A) Cells were lysed for immunoblotting with antibodies against STAT3, STAT1, and actin. (B) Top: following the transfection procedure, MCF-7 cells were treated with or without leptin (10 nM) for 2 h and then subjected to immunoblotting with antibodies against survivin and actin. Bottom: densitometric analysis of the level of survivin protein. (C) Following the transfection procedure, MCF-7 cells were subjected to leptin treatment for 30 min. Then RNA was isolated for quantitative real-time RT-PCR analysis. All data were presented as a fold induction relative to the control group. Mean ± SEM ($N = 3$–4).

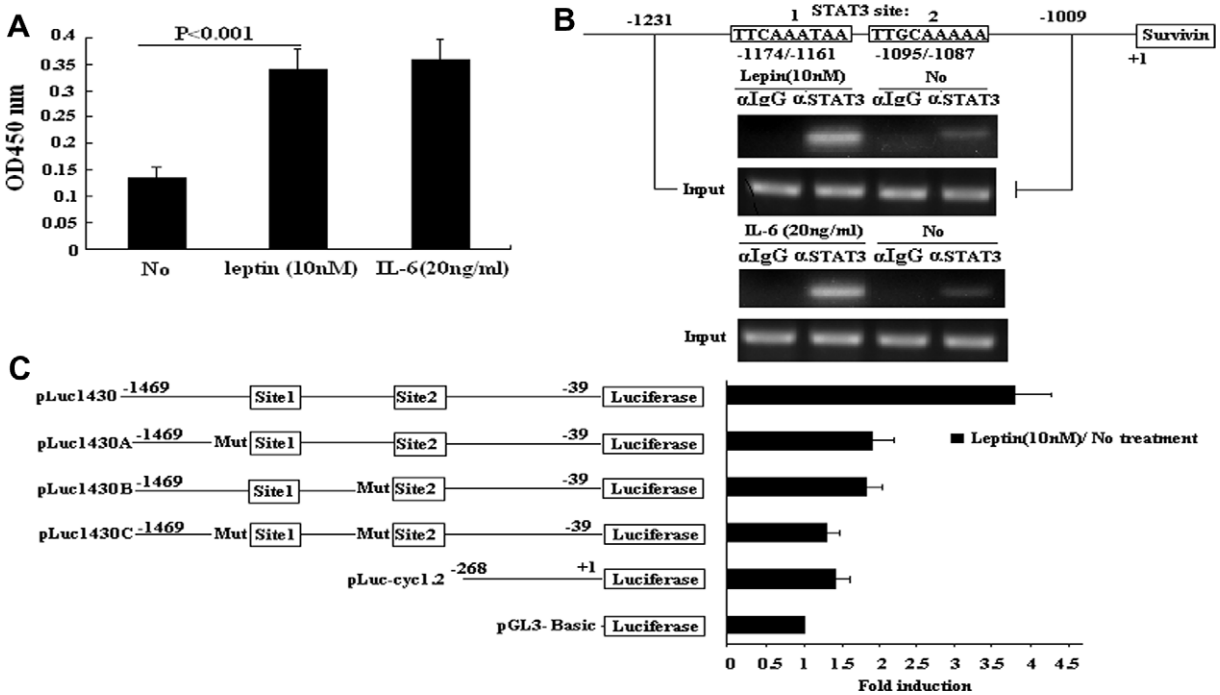

Fig. 4. Leptin enhances the binding of STAT3 to survivin promoter. (A) Active STAT3 in nuclear extracts were quantified by ELISA. OD value (450 nm) is proportional to the concentration of STAT3. Mean ± SEM ($N = 4$). (B) Chromatin immunoprecipitation (ChIP) assay was performed using anti-STAT3 antibody (αSTAT3) or irrelevant anti-immunoglobin G (αIgG) antibody as negative control. PCR primers covering the STAT3 binding sites of survivin promoter region were used to detect promoter fragment in immunoprecipitates. Input: total genomic DNA used as control for the PCR. (C) Luciferase assay. Cells were transfected with the various promoter constructs (mutant forms: TTN_5AA to TTN_5TT) and then incubated with or without leptin (10 nM) for 2 h. Luciferase activity was normalized for transfection efficiency using a *Renilla* reporter plasmid. Results were expressed as a fold induction relative to the no treatment group. Mean ± SEM ($N = 3$).

the survivin promoter in live cells, we performed a ChIP assay using the primers covering the suggested STAT3 binding sites. As shown in Fig. 4B, leptin (10 nM) did augment the binding of STAT3 to survivin promoter after treatment for 30 min, which is similar to the efficacy of IL-6 (20 ng/ml).

To further investigate whether the STAT3 binding sites are involved in leptin-mediated survivin expression, we transfected survivin promoter luciferase constructs into MCF-7 cells. As shown in Fig. 4C, the survivin promoter construct containing both of the two STAT3 binding sites, pLuc1430, raised luciferase reporter activity to ~4-fold after treatment by leptin (10 nM). However, when introducing mutations into any of the two sites (pLuc1430A or B), reporter gene induction by leptin was substantially reduced, indicating that both sites work in leptin-mediated survivin expression. The combinatorial mutant construct (pLuc1430C) showed a further decrease in luciferase activity below the single mutant levels. Leptin did not upregulate luciferase activity from empty pGL3-Basic plasmids. It should be of note that leptin-induced upregulation was still present after the two STAT3 binding sites were muted. Also, leptin augmented the activity of the survivin core promoter, pLuc-cyc1.2 [12], which does not contain potential STAT3 binding sites. These findings suggest that other transcription factors are involved in leptin-induced survivin transcription. One candidate gene is c-myc. c-Myc is a target gene of STAT3 [23] and has been shown to transactivate survivin expression in breast cancer cells [24]. Since c-myc has been reported to be activated by leptin through STAT3 [6], we postulate that leptin/STAT3 signaling may also upregulate survivin expression through c-myc. It is also possible that STAT3-independent pathways were responsible for the remaining leptin-induced reporter activity.

In conclusion, we identify leptin/STAT3 signaling as a novel pathway that upregulates survivin expression and mediates resistance of apoptosis in MCF-7 breast cancer cells. These findings gain insight into the molecular link between obesity and breast cancer.

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