Biochemical and Biophysical Research Communications 394 (2010) 59–63

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Leptin upregulates telomerase activity and transcription of human telomerase reverse transcriptase in MCF-7 breast cancer cells

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ARTICLE INFO

Article history:
Received 11 February 2010
Available online 18 February 2010

Keywords:
Leptin
Telomerase
hTERT
STAT3

ABSTRACT

The aim was to analyze the mechanism of leptin-induced activity of telomerase in MCF-7 breast cancer cells. We found that leptin activated telomerase in a dose-dependent manner; leptin upregulated the expression of Human Telomerase Reverse Transcriptase (hTERT) at mRNA and protein levels; blockade of signal transducer and activator of transcription 3 (STAT3) phosphorylation significantly counteracted leptin-induced hTERT transcription and protein expression; chromatin immunoprecipitation analysis showed that leptin enhanced the binding of STAT3 to the hTERT promoter. This study uncovers a new mechanism of the proliferative effect of leptin on breast cancer cells and provides a new explanation of obesity-related breast cancer.

Introduction

Obesity is a risk factor in breast cancer [1]. Obesity breast cancer patients face a higher risk for lymph node metastasis, larger tumor burden, and mortality compared with those non-obese patients. At present, the mechanism of obesity-related breast cancer remains to be defined. Previous studies have shown that overweight is associated with overexpression of many hormones including leptin [2]. Leptin is an adipose-secreted hormone. It functions through binding to its specific receptors. Evidence has accumulated that leptin is a link between obesity and higher incidence of breast cancer. Leptin induces the growth of breast cancer through multiple mechanisms, including regulating the cell cycle, apoptosis, and the extracellular environment [3]. For instance, leptin enhances the expression of cell cycle regulators (Cyclin D, Cyclin G, and cdk2) and anti-apoptotic protein (Bcl-2 and survivin).

Telomerase is a ribonucleoprotein complex composed of the catalytic subunit human telomerase reverse transcriptase (hTERT) and RNA subunit human telomerase RNA [4]. Overactivity of telomerase occurs in about 90% cancer cells whereas normal cells exhibit undetectable level of activity [5]. Activation of telomerase is required for cancer cells to maintain their malignant phenotype and therefore provides a potential target for the treatment of cancer. Importantly, hTERT expression is specific to cancer cells and tightly associated with telomerase activity [6]. Therefore, numerous studies have focused on the cancer-specific regulation of hTERT and its application of tumor diagnosis and treatment. hTERT promoter has numerous transcription factor binding sites such as c-Myc, SP1, NF-kB, MZF2, AP1. Recently, signal transducer and activator of transcription 3 (STAT3) has been identified as a critical regulator of hTERT [7]. STAT proteins are critical mediators of cytokine signaling in cancer cells [8]. After phosphorylation, STAT3 is activated and translocated to the nucleus to regulate target gene. Furthermore, previous studies have shown that leptin signaling is mainly mediated by the Janus kinase 2 (JAK2)/STAT3 pathway [9,10]. Based on these studies, we postulate that leptin may upregulate the expression of hTERT.

In this study, we aimed (1) to evaluate the effect of leptin on the regulation of telomerase activity in MCF-7 cells, (2) to evaluate the expression of hTERT in response to leptin (3) to elicit the role of STAT3 in leptin-induced expression of hTERT. Above all, we try to understand the mechanism of leptin-induced proliferation in MCF-7 breast cancer cells and provide a new explanation for obesity-related breast cancer.

Materials and methods

Cell culture, reagents and treatments. Human MCF-7 breast cancer cells were obtained from Tianjin Medical University Cancer Institute and Hospital (Tianjin, China) and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM) at 37 °C, under 5% CO_2 atmosphere. For treatment, cells were plated at a density of 3×10^5 cells/well in six-well plates containing complete medium and then serum-starved overnight. Subsequently, cells were treated with different concentrations of recombinant human Leptin (BioVision, Mountain View, USA) and/or STAT3 inhibitor AG490 (Calbiochem, Darmstadt, Germany) 100 μM.

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doi:10.1016/j.bbrc.2010.02.093

Western blot analysis. For Western blot analysis, 50 μg of total extracted protein from cell lysates was applied per lane before SDS–PAGE. Following transfer to PVDF membranes, protein expression levels were detected using anti-hTERT anti-phosphorylated-STAT3 and anti-β-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, USA). Western blot strips were examined by reflectance densitometry with Image J Software.

Quantitative real-time RT-PCR. Total RNA was extracted using TRIzol reagent (Invitrogen, Paisley, UK), Complementary DNA (cDNA) was synthesized using a first strand cDNA synthesis kit (TaKaRa, Dalian, China). Two microliters sample of the cDNA was then quantified by real-time PCR using primer pairs for hTERT or β-microglobulin with SYBR Green PCR Master mix. Real-time PCR was performed using the ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, USA). Primer sequences for hTERT mRNA were 5′-CGG AAG AGT GTC TGG AGC AA-3′ for the upstream primer and 5′-GGA TGA AGC GGA GTC TGG A-3′ for the downstream primer. In addition, specific primers for the β-microglobulin were used as control. Primer sequences for β-microglobulin mRNA were 5′-ACC CCC ACT GAA AAA GAT GA-3′ for the upstream primer and 5′-ATC TTC AAA CCT CCA TGA TG-3′ for the downstream primer. The quantity of PCR product generated from amplification of the hTERT gene was standardized using the quantity of β-microglobulin product for each sample to obtain a relative level of gene expression.

Small interfering RNA treatment. Cells were treated with small interfering RNA (siRNA) targeting STAT3 for 48 h as described previously [7]. Briefly, transfections with siRNAs were done overnight with lipofectamine 2000 (Invitrogen, Carlsbad, CA) and supplemented with serum the next morning. The sequences for siSTAT3 were previously published [7].

Telomerase ELISA. MCF-7 cells (3×10^5) were plated per well using six-well plates, and cells were grown to 70% confluence. They were subsequently serum-starved overnight before treatment with leptin for 24 h. Then cells were lysed and telomerase activity was determined by the telomerase PCR ELISA kit (Roche Diagnostics, Basel, Switzerland) according to the manufacture's protocol. HeLa cell extracts were used as positive controls.

Chromatin immunoprecipitation assay (ChIP). ChIP assay was performed using a ChIP assay kit (Upstate Biotechnology, Waltham, USA) according to the manufacturer's instructions. Briefly, MCF-7 cells were cross-linked by 1% formaldehyde and lysed in SDS Lysis buffer. Lysate was sonicated to shear DNA. Then the cross-linked protein (STAT3) was immunoprecipitated using anti-STAT3 antibodies (Santa Cruz Biotechnology, Santa Cruz, USA). The immunoprecipitates were pelleted by centrifugation and incubated at 65 °C to reverse the protein-DNA cross-linking. The DNA was extracted from the elute by the phenol/chloroform method and precipitated by ethanol. Purified DNA was subjected to PCR with primers covering putative STAT3-binding sites in the human hTERT promoter. The sequences of the PCR primers are described previously [7].

Transient transfection and luciferase assay. The full-length (3.3 kb) hTERT promoter-luciferase reporter plasmids (pGL3-hTERT) were constructed as described [11] and transfected into MCF-7 cells using lipofectamin 2000 (Invitrogen, Paisley, UK) following the manufacture's protocols. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, USA). *Renilla* luciferase plasmids (pRL-SV40) were co-transfected as a control.

Statistic analysis. All data were derived from at least three independent experiments and statistical analyses were performed using Student *t*-test. Values were presented as means ± SEM. *P* value <0.05 was considered statistically significant.

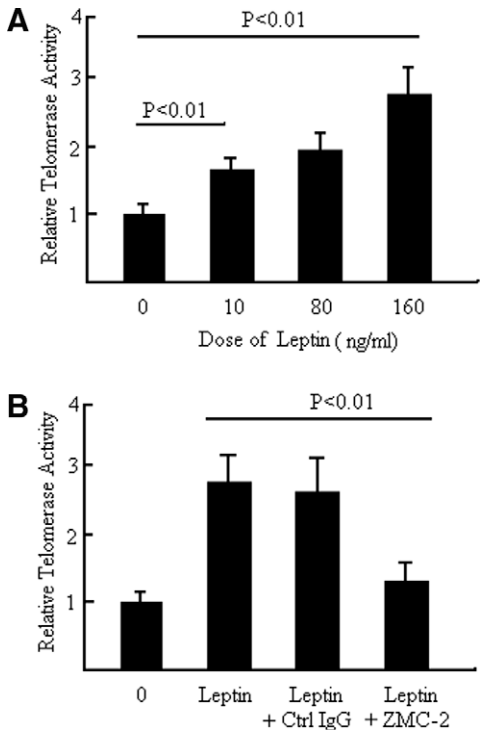

Fig. 1. Leptin activates telomerase in MCF-7 cells. Telomerase activity was quantified by ELISA. All data were presented as fold induction relative to the untreated group (assigned as 1). Mean ± SEM ($N = 4–6$). (A) Cells were treated with 10, 80, or 160 ng/ml of leptin for 24 h. (B) Cells were pretreated with leptin receptor monoclonal antibody, ZMC-2 (15 μg/ml) or irrelevant IgG antibody (15 μg/ml). Then 160 ng/ml of leptin was added to the culture system.

Results

Leptin activates telomerase

Telomerase activity was detected in MCF-7 breast cancer cells after treatment with different concentrations of leptin (10, 80, 160 ng/ml) for 24 h. As shown in Fig. 1A, leptin upregulated telomerase activity in a dose-dependent manner. Telomerase activity was increased 1.7-fold ($p < 0.01$) by 10 ng/ml of leptin and 2.8-fold ($p < 0.01$) by 160 ng/ml of leptin. For Blocking experiments, a leptin receptor monoclonal antibody, ZMC-2 (kindly gifts from Dr. Zida Wu, University Medicine Berlin) was used to preincubate with MCF-7 cells for 24 h at a concentration of 15 μg/ml, before leptin was added to the culture medium. The control was irrelevant IgG antibody (Sigma–Aldrich, Steinheim, Germany). Expectedly, ZMC-2 significantly inhibited leptin-induced activation of telomerase ($p < 0.01$, Fig. 1B), suggesting that this effect was mediated by the leptin receptor.

Leptin upregulates hTERT expression

Since telomerase activity is tightly associated with the expression of hTERT, the catalytic reverse transcriptase of telomerase, we evaluated the levels of hTERT mRNA in MCF-7 cells by real-time RT-PCR. In agreement with the effect on telomerase activity, leptin also enhanced the expression of hTERT mRNA in a dose-dependent fashion (Fig. 2A). The levels of hTERT mRNA were increased by ~1.4 ($p < 0.05$) to 2.3-fold ($p < 0.01$) with 10 and 160 ng/ml of leptin. To confirm that the upregulated hTERT mRNA translated to the increased hTERT protein, total cell extracts were subject to Western blot analysis using an antibody specific for hTERT. As expected, a dose-dependent upregulation of hTERT protein was achieved and 160 ng/ml of leptin induced the hTERT protein by ~2.9-fold ($p < 0.01$, Fig. 2B). Therefore, we suggests that the upregulation of telomerase activity by leptin may, at least in part, be secondary to the increase of hTERT mRNA and protein. Previous

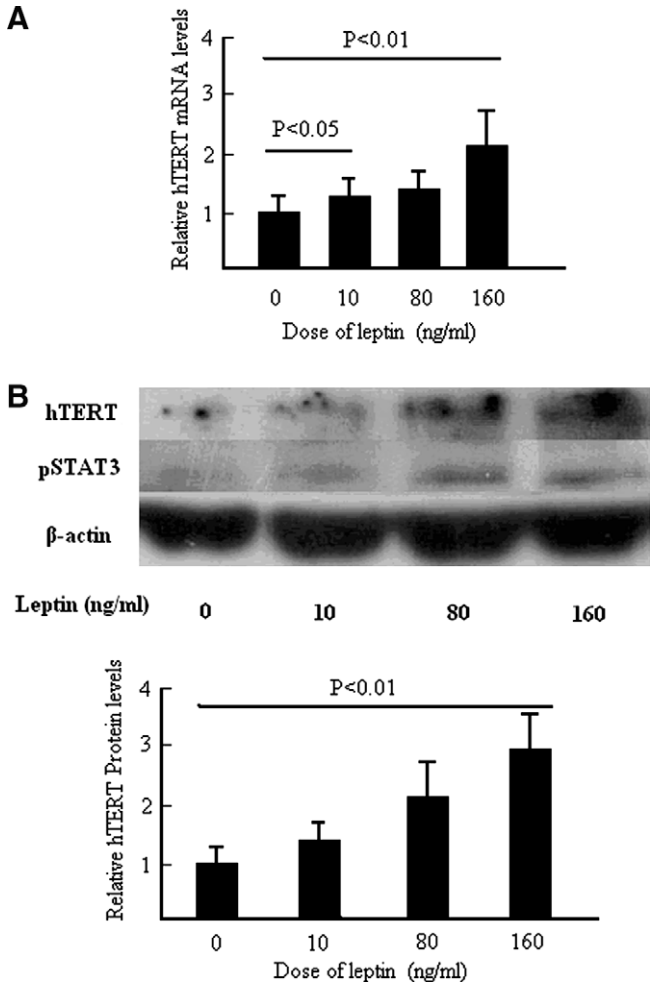

Fig. 2. Leptin upregulates the expression of hTERT. All Data were presented as fold induction relative to the untreated group (assigned as 1). Cells were cultured in complete medium for 24 h and then serum-starved overnight. 10, 80, 160 ng/ml of Leptin was added for 30 min. (A) Total RNA was isolated and examined by quantitative real-time RT-PCR as described under Materials and methods, to determine changes in the level of hTERT mRNA expression after normalization to β-microglobulin expression. (B) Total cell lysates (50 μg of protein) were examined by Western blot analysis to determine the levels of hTERT protein and pSTAT3 levels after normalization to β-actin expression.

studies have shown that STAT3 is an important regulator of hTERT transcription in cancer cells [7]. We also found that hTERT levels and phosphorylated STAT3 increased concomitantly in response to leptin (Fig. 2B), suggesting that STAT3 is involved in leptin-induced hTERT expression.

STAT3 is involved in leptin-induced hTERT transcription

To assess whether STAT3 is a critical mediator of hTERT expression by leptin treatment, we transfected siRNA against STAT3 into MCF-7 cells. As shown in Fig. 3A, two different siRNA effectively down-regulated the expression of STAT3 while the expression of STAT1 was not affected. Real-time RT-PCR analysis showed that mock transfection did not affect leptin-induced hTERT transcription. In contrast, knockdown of STAT3 significantly reduced leptin-induced transcription of hTERT. To further evaluate the role of STAT3 phosphorylation on hTERT expression, MCF-7 cells were pretreated with or without JAK2/STAT3 phosphorylation specific inhibitor AG490 before treatment with leptin at 160 ng/ml (Fig. 3B). Blockade of STAT3 activation by AG490 reduced the baseline hTERT expression in MCF-7 breast cancer cells. Importantly, AG490 significantly counteracted leptin-induced increase of hTERT expression ($p < 0.01$). Furthermore, hTERT transcriptional activity was evaluated by transfecting MCF-7 cells with a full-length (3.3 kb) hTERT promoter. We found that leptin (160 ng/ml) increased hTERT promoter activity by ~4-fold ($p < 0.01$). Inhibition of STAT3 activation significantly decreased basal and leptin-induced transcription of hTERT promoter (Fig. 3C). It has been suggested that STAT3 enhances the hTERT transcription through directly binding to the STAT3-binding site located at −3308 bp of hTERT promoter [7]. To further elucidate whether leptin could enhance the binding of STAT3 to the hTERT promoter in live cells, we performed a ChIP assay using the primers flanking the suggested STAT3 binding site. As shown in Fig. 3D, leptin (160 ng/ml) did increase the binding of STAT3 to hTERT promoter by ~3.3-fold after treatment for 30 min. The efficacy of leptin is similar to that of IL-6 (20 ng/ml), a known cytokine to activate STAT3 signaling. Collectively, these results suggest that leptin enhances the interaction of STAT3 with hTERT promoter and then upregulates the transcription of hTERT gene.

Discussion

It is generally believed that obesity is a reverse prognostic factor in breast cancer. One compelling study evaluated 495,477 US women over a 16-year period, and demonstrated that the death rate of women from breast cancer in the highest quantile of body mass index (BMI) is 2-fold as high as that in the lowest BMI [12]. Obesity is considered as an endocrine disorder exerting the biological effects through cytokines released by adipocytes. Among these cytokines, leptin has been characterized as a growth factor for breast cancer. Treatment with leptin activity by pegylated leptin peptide receptor antagonist 2 (PEG-LPrA2) demonstrated a therapeutic effect on breast cancer xenografts [13], suggesting that leptin signaling may be a potential target for the treatment of breast cancer.

For detecting the role of leptin on telomerase activity, MCF-7 cells were preferentially chosen because several studies from different laboratories have demonstrated that leptin effectively stimulates the growth of MCF-7 cells [9,14,15]. Moreover, the MCF-7 cell line has recently been characterized as a good model to reflect molecular events in human breast carcinomas [16]. It is worth mentioning that MCF-7 cells are derived from estrogen receptor alpha positive (ER+) breast cancer cells. In MDA-MB231 cells (ER− breast cancer cells), leptin did not induce a significant increase of hTERT expression (Supplementary Fig. 1), suggesting that ER may be involved in this effect. We also showed that leptin significantly increased the hTERT expression in SMMC-7721 liver cancer cells, but inhibited that in MIA-paca 2 pancreatic cancer cells, suggesting that the role of leptin on hTERT transcription is cell type specific. In accordance with this effect, previous data also showed that leptin stimulated the growth of liver cancer cells [17] but inhibited the growth of pancreatic cancer cells [18].

In this report, we demonstrated that leptin increased telomerase activity by 1.7- to 2.8-fold in MCF-7 breast cancer cells. Up to now, there are no uniform standards for telomerase abnormal activity. It should be mentioned that telomerase is not only an on/off switch, and a relative level of activity is important. According to previous literature, IGF-1 (1000 ng/ml) increased telomerase activity to ~2-fold in DU-145 cells [19]; Genistein (1 μM) induced telomerase activity to ~1.5-fold in DU-145 and LNCaP cells [20]. These published abnormal telomerase activities are comparable with our results.

In agreement with this study, we and others have shown that leptin could increase the colony formation of breast cancer cells ([21]; Supplementary Fig. 2). Elevation of telomerase in tumor cells

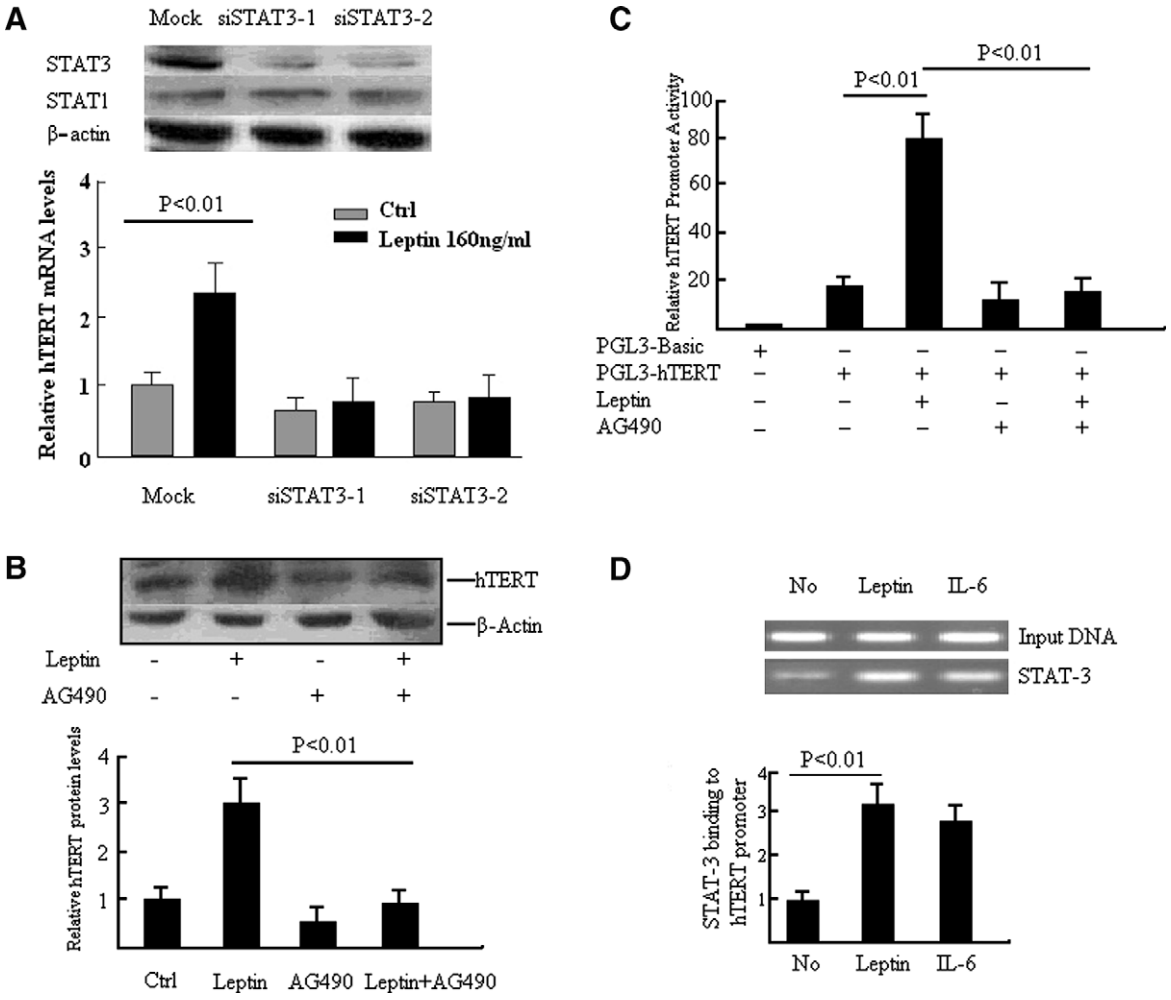

Fig. 3. STAT3 is involved in leptin-induced hTERT transcription. (A) The upper panel: MCF-7 breast cancer cells were mock transfected with lipofectamine 2000 or with one of two of STAT3 siRNA for 48 h. Then cells were lysed for Western blot analysis of STAT3, STAT1, and β-actin expression; the lower panel: quantitative real-time RT-PCR analysis of hTERT mRNA in MCF-7 cells after STAT3 siRNA treatment. Relative hTERT expression is shown after normalization to β–microglobulin expression. (B) Cells were pretreated with 100 μM AG490 for 24 h in free-serum medium. Then 160 ng/ml of Leptin was added for 30 min. Total cell lysates (50 μg) of protein) were examined by Western blot analysis to determine the levels of hTERT, after normalization to β-actin expression. (C) Cells were transfected with the negative control plasmids (PGL3-Basic) or the full-length hTERT promoter-luciferase plasmids (PGL3-hTERT). The *Renilla* luciferase plasmids (pRL-SV40) were co-transfected into cells then incubated with leptin (160 ng/ml) or AG490 (100 μM) 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 ($N = 3$). (D) Chromatin immunoprecipitation (ChIP) assay was performed using anti-STAT3 antibody. PCR primers covering the STAT3 binding sites of hTERT promoter region were used to detect promoter fragment in immunoprecipitates. Input: total genomic DNA used as control for the PCR reaction.

is vital and associated with more aggressive phenotype. Specifically in breast cancer cells, increased telomerase activity has been demonstrated to be associated with tumor size, lymph node status and decreased free-survival rate [22]. Therefore, the significance of leptin on telomerase induction in cancer cell lines may be involved in the promotion of malignant phenotype.

We and others also showed that leptin could not increase the colony formation in normal breast epithelial cells ([21], Supplementary Fig. 2). This result suggests that leptin along at the given dose may not be enough to induce the malignant transformation. However, recent evidence showed that obesity and the associated mediators leptin, estrogen and IGF-I enhanced the cell proliferation and early tumorigenesis of breast cancer cells [23]. Since IGF-1 and estrogen has been reported as a enhancer of telomerase activity [19,24], we suggest that these cytokines may cross-talk to enhance hTERT transcription and telomerase activity, thus increasing the risk for breast carcinogenesis.

. Recently, several studies suggest that the function of hTERT is not limited to the maintenance of telomeres. Lee et al. reported that hTERT promoted cellular survival independent of telomerase activity [25]. Interestingly, hTERT regulates the expression of cyclinD1 (an important cell cycle protein) and vascular endothelial growth factor (VEGF, a key angiogenic factor), the two crucial downstream targets of leptin. Combined with these studies, we postulate that hTERT may, at least in part, mediate the function of leptin in tumor growth and angiogenesis.

To further elucidate the mechanism of leptin-induced hTERT transcription, we chose STAT3 as a possible transcription factor because it is downstream of leptin and upstream of hTERT. Recent report has shown that blocking STAT3 phosphorylation significantly reduced the mitogenic effect of leptin on breast cancer cells [10]. In agreement with these findings, we showed that knockdown of STAT3 expression by siRNA or Inhibition of STAT3 phosphorylation by AG490 significantly decreased leptin-induced hTERT expression at protein and transcriptional levels, suggesting that leptin upregulates the expression of hTERT through STAT3.

Most interestingly, we observed that leptin could directly upregulate hTERT transcription through enhancing the binding of STAT3 to the hTERT promoter. In addition, it is possible that STAT3 might indirectly regulate hTERT transcription through c-Myc [26].

Constitutive activation of STAT3 occurs in diverse human tumors such as breast and prostate cancer [27]. STAT3 signaling promotes tumor survival, angiogenesis, thereby contributing to malignant transformation and tumor progression. Recent investigations have demonstrated that STAT3 may be an attractive target for tumor therapy. AG490 has been used to inhibit the proliferation of acute lymphocytic leukemia [28]. In breast cancer, a STAT3 small molecule inhibitor-STA21 has shown a therapeutic potential [29]. Our study indicates that therapeutic strategies targeting STAT3 activation may be effective in breast cancer patients with high leptin levels.

Recent evidence showed that estrogen increased leptin-induced STAT3 phosphorylation [30]. In accordance with this finding, we found estrogen and leptin synergistically increased STAT3 phosphorylation and hTERT expression and blocking ER binding by tamoxifen could counteract leptin-induced STAT3 phosphorylation and hTERT expression (Supplementary Fig. 3). Contrary to our report, Binai et al. suggests that ER enhances leptin-induced STAT3 activation independent of estrogen binding, which was not inhibited by the anti-hormones [31]. Further studies are needed to identify the regulatory mechanism of ER on leptin-induced STAT3 activation. Anyway, all these studies demonstrate that ER plays a crucial role in leptin-induced STAT3 phosphorylation and the downstream effect.

In conclusion, this study shows for the first time that leptin activates telomerase and transcription of hTERT by enhancing the binding of STAT3 to the hTERT promoter. This finding uncovers a new mechanism that elevated transcription of hTERT triggered by leptin as well as other hormones may be associated with obesity-related breast cancer. Blocking leptin signaling may be valuable for the treatment of breast cancer with elevated leptin levels.

Acknowledgments

This work was supported by the grants from National Natural Science Foundation of China (30701013, 30900596, 30973490, 30901448) and from China Postdoctoral Science Foundation (20090450773).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.02.093.

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