Biochimica et Biophysica Acta 1782 (2008) 593–604

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

c-Jun NH_2-terminal kinase mediates leptin-stimulated androgen-independent prostate cancer cell proliferation via signal transducer and activator of transcription 3 and Akt

Toshiaki Miyazaki [a,b], Jeffrey D. Bub [b], Yoshiki Iwamoto [a,b,*]

[a] Department of Surgical Research, Beckman Research Institute of the City of Hope, Duarte, CA 91010-3000, USA
[b] Department of Urology, Human and Molecular Genetics Center, Medical College of Wisconsin, Milwaukee, WI 53226-0509, USA

ARTICLE INFO

Article history:
Received 6 March 2008
Received in revised form 6 July 2008
Accepted 24 July 2008
Available online 5 August 2008

Keywords:
Prostate cancer
Obesity
Leptin
JNK
STAT3
Akt

ABSTRACT

Obesity is associated with advanced prostate cancer. Here we demonstrate that in mouse prostate cancer TRAMP-C1 cells epididymal fat extracts from high-fat diet-fed obese mice stimulate androgen-independent cell growth more significantly than those from low-fat diet-fed lean mice or genetically obese leptin-deficient *ob*/*ob* mice in correlation with leptin concentrations. This result suggests that obesity promotes androgen-independent prostate cancer cell growth via adipose leptin. We have reported that added leptin stimulates androgen-independent prostate cancer cell proliferation through c-Jun NH_2-terminal kinase (JNK). As with JNK, signal transducer and activator of transcription 3 (STAT3) and Akt are implicated in androgen-independent prostate cancer. In this study, we identify novel interaction of these three molecules in leptin-stimulated androgen-independent cell proliferation. Leptin activates JNK, STAT3 and Akt in a biphasic manner with a similar time-course. Pharmacological JNK inhibition suppresses leptin-stimulated DNA binding activity, as well as Ser-727 phosphorylation, of STAT3. Since JNK upregulates STAT3 activity via Ser-727 phosphorylation, JNK mediates leptin-stimulated STAT3 activation through Ser-727 phosphorylation. Moreover, JNK inhibition impairs leptin-stimulated Ser-473 phosphorylation of Akt that is required for its activation. Thus, JNK is involved in leptin-stimulated Akt activation. These findings together indicate that JNK mediates leptin-stimulated androgen-independent prostate cancer cell proliferation via STAT3 and Akt.

1. Introduction

Prostate cancer is the second leading cause of cancer death among American men. Development of androgen independence by cancer cells is a fatal event during the natural history of the disease. Thus, the molecular basis of androgen-independent prostate cancer (AIPC) needs to be clarified to develop cures and preventives for reducing prostate cancer mortality.

Extensive evidence indicates that obesity, another serious health problem, is associated with aggressive progression and mortality of prostate cancer [1–5] though it appears to reduce the risk of non-aggressive disease [4–6]. It is interesting to note that abdominal adiposity is related with prostate cancer even in a lean population [7]. Adipocytes secrete various factors, including cytokines, growth factors and hormones [8]. We have demonstrated that conditioned medium from adipocyte culture augments AIPC cell proliferation [9]. This finding indicates that adipose factors stimulate AIPC cell growth.

Leptin is one of the major adipose cytokines controlling body weight homeostasis and insulin secretion [10,11]. A recessive mutation in the leptin (obese, *ob*) gene causes obesity and type II diabetes mellitus [12]. Plasma leptin levels correlate with total amounts of body fat [13]. Furthermore, consistent with the fact that over-expressing leptin genetic polymorphism is related to prostate cancer [14], circulating leptin levels are associated with prostate cancer risk [15–17] although some controversies exist [18–20]. Our previous study has shown that prostate cancer cells express functional forms of the leptin receptor and that leptin stimulates cell proliferation in AIPC DU145 and PC-3 cells but not in androgen-dependent LNCaP-FGC cells [9]. Leptin has also been demonstrated to mediate cell survival [21] and migration [22] in AIPC cells. Intriguingly, leptin interacts with other adipose cytokines insulin-like growth factor-I (IGF-I) and interleukin-6 (IL-6) whose levels increase in obesity to stimulate AIPC cell growth [9] whereas adiponectin, an adipose cytokine that decreases in obesity, competes with leptin and IGF-I to inhibit AIPC cell growth [23]. These observations suggest that adipose cytokines are mediators between obesity and AIPC.

Abbreviations: AIPC, androgen-independent prostate cancer; IGF-I, insulin-like growth factor-I; IL-6, interleukin-6; STAT3, signal transducer and activator of transcription 3; PI3K, phosphatidylinositol-3 kinase; JNK, c-Jun NH_2-terminal kinase; JAK, Janus kinase; SIE, sis-inducible element; PIP3, phosphatidylinositol-3,4,5-triphosphate; PDK, 3'-phosphoinositide-dependent protein kinase; PTEN, phosphatase and tensin homologue deleted on chromosome 10; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; EMSA, electromobility shift assay

* Corresponding author. Department of Surgical Research, Beckman Research Institute of the City of Hope, 1500 East Duarte Road, Duarte, CA 91010-3000, USA. Tel.: +1 626 256 4673; fax: +1 626 301 8972.
E-mail address: yiwamoto@coh.org (Y. Iwamoto).

0925-4439/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbadis.2008.07.005

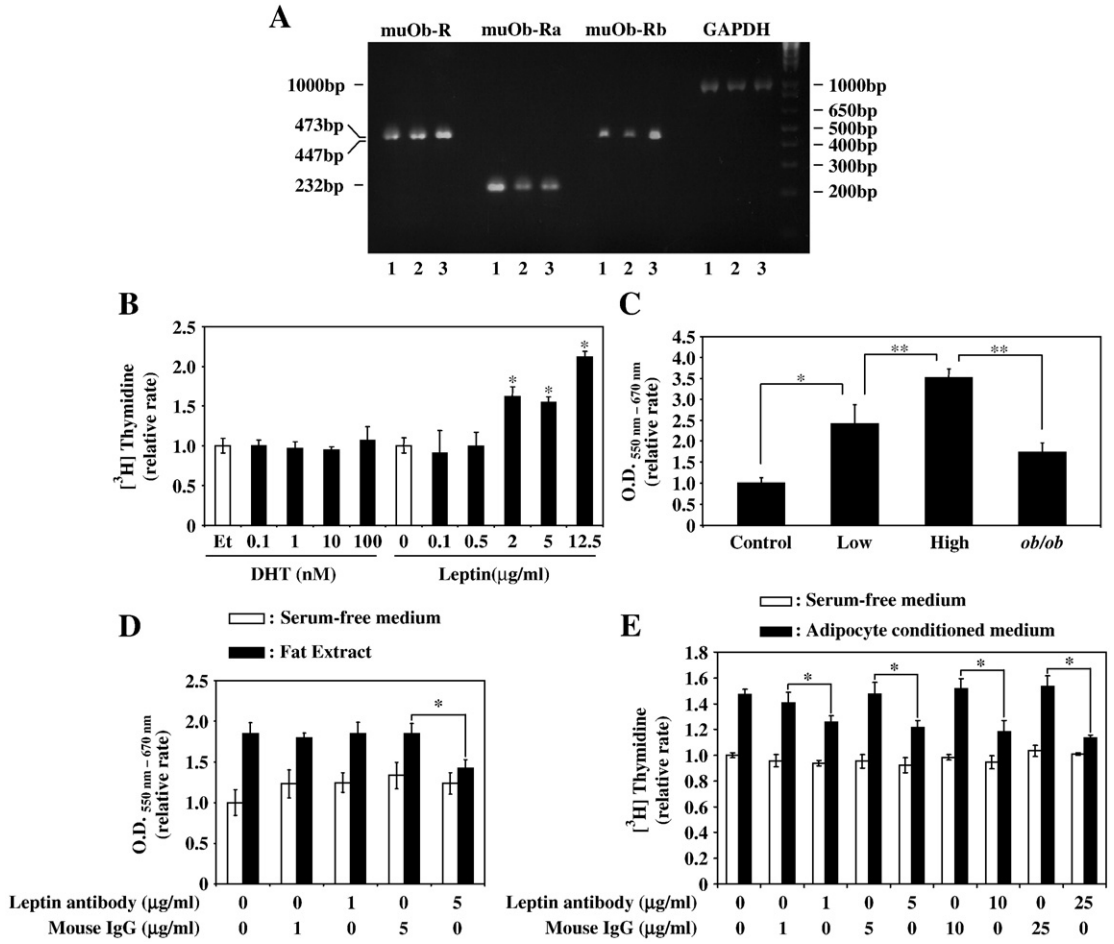

Fig. 1. Adipose leptin mediates cell proliferation in androgen-independent TRAMP-C1 prostate cancer cells that express leptin receptor. (A) mRNA expression of the two signaling-competent leptin receptor isoforms muOB-Ra and muOB-Rb was profiled in TRAMP-C1 cells. RT-PCR analysis identified expression of these isoforms with primers that detect each of them specifically (*muOB-Ra* and *muOB-Rb*). PCR also amplified the common extracellular portion of the two isoforms (*muOB-R*). GAPDH served as a control. Presented are the reproducible results from one of the four repeated experiments. The experiment was performed using three independently prepared samples (samples 1, 2 and 3): three PCR were carried out simultaneously with each primer set, and PCR products were analyzed on the same gel. Three PCR showed consistent results with all primer sets. (B) TRAMP-C1 cells were serum-starved for 48 h and stimulated with DHT or leptin at indicated concentrations for 20 h. [^{3}H] thymidine incorporation was measured during the last 5 h. Ethanol (*Et*) was used as a vehicle control for DHT. Values represent the mean ± SD of quadruplicate samples of a representative experiment. *P< 0.0005 versus the controls (open bars). (C) Fat extracts were prepared from low-fat (Low) and high-fat (High) diet-fed male C57BL/6J mice, as well as regular diet-fed *ob*/*ob* mice (*ob*/*ob*), as described in Materials and methods. TRAMP-C1 cells were deprived of serum for 24 h and treated for 5 days with these fat extracts using serum-free medium as a control. Cell viability was measured by the enzymatic reduction of MTT (OD 550-670 nm) during the last 3 h. Values represent the mean ± SD of quadruplicate samples of a representative experiment. *P< 0.01, **P< 0.0005. (D) Serum-starved TRAMP-C1 cells were incubated for 5 days with the control serum-free medium (open bars) or fat extracts from high-fat diet-fed male C57BL/6J mice (solid bars) in the absence and presence of indicated concentrations of anti-leptin neutralizing antibody or non-specific mouse IgG; and cell viability was evaluated by the MTT assay. Values represent the mean ± SD of quadruplicate samples of a representative experiment. *P= 0.002. (E) Conditioned medium was prepared from in vitro differentiated human primary adipocytes as described before [9]. Serum-deprived DU145 cells were treated for 20 h with the control serum-free medium (open bars) or adipocyte condition medium (solid bars) in the absence and presence of anti-leptin neutralizing antibody or non-specific mouse IgG at indicated concentrations. [^{3}H] thymidine incorporation was measured during the last 5 h. Values represent the mean ± SD of quadruplicate samples of a representative experiment. *P< 0.02.

Leptin exerts cellular functions via activation of intracellular signaling pathways upon binding to its cell-surface receptor. Several splice variants of leptin receptor have been identified in mice (muOB-Ra, b, c, d and e) [24] and humans (huOB-R and huB219.1 to huB219.3) [25]. Only the full-length (muOB-Rb/huOB-R) and the second longest (muOB-Ra/huB219.3) isoforms have been shown to mediate signal transduction. These two isoforms demonstrate divergent signaling capability: OB-Rb/OB-R mediates activation of both signal transducer and activator of transcription 3 (STAT3) and extracellular regulating kinase (ERK) 1/2 pathways while muOB-Ra/huB219.3 activates ERK1/2 but not STAT3 [26-28]. In addition, leptin is potent to activate such other signaling pathways as the phosphatidylinositol-3 kinase (PI3K) [29] and c-Jun NH_2-terminal kinase (JNK) pathways [9,30].

JNK is a Ser/Thr kinase that constitutes one of the mammalian mitogen-activated protein kinase families. Upon activation JNK translocates to the nucleus, and phosphorylates and activates transcription factors including activator protein-1 components, c-Jun and activating transcription factor-2 to mediate various cellular processes such as cell proliferation, cell survival, apoptosis and malignant transformation [31]. JNK has been shown to be involved in cell proliferation and survival in PC-3 cells in vitro [32] and PC-3 xenograft growth in vivo [33]. Furthermore, we have recently reported that JNK is activated by leptin and required for leptin-stimulated androgen-independent cell proliferation in DU145 and PC-3 cells [9].

STAT3 is an oncogenic transcription factor that mediates cellular events including cell transformation, proliferation, survival and migration [34]. Cytokines, such as leptin and IL-6, regulate STAT3 activation through phosphorylation at Tyr-705 and Ser-727 in the C-terminus [34]. Upon ligand binding, cytokine receptors mediate STAT3 phosphorylation at Tyr-705 via receptor-associated Janus kinase (JAK). Tyr-705 phosphorylation allows STAT3 to dimerize, translocate to the nucleus and activate transcription from target gene promoters

containing a sis-inducible element (SIE) [35]. Following Tyr-705 phosphorylation, Ser-727 phosphorylation occurs in the nucleus. Ser-727 phosphorylation is mediated by Ser/Thr kinases including JNK [36,37] and required for maximum, sustained STAT3 activation that is crucial in cancer pathogenesis [34]. Constitutive STAT3 activation is observed in primary prostate adenocarcinoma more prominently than non-malignant prostate glands [38]. Furthermore, levels of STAT3 activation in tumor cells correlate with higher malignancy based on Gleason scores [39]. Moreover, inhibition of constitutive STAT3 activation causes cell growth arrest and apoptosis in DU145 cells, indicating that STAT3 is implicated in cell proliferation and survival in AIPC cells [39]. Although leptin has been demonstrated to induce STAT3 activation in several cell types, leptin-mediated STAT3 activation has not been reported in prostate cancer cells.

PI3K is a lipid kinase that phosphorylates inositol ring 3′-OH group in inositol phospholipids to produce phosphatidylinositol-3,4,5-triphospate (PIP3), leading to Akt, a Ser/Thr kinase, activation through phosphorylation at Thr-308 and Ser-473 via 3′-phosphoinositide-dependent protein kinases (PDKs) [40]. This PI3K-Akt pathway controls cellular activities such as proliferation, survival, malignant transformation, migration and morphogenesis [40]. Elevated Akt activation is detected in human prostate cancer specimens including almost all prostate intraepithelial neoplasia and invasive cancer samples; and activation levels correlate with higher malignancy [41]. Furthermore, the PI3K-Akt pathway mediates the development and progression of AIPC [42–44]. In addition, the PI3 kinase-Akt pathway has been reported to mediate AIPC cell migration in vitro [22]. The PI3K-Akt pathway can be activated through multiple mechanisms in cancer cells. Phosphatase and tensin homologue deleted on chromosome 10 (PTEN), a phosphatase that dephosphorylates PIP3 to negatively regulate PI3K signaling, is frequently mutated in prostate cancer, resulting in constitutive Akt activation in cancer cells [41]. Paracrine and/or autocrine cytokines and growth factors, such as IGF-I [45], can also activate the PI3K-Akt pathway. Leptin has been shown to induce Akt activation during cell proliferation in DU145 cells that express wild-type PTEN [21].

In this study, we confirm the crucial role of adipose leptin in obesity-stimulated AIPC cell growth using the in vitro reconstitution system in which mouse AIPC TRAMP-C1 cells are treated with epididymal fat extracts from high-fat diet-fed obese and low-fat diet-fed lean C57BL/6J mice and genetically obese leptin-deficient *ob*/*ob* mice. Furthermore, we demonstrate that JNK mediates leptin-stimulated AIPC cell proliferation through STAT3 and Akt in DU145 cells. We also show that leptin activates JNK, STAT3 and Akt in not only DU145 but also TRAMP-C1 cells.

2. Materials and methods

2.1. Materials

Recombinant human leptin, IL-6 and IGF-I, and anti-leptin neutralizing antibody were purchased from R & D Systems Inc. (Minneapolis, MN). SP600125 and LY294002 were bought from Tocris Cookson, Inc. (Ellisville, MO). The stress-activated protein kinase/JNK assay kit, anti-JNK, -c-Jun, -phospho-c-Jun (Ser-63), -phospho-c-Jun (Ser-73), -STAT3, -phospho-STAT3 (Tyr-705), -phospho-STAT3 (Ser-727) and -phospho-Akt (Ser-473) rabbit polyclonal antibodies, and immobilized anti-Akt (1G1) mouse monoclonal antibody were from Cell Signaling Technology, Inc. (Beverly, MA). Anti-STAT3 rabbit polyclonal antibody for the electromobility shift assay (EMSA) was obtained from Chemicon International (Temecula, CA). Anti-Akt rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mouse monoclonal antibody was a product of AbD Serotec (Oxford, UK). The Mouse and Rat Leptin ELISA kit was bought from BioVendor Laboratory Medicine, Inc. (Modrice, Czech Republic). C57BL/6J and *ob*/*ob* mice were obtained from Jackson laboratory (Bar Harbor, ME). High-fat (58 kcal% fat, 5.55 kcal/g) and low-fat (11 kcal% fat, 4.07 kcal/g) diets were from Research Diets, Inc. (New Brunswick, NJ).

2.2. Prostate cancer cells and culture conditions

Human prostate cancer cell line DU145 and mouse prostate cancer cell line TRAMP-C1 were purchased from the American Type Culture Collection (Manassas, VA). DU145 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) plus 100 units/ml penicillin and 100 μg/ml streptomycin. TRAMP-C1 cells were grown in DMEM containing 5% FBS, 5% Nu-Serum IV, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.005 mg/ml bovine insulin and 10 nM dehydroisoandrosterone, according to a previous report [46]. All cultures were maintained at 37 °C in a 5 % CO_2 atmosphere.

2.3. Reverse transcriptase (RT)-PCR analysis for leptin receptor isoforms

Seventy percent confluent TRAMP-C1 cells were deprived of serum for 24 h, and total RNA was prepared using the TRIZOL standard technique. cDNA was generated from 1 μg of total RNA and subjected to PCR analysis with primers amplifying each of the muOB-Ra and muOB-Rb isoforms specifically, as well as their common extracellular domain (muOB-R), following previous reports with some modifications [47,48]. The reaction mixture for muOB and muOB-Ra amplification contained 5 % of the cDNA product, 10 mM Tris–HCl (pH 9), 50 mM KCl, 0.01% Triton X-100, 5 μM each primer, 200 μM dNTP (Roche Applied Science), 1.5 mM $MgCl_2$ and 1.5 U Taq polymerase (Invitrogen), in a final volume of 20 μl. The reaction mixture for muOB-Rb amplification had the same composition except that each primer concentration was reduced to 1 μM. PCR for muOB and muOB-Rb was performed with an initial denaturing at 94 °C for 4 min, followed by 35 cycles consisting of denaturing at 94 °C for 1 min, annealing at 55 °C for 1 min, and extending at 72 °C for 1 min. PCR for muOB-Ra was carried out under the same condition except for changing the annealing temperature to 54 °C. The following forward and reverse primers were used for leptin receptor amplification: muOB-R (5′-CAG ATT CGA TAT GGC TTA ATG GG-3′ and 5′-GTT AAA ATT CAC AAG GGA AGC G-3′) [47], muOB-Ra (5′-ACA CTG TTA ATT TCA CAC CAG AG-3′ and 5′-AGT CAT TCA AAC CAT TAG TTT AGG-3′) [47], and muOB-Rb (5′-ACA CTG TTA ATT TCA CAC CAG AG-3′ and 5′-TGG ATA AAC CCT TGC TCT TCA-3′) [48]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed as an internal standard. Forward and reverse primers utilized for GAPDH amplification were 5′-TGA AGG TCG GTG TCA ACG GAT TTG GC-3′ and 5′-CAT GTA GGC CAT GAG GTC CAC CAC-3′, and PCR was performed as described before [9]. PCR for muOB-R, muOB-Ra, muOB-Rb and GAPDH generated products of 473, 237, 447 and 1000 bp, respectively. PCR products were separated by electrophoresis on 2 % agarose gels with ethidium bromide staining. All experiments were repeated at least four times, each including three independently prepared samples.

2.4. Preparation of fat extracts from mice

Three-week-old male C57BL/6J mice were placed on a low-or high-fat diet (n= 9 each) and continued to be fed ad libitum the same diet until sacrificed at 15 weeks of age. Immediately after euthanization by CO_2 asphyxiation, mice were weighed; and epididymal fat pads were harvested, weighed and kept frozen at −80 °C until further processed. For fat extract preparation, epididymal fat tissue (0.4 g) from each mouse was minced extensively with surgical scissors and incubated for 24 h in 10 ml of phenol red-free DMEM supplemented with 2.5 μg/ml fungizone, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.11 g/l sodium pyruvate, and 0.584 g/l L-glutamine. The medium alone

was also mock-incubated and used as a control for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and ELISA described below. The conditioned medium (referred to as a fat extract) was then collected, cleared by centrifugation to remove tissue debris and stored at −80 °C until use. Fat extracts from *ob*/*ob* mice were prepared following the same procedure except for feeding mice on a regular diet.

2.5. Preparation of human adipocyte conditioned medium

Human primary adipocytes were purchased from BioWhittaker Inc. (Walkersville, MD) and differentiated into mature adipocytes in vitro; and the conditioned medium was prepared as previously described [9].

2.6. MTT assay

TRAMP-C1 cells (0.5×10^3/well) were seeded in 96-well plates, followed by serum starvation for 24 h. Cells were then grown in fat extracts or the control mock-incubated medium for 5 days and subjected to MTT assays as previously reported [9]. Values were shown as means±SD of quadruplicated determinations of a representative experiment (repeated three times).

2.7. Measurement of leptin concentrations

Leptin concentrations of fat extracts were measured by the Mouse and Rat Leptin ELISA kit according to the manufacturer's protocol.

2.8. Cell lysate preparation

DU145 and TRAMP-C1 cells were seeded into 10-cm plates and grown to a 70% confluent state. In experiments profiling the time-dependent effect of cytokine treatment on signal transduction, cells were starved in serum-free medium for 24 h and treated with cytokine for indicated periods. Cytokine treatment was carried out by changing the starvation medium with fresh serum-free medium containing 12.5 μg/ml leptin, 100 ng/ml IL-6 or 100 ng/ml IGF-I. In experiments testing the effect of JNK or PI3K inhibition on cytokine-mediated signal transduction, following serum starvation cells were pretreated with 10 μM SP600125 or 5 μM LY294002 for 30 min before cytokine treatment. Pretreatment was performed by replacing the starvation medium with fresh serum-free medium with and without Me_2SO (vehicle control) or each of the inhibitors. One hundred-fold concentrated leptin, IL-6 or IGF-I solution was then added to cultures to make a final concentration of 12.5 μg/ml, 100 ng/ml or 100 ng/ml, respectively. We tested the effect of vehicle solutions for these cytokines (20 mM Tris–HCl, pH 8.0 for leptin; phosphate-buffered saline containing 0.1% bovine serum albumin for IL-6 and IGF-I) on JNK, STAT3 and Akt activation, and found no differential effect with and without addition of either vehicle solution (data not shown). Therefore, controls for cytokine treatment were sampled without adding any vehicle solution. Cell lysates were prepared as previously described [9], frozen on dry ice and stored at −80 °C until use.

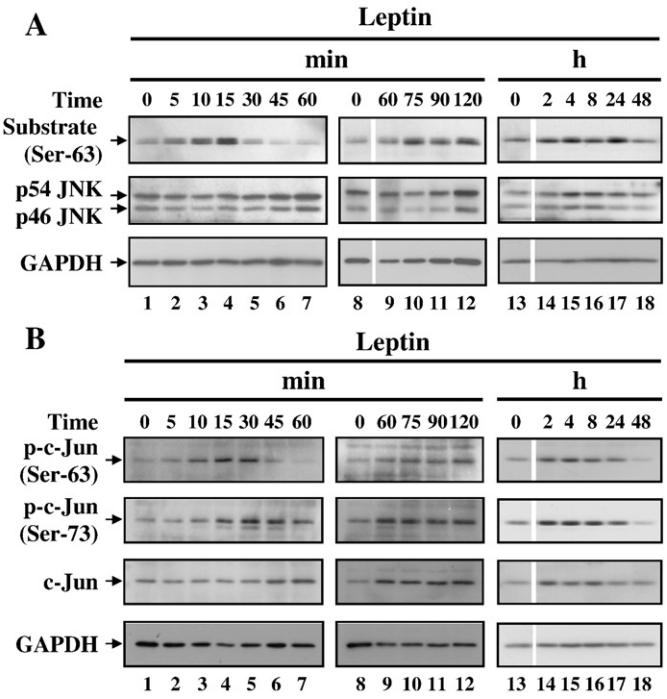

Fig. 2. Leptin activates the JNK-c-Jun pathway biphasically in androgen-independent DU145 prostate cancer cells. DU145 cells were serum-deprived for 24 h and incubated in serum-free medium (lanes 1, 8, and 13) or the serum-free medium containing 12.5 μg/ml leptin (lanes 2–7, 9–12, and 14–18) for indicated periods. (A) Cell lysates (250 μg protein) were subjected to the in vitro JNK assay with N-terminal c-Jun fusion protein as a substrate. Phosphorylation of the substrate protein on Ser-63 was detected by Western blot analysis using the specific antibody (*Substrate (Ser-63)*). To assess total JNK protein levels, cell lysates (50 μg protein) were applied to Western blot analysis using anti-JNK antibody that detects both active and inactive forms of JNK (*p54 JNK* and *p46 JNK*). Membranes probed with this antibody were stripped and re-probed with anti-GAPDH antibody to normalize variations in sample loading (*GAPDH*). (B) Cell lysates (50 μg protein) were analyzed by Western blotting. c-Jun phosphorylation at Ser-63 and-73 was assessed using anti-phospho-c-Jun (Ser-63) and (Ser-73) antibodies (*p-c-Jun (Ser-63)* and *p-c-Jun (Ser-73)*). To determine total c-Jun protein levels, membranes probes with these antibodies were stripped and re-probed with anti-c-Jun antibody that recognizes both phosphorylated and non-phosphorylated forms of c-Jun (*c-Jun*). The same membranes were stripped again and re-hybridized with anti-GAPDH antibody to normalize variations in sample loading (*GAPDH*). In some gels, a lane is removed and the remaining lanes are spliced together. Such alterations are indicated by leaving a white line between the gel pieces.

Table 1
Comparison of body weight, fat weight and leptin concentrations of fat extracts among high-and low-fat diet-fed C57BL/6J mice and ob/ob mice. Body weight and epididymal fat weight were measured at sacrifice

	Control	Low	High	ob /ob mice	*P*-value (Low:High)	*P*-value (High:ob/ob)
Body weight (g)	N/A	27.79 ±2.01	37.02 ±3.80	42.74 ±2.02	0.000002	0.034834
Fat weight (g)	N/A	0.80 ±0.18	2.25 ±0.40	4.01 ±0.25	$<10^{-6}$	0.000038
Leptin concentration of fat extract (ng/ml)	1.38 ±0.01	10.56 ±2.73	15.18 ±5.03	6.50 ±0.60	0.017250	0.016082

Fat extracts were prepared from epididymal fat pads as described in Materials and methods, and leptin concentrations were determined by ELISA.
N/A, not applicable.

2.9. Western blot analysis of c-Jun, STAT3 and Akt

To measure phosphorylation states of c-Jun and STAT3, cell lysates containing 50 μg of protein were subjected to Western blot analysis using anti-phospho-c-Jun (Ser-63 and -73) and-phospho-STAT3 (Tyr-705 and Ser-727) antibodies as described before [9,49]. Membranes probed with these antibodies were stripped and re-probed with anti-c-Jun and -STAT3 antibodies to determine c-Jun and STAT3 protein levels. The same membranes were stripped again and re-probed with anti-GAPDH antibody for normalizing variations in sample loading. To assess Akt phosphorylation at Ser-473, phosphorylated Akt was immunoprecipitated from cell lysates (1 mg protein) with anti-immobilized anti-Akt (1G1) monoclonal antibody and detected by Western blot analysis using anti-phospho-Akt (Ser-473) polyclonal antibody. Total protein levels of Akt were determined in cell lysates by

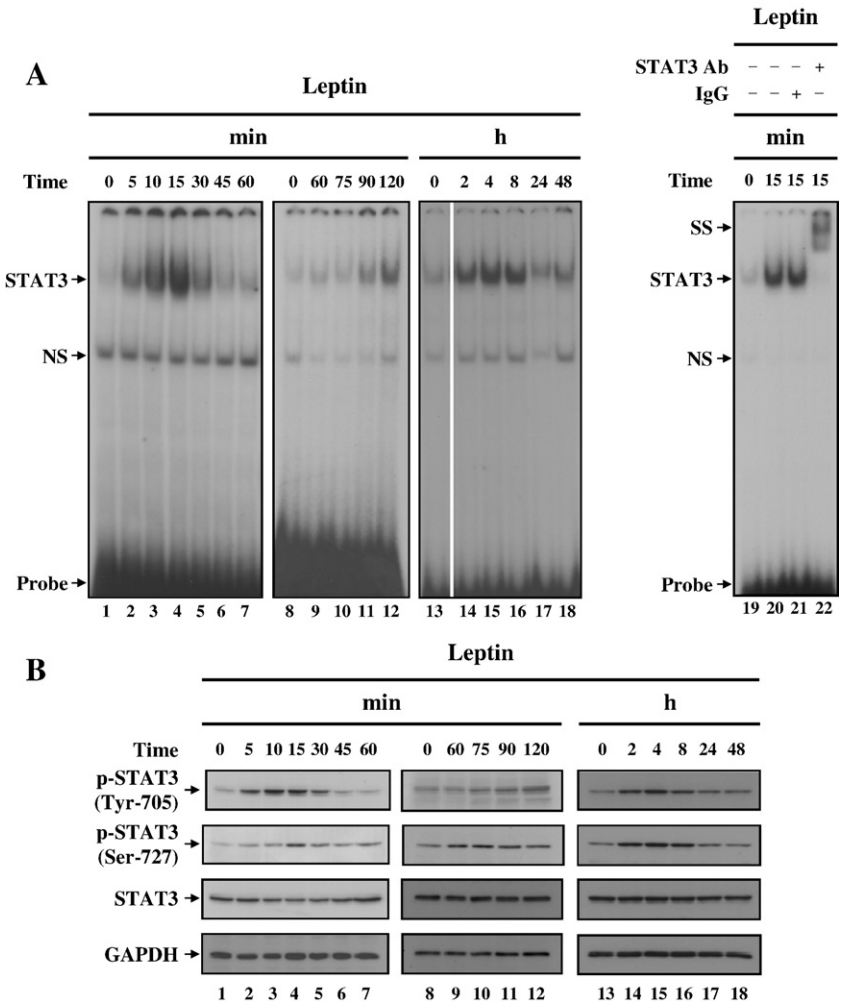

Fig. 3. Leptin biphasically stimulates the DNA binding activity and Tyr-705 and Ser-727 phosphorylation of STAT3 in androgen-independent DU145 prostate cancer cells. Following 24-h serum starvation, DU145 cells were incubated in serum-free medium in the absence (lanes 1, 8, 13, and 19) and presence (lanes 2–7, 9–12, 14–18, and 20–22) of 12.5 μg/ml leptin for indicated periods. (A) To assay STAT3 DNA binding activity, cell lysates (10 μg protein) were subjected to EMSA using ^{32}P-end-labeled M67-SIE as a probe. The STAT3–DNA complex was confirmed by the supershift assay using anti-STAT3 antibody (lanes 19–22). A lane is removed from one of the gels, and the remaining lanes are spliced together. This alteration is indicated by putting a white line between the gel pieces. *STAT3*, STAT3–DNA complex; *STAT3 Ab*, anti-STAT3 antibody; *SS*, STAT3–DNA complex supershifted by the anti-STAT3 antibody; *NS*, non-specific. (B) Cell lysates (50 μg protein) were applied to Western blot analysis. STAT3 phosphorylation at Tyr-705 and Ser-727 was assessed with anti-phospho-STAT3 (Tyr-705) and (Ser-727) antibodies (*p-STAT3 (Tyr-705)* and *p-STAT3 (Ser-727)*). To determine total amounts of STAT3 protein, membranes hybridized with these antibodies were stripped and re-hybridized with anti-STAT3 antibody that detects both phosphorylated and non-phosphorylated forms of STAT3 (*STAT3*). The same membranes were stripped again and re-hybridized with anti-GAPDH antibody to normalize variations in sample loading (*GAPDH*).

Western blot analysis with anti-Akt antibody. Membranes hybridized with anti-Akt antibody were stripped and re-hybridized with anti-GAPDH antibody to normalize variations in sample loading. All experiments were repeated at least three times, and representative results were presented.

2.10. In vitro JNK assay

Cell lysates (250 μg protein) were applied to the in vitro JNK assay utilizing the stress-activated protein kinase/JNK assay kit following the manufacturer's instructions with some modifications as reported before [9]. Briefly, JNK was precipitated in cell lysates with glutathione-Sepharose beads immobilized by a recombinant fusion protein of the c-Jun N-terminus (1–89) and glutathione S-transferase as a JNK substrate. The kinase reaction was then carried out in the presence of 200 μM ATP at 30 °C for 30 min. Substrate phosphorylation at Ser-63 was determined by Western blot analysis using anti-phospho-c-Jun (Ser-63) polyclonal antibody. Cell lysates were also assessed for JNK protein levels by Western blot analysis with anti-JNK antibody. Membranes probed with the anti-JNK antibody were stripped and re-probed with anti-GAPDH antibody to normalize variations in sample loading. All results were representative of three or more independent data sets.

2.11. EMSA

Cell lysates (10 μg protein) were subjected to EMSA with ^{32}P-end-labeled double-stranded oligonucleotide M67-SIE as a probe as previously described [50]. Supershift assays were performed using anti-STAT3 antibody to confirm the STAT3–DNA complex.

2.12. Thymidine incorporation assay

DU145 (1.5×10^5/well) and TRAMP-C1 (1.0×10^5/well) cells were seeded into 24-well plates, deprived of serum for 48 h, treated with leptin or dihydrotestosterone (DHT) at indicated concentrations for 20 h, and subjected to thymidine incorporation assays as reported previously [9]. DHT was used to determine whether TRAMP-C1 cells are androgen-responsive. In experiments with PI3K inhibition, cells were pretreated with 5 μM LY294002 for 30 min prior to leptin

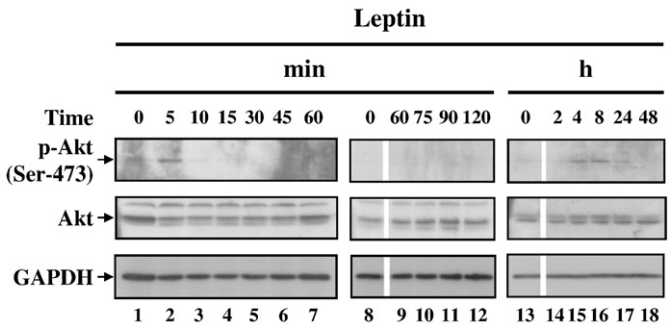

Fig. 4. Leptin stimulates Akt phosphorylation at Ser-473 in a biphasic manner in androgen-independent DU145 prostate cancer cells. DU145 cells were serum-deprived for 24 h and incubated in serum-free medium in the absence (lanes 1, 8, and 13) and presence (lanes 2–7, 9–12, and 14–18) of 12.5 μg/ml leptin for indicated periods. Akt phosphorylated at Ser-473 was immunoprecipitated in cell lysates (1 mg protein) with immobilized anti-Akt (1G1) antibody and detected by Western blot analysis using anti-phospho-Akt (Ser-473) antibody (*p-Akt (Ser-473)*). To determine total levels of Akt protein, cell lysates (50 μg protein) were applied to Western blot analysis using anti-Akt antibody that recognizes both phosphorylated and non-phosphorylated forms of Akt (*Akt*). Membranes probed with this antibody were stripped and re-probed with anti-GAPDH antibody to normalize variations in sample loading (*GAPDH*). In some gels, a lane is removed and the remaining lanes are spliced together. Such alterations are indicated by putting a white line between the gel pieces.

stimulation to inhibit PI3K activity. All experiments were repeated at least three times. Values were reported as means±SD of quadruplicated measurements of a representative experiment.

3. Results

3.1. Adipose leptin stimulates AIPC cell growth in obesity

Our previous study has demonstrated that added recombinant leptin augments androgen-independent cell proliferation in human prostate cancer DU145 and PC-3 cells [9]. However, it has not been clear whether obesity stimulates AIPC cell growth through adipose leptin. Here we address this question with a newly developed in vitro system utilizing mouse prostate cancer cells and fat extracts.

RT-PCR analysis demonstrated that mouse prostate cancer TRAMP-C1 cells, as well as human prostate cancer cells [9], expressed signaling-competent leptin receptor isoforms (Fig. 1A). This result suggests that leptin may mediate cell proliferation in TRAMP-C1 cells. We employed the thymidine incorporation assay to test this hypothesis. As with DU145 and PC-3 cells [9] TRAMP-C1 cells proliferated in response to added leptin but not DHT: leptin stimulated cell proliferation in a dose-dependent manner at concentrations up to 12.5 μg/ml while DHT did not influence it at 0.1, 1, 10 or 100 nM (Fig. 1B). This finding allowed developing an in vitro reconstitution system to investigate the effect of such adipose factors as leptin contained in mouse epididymal fat extracts on androgen-independent cell growth in TRAMP-C1 cells. To define whether obesity stimulates AIPC cell growth via adipose leptin, we employed the MTT assay to compare the effect of fat extracts on TRAMP-C1 cell growth among high-fat diet-fed obese and low-fat diet-fed lean male C57BL/6J mice and genetically obese leptin-deficient male *ob*/*ob* mice. As shown in Table 1, the high-fat diet markedly increased body weight, epididymal fat pad weight and fat extract leptin levels in C57BL/6J males whereas the low-fat diet maintained them at low levels. Fat extracts from high-fat diet-fed C57BL/6J males facilitated TRAMP-C1 cell growth more significantly than those from low-fat diet-fed males in correlation with leptin concentrations (Fig. 1C). In addition, fat extracts from leptin-deficient *ob*/*ob* males were remarkably less stimulatory to cell growth than those from high-fat diet-fed C57BL/6J males (Fig. 1C) although the former mice were more obese than the latter (Table 1). These results indicate that obesity augments AIPC cell growth through adipose leptin. To further confirm the involvement of adipose leptin in AIPC cell growth, we neutralized leptin with anti-leptin antibody in fat extracts from high-fat diet-fed mice to examine the effect on fat extract-stimulated TRAMP-C1 cell growth. Addition of 5 μg/ml anti-leptin neutralizing antibody significantly inhibited fat extract-facilitated TRAMP-C1 cell growth while control non-specific mouse IgG did not influence it (Fig. 1D), providing direct evidence for leptin involvement. Moreover, the thymidine incorporation assay showed that consistent with our previous report [9] conditioned medium from in vitro differentiated human adipocytes stimulated androgen-independent cell proliferation in DU145 cells and that anti-leptin antibody, but not the control IgG, suppressed conditioned

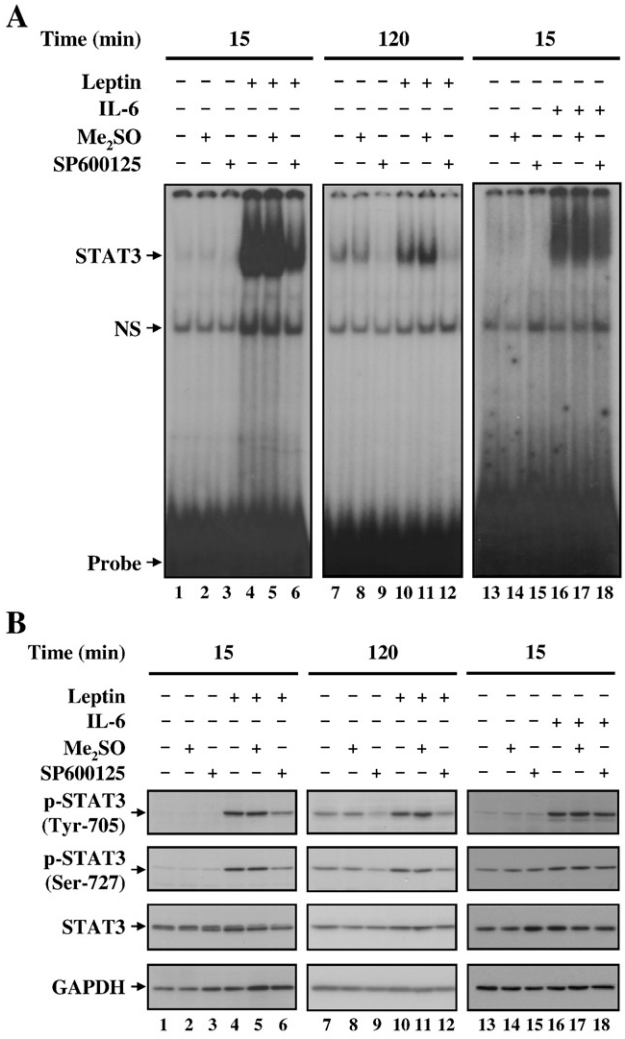

Fig. 5. JNK mediates the leptin-stimulated DNA binding activity and phosphorylation of STAT3 in androgen-independent DU145 prostate cancer cells. Following 24-h serum deprivation, DU145 cells were treated with 12.5 μg/ml leptin for 15 or 120 min or 100 ng/ml IL-6 for 15 min with and without pretreatment with Me_2SO (vehicle control) or 10 μM SP600125 (pharmacological JNK inhibitor) for 30 min. Cells without any treatment (lanes 1, 7 and 13) and treated with Me_2SO alone (lanes 2, 8 and 14) were included as controls. (A) To assess STAT3 DNA binding activity, cell lysates (10 μg protein) were subjected to EMSA with ^{32}P-end-labeled M67-SIE as a probe. *STAT3*, STAT3–DNA complex; *NS*, non-specific. (B) Cell lysates (50 μg protein) were analyzed by Western blotting. STAT3 phosphorylation at Tyr-705 and Ser-727 was determined using anti-phospho-STAT3 (Tyr-705) and (Ser-727) antibodies (*p-STAT3 (Tyr-705)* and *p-STAT3 (Ser-727)*). To measure total STAT3 protein levels, membranes hybridized with these antibodies were stripped and re-hybridized with anti-STAT3 antibody that recognizes both phosphorylated and non-phosphorylated forms of STAT3 (*STAT3*). The same membranes were stripped again and re-hybridized with anti-GAPDH antibody to normalize variations in sample loading (*GAPDH*).

medium-stimulated cell proliferation in a dose-dependent fashion (1–25 μg/ml) (Fig. 1E). This observation suggests that adipose leptin stimulates AIPC cell growth not only in mice but also in humans. These results together lead to the conclusion that adipose leptin mediates obesity-stimulated AIPC cell growth.

3.2. Leptin activates JNK, STAT3 and Akt in a biphasic manner during AIPC cell proliferation

We have shown that leptin activates JNK rapidly in AIPC cells within 15-min treatment and that this activation is required for leptin-stimulated AIPC cell proliferation [9]. STAT3 [39] and Akt [42–44], as well as JNK [9,32,33], play an important role in AIPC pathogenesis. Leptin has been shown to be capable of activating each of these molecules in various cell types [21,26–29]. However, there is no evidence for simultaneous activation/interaction of the three molecules by leptin in any cell types including AIPC cells. Both rapid and persistent activation of these molecules are important in cancer pathogenesis. Here we treated DU145 cells with 12.5 μg/ml leptin over extended periods (up to 48 h) to determine whether leptin stimulates activation of JNK, STAT3 and Akt rapidly and/or persistently.

Consistent with our previous report [9], JNK activity was constitutively detected at a low level and stimulated by leptin, peaking at 15 min after addition of leptin and returning to the basal level 45 min after (Fig. 2A). JNK was then re-activated starting at 75 min, and the second phase of activation sustained at least until after 24 h (Fig. 2A). JNK mediates c-Jun activation through Ser-63 and-73 phosphorylation [31]. We profiled c-Jun phosphorylation at these Ser residues in response to leptin. Leptin stimulated c-Jun phosphorylation at both Ser residues in correlation with JNK activation (Fig. 2B).

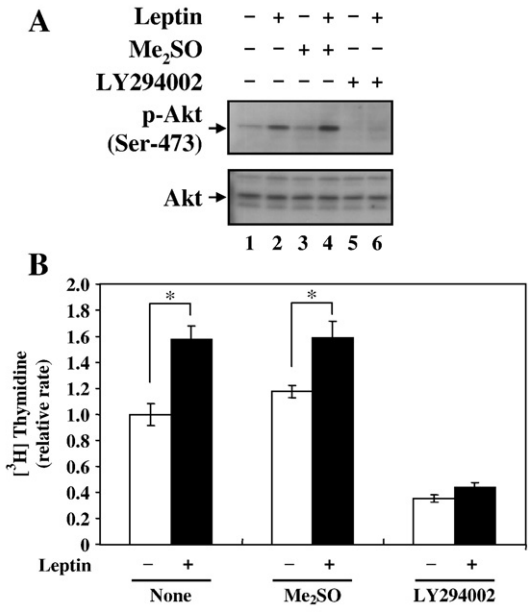

Fig. 6. The PI3K-Akt pathway mediates leptin-stimulated androgen-independent cell proliferation in DU145 cells. (A) Androgen-independent DU145 prostate cancer cells were serum-starved for 24 h and treated with 12.5 μg/ml leptin for 5 min with and without pretreatment with Me_2SO (vehicle control) or 5 μM LY294002 (pharmacological PI3K inhibitor) for 30 min. Cells without any treatment (lanes 1 and 2) and treated with Me_2SO alone (lanes 3 and 4) served as negative controls. Ser-473-phosphorylated Akt was immunoprecipitated in cell lysates (1 mg protein) with immobilized anti-Akt (1G1) antibody and assessed by Western blot analysis using anti-phospho-Akt (Ser-473) antibody (*p-Akt (Ser-473)*). To determine total Akt protein levels, cell lysates (50 μg protein) were subjected to Western blot analysis using anti-Akt antibody that detects both phosphorylated and non-phosphorylated forms of Akt (*Akt*). Membranes probed with this antibody were stripped and re-probed with anti-GAPDH antibody to normalize variations in sample loading (*GAPDH*). (B) After 48-h serum deprivation, DU145 cells were incubated for 20 h in serum-free medium in the absence (open bars) and presence (solid bars) of 12.5 μg/ml leptin with and without pretreatment with Me_2SO or 5 μM LY294002 for 30 min. Cell proliferation was measured by [^{3}H] thymidine incorporation during the last 5 h. Values represent the mean ± SD of quadruplicate samples of a representative experiment. *$P<$ 0.005 versus the controls (*open bars*).

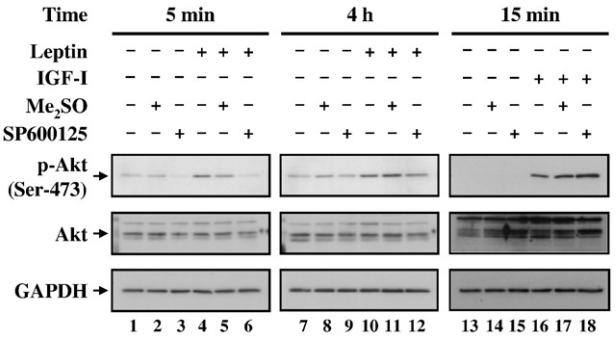

Fig. 7. JNK is indispensable for leptin-stimulated Akt phosphorylation at Ser-473 in androgen-independent DU145 prostate cancer cells. DU145 cells were deprived of serum for 24 h and treated with 12.5 μg/ml leptin for 5 min or 4 h or 100 ng/ml IGF-I for 15 min with and without pretreatment with Me_2SO (vehicle control) or 10 μM SP600125 (pharmacological JNK inhibitor) for 30 min. Cells without any treatment (lanes 1, 7 and 13) and treated with Me_2SO alone (lanes 2, 8 and 14) were included as controls. Ser-473-phosphorylated Akt was immunoprecipitated in cell lysates (1 mg protein) with immobilized anti-Akt (1G1) antibody and detected by Western blot analysis using anti-phospho-Akt (Ser-473) antibody (*p-Akt (Ser-473)*). To determine total levels of Akt protein, cell lysates (50 μg protein) were subjected to Western blot analysis using anti-Akt antibody that recognizes both phosphorylated and non-phosphorylated forms of Akt (*Akt*). Membranes probed with this antibody were stripped and re-probed with anti-GAPDH antibody to normalize variations in sample loading (*GAPDH*).

The DNA binding activity of STAT3 was constitutively observed in DU145 cells as previously reported [39] and further stimulated by leptin treatment in a biphasic fashion, peaking 15 min and 2–8 h after leptin addition (Fig. 3A). This is the first evidence for leptin-mediated STAT3 activation in prostate cancer cells. STAT3 activation is regulated through Tyr-705 and Ser-727 phosphorylation [34]. We found that STAT3 was constitutively phosphorylated at these residues and that leptin biphasically augmented STAT3 phosphorylation at both residues correlating to the DNA binding activity (Fig. 3B).

Phosphorylation at Ser-473 is required for Akt activation [40]. Leptin stimulated Ser-473 phosphorylation, peaking at 5 min and 4–8 h after addition of leptin (Fig. 4). Thus, leptin activated Akt, as well as JNK-c-Jun and STAT3, in a biphasic manner.

3.3. JNK mediates leptin-stimulated STAT3 DNA binding activity and phosphorylation

JAK-mediated Tyr-705 phosphorylation is a cytosolic event obligatory for STAT3 activation whereas Ser-727 phosphorylation that occurs in the nucleus after Tyr-705 phosphorylation is required for maximal, prolonged STAT3 activation [34]. JNK is potent to regulate STAT3 activity through Ser-727 phosphorylation [37]. Since leptin stimulates JNK and STAT3 in a biphasic fashion showing a similar time-course in DU145 cells (Figs. 2 and 3), JNK may mediate leptin-stimulated STAT3 activation via Ser-727 phosphorylation during AIPC cell proliferation. To determine whether JNK is involved in the first and/or second phases of leptin-stimulated STAT3 activation, we examined the effect of pharmacological JNK inhibition with 10 μM SP600125 on the DNA binding activity, as well as Ser-727 and Tyr-705 phosphorylation, of STAT3 after leptin treatment (12.5 μg/ml) for 15 and 120 min. The SP600125 concentration (10 μM) was chosen that completely blocks leptin-induced JNK activation in DU145 cells [9]. JNK inhibition remarkably attenuated leptin-stimulated DNA binding activity and Ser-727 phosphorylation of STAT3 at both time points (Fig. 5A and B). These data suggest that leptin stimulates STAT3 DNA binding activity through JNK-mediated

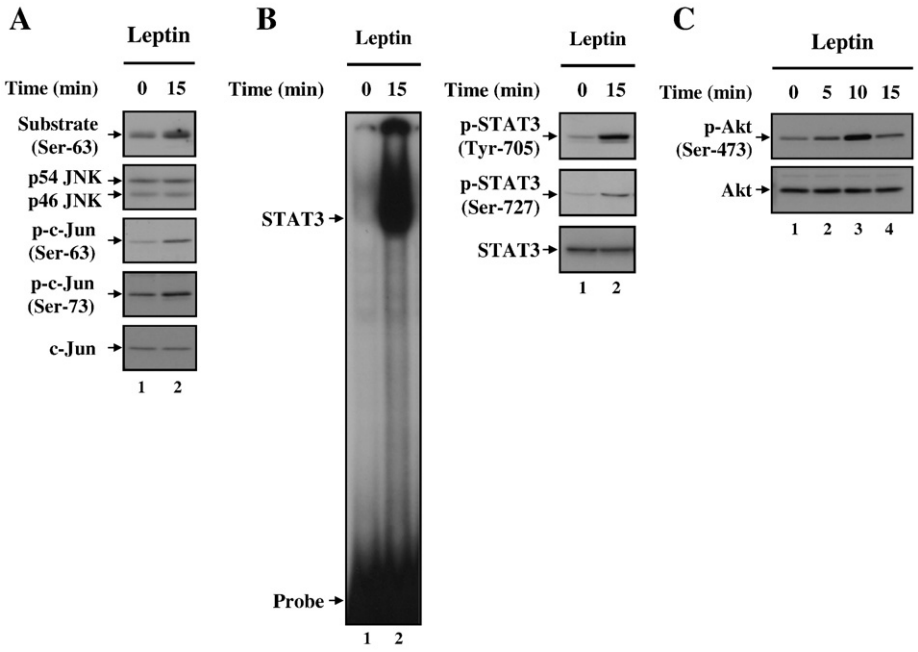

Fig. 8. Leptin activates JNK, STAT3 and Akt pathways in androgen-independent prostate cancer TRAMP-C1 cells. TRAMP-C1 cells were starved of serum for 24 h and stimulated with 12.5 μg/ml leptin for indicated periods. (A) Cell lysates were subjected to the in vitro JNK assay with N-terminal c-Jun fusion protein as a substrate (*Substrate (Ser-63)*) and Western blot analysis using anti-phospho-c-Jun (Ser-63) and (Ser-73) antibodies (*p-c-Jun (Ser-63)* and *p-c-Jun (Ser-73)*). The membrane probed with anti-phospho-c-Jun (Ser-63) antibody was stripped and re-probed with anti-c-Jun antibody to normalize c-Jun phosphorylation to total c-Jun protein levels (*c-Jun*). (B) Left panel, Cell lysates were applied to EMSA to assess STAT3 DNA binding activity using ^{32}P-end-labeled M67-SIE as a probe. *STAT3*, STAT3–DNA complex. Right panel, STAT3 phosphorylation on Tyr-705 and Ser-727 was determined in cell lysates by Western blot analysis using anti-phospho-STAT3 (Tyr-705) and (Ser-727) antibodies (*p-STAT3 (Tyr-705)* and *p-STAT3 (Ser-727)*). To normalize STAT3 phosphorylation to total STAT3 protein levels, the membrane hybridized with anti-phospho-STAT3 (Tyr-705) antibody was stripped and re-hybridized with anti-STAT3 antibody (*STAT3*). (C) Akt phosphorylated at Ser-473 was immunoprecipitated with immobilized anti-Akt (1G1) antibody in cell lysates and analyzed by Western blotting using anti-phospho-Akt (Ser-473) antibody (*p-Akt (Ser-473)*). To normalize Akt phosphorylation to total amounts of Akt protein, cell lysates were subjected to Western blot analysis with anti-Akt antibody (*Akt*).

Ser-727 phosphorylation. It is notable that JNK inhibition suppressed the first phase of STAT3 activation and Ser-727 phosphorylation partially but the second phase thoroughly. This observation suggests that Ser-727 phosphorylation via JNK is essential for leptin-stimulated, not rapid but sustained activation of STAT3 and that the rapid, maximal activation involves both JNK-dependent and-independent Ser phosphorylation. Furthermore, suppression of Ser-727 phosphorylation was accompanied by a decrease in Tyr-705 phosphorylation (Fig. 5B). Therefore, JNK-mediated Ser-727 phosphorylation may augment STAT3 activity through stabilizing Tyr-705 phosphorylation. In addition, we tested the effect of SP600125 on IL-6-mediated STAT3 DNA binding activity and phosphorylation. Unlike leptin-mediated STAT3 activation, SP600125 pretreatment did not significantly suppress either DNA binding activity (Fig. 5A) or Ser-727/Tyr-705 phosphorylation (Fig. 5B) of STAT3 in DU145 cells when treated with 100 ng/ml IL-6 for 15 min. This result indicates that SP600125 does not inhibit STAT3 activation in a non-specific fashion and further confirms the implication of JNK in leptin-stimulated STAT3 activation.

3.4. Akt is implicated in leptin-stimulated AIPC cell proliferation

Since leptin stimulates Ser-473 phosphorylation of Akt during AIPC cell proliferation (Fig. 4), Akt may mediate leptin-stimulated AIPC cell proliferation. To confirm this, we blocked activation of Akt by pharmacologically inhibiting its upstream effector PI3K with LY294002 and determined the effect on cell proliferation stimulated by 12.5 μg/ml leptin in the absence of androgen. LY294002 completely suppressed leptin-stimulated cell proliferation, as well as Akt phosphorylation, at 5 μM (Fig. 6A and B). Thus, the PI3K-Akt pathway mediates leptin-stimulated AIPC cell proliferation. In addition, LY294002 inhibited both cell proliferation and Akt phosphorylation in leptin-untreated cells, indicating that the PI3K-Akt pathway is important in the basal level of AIPC cell proliferation.

3.5. JNK mediates leptin-stimulated Akt activation

As with JNK and STAT3, Akt is biphasically activated by leptin (Fig. 4) and plays a crucial role in leptin-stimulated AIPC cell proliferation (Fig. 6). Since JNK mediates leptin-stimulated STAT3 activation (Fig. 5), JNK may also be involved in leptin-stimulated Akt activation. To define whether JNK mediates the first and/or second phase of leptin-stimulated Akt activation, we tested the effect of pharmacological JNK inhibition on Ser-473 phosphorylation of Akt after treatment with 12.5 μg/ml leptin for 5 min and 4 h. JNK inhibition with 10 μM SP600125 suppressed the first phase of leptin-stimulated Akt phosphorylation completely and the second phase partially (Fig. 7). Thus, JNK is indispensable for rapid Akt activation by leptin while the late phase of sustained activation implicates a JNK-independent, as well as-dependent, mechanism. In addition, in contrast to leptin-mediated Akt phosphorylation SP600125 pretreatment did not affect Akt phosphorylation in DU145 cells when treated with 100 ng/ml IGF-I for 15 min (Fig. 7). Therefore, SP600125 does not inhibit Akt activation in a non-specific manner, further confirming the involvement of JNK in leptin-stimulated Akt activation.

3.6. Leptin activates JNK, STAT3 and Akt in not only DU145 but also TRAMP-C1 cells

To confirm whether leptin activates JNK, STAT3 and Akt pathways not only in DU145 cells but also in other AIPC cells, we examined the effect of short-term leptin treatment on activation of these pathways in TRAMP-C1 cells. All the three pathways were constitutively activated in TRAMP-C1 cells (Fig. 8). Treatment with 12.5 μg/ml leptin

for 15 min stimulated JNK activity, as well as c-Jun phosphorylation on Ser-63 and-73 (Fig. 8A). In addition, leptin augmented the DNA binding activity and Tyr-705 and Ser-727 phosphorylation of STAT3 after 15-min treatment (Fig. 8B). Furthermore, Akt phosphorylation at Ser-473 was rapidly up-regulated by leptin in a time-dependent fashion, peaking at 10 min after leptin addition (Fig. 8C). Therefore, TRAMP-C1 and DU145 cells exhibit similar activation profiles of JNK, STAT3 and Akt pathways in response to leptin.

3.7. JNK mediates leptin-stimulated STAT3 and Akt activation in not only DU145 but also TRAMP-C1 cells

Since leptin stimulates JNK, STAT3 and Akt in both TRAMP-C1 and DU145 cells, JNK may be involved in leptin-mediated STAT3 and Akt activation in TRAMP-C1, as well as DU145, cells. To confirm this, we first examined the effect of JNK inhibition with 10 μM SP600125 on the DNA binding activity and Ser-727 and Tyr-705 phosphorylation of STAT3 in TRAMP-C1 cells when treated with 12.5 μg/ml leptin for 15 min. As observed in DU145 cells (Fig. 5A and B), SP600125 pretreatment remarkably attenuated leptin-stimulated DNA binding activity (Fig. 9A), as well as Ser-727 and Tyr-705 phosphorylation (Fig. 9B), of STAT3. We then tested the effect of JNK inhibition on Akt phosphorylation at Ser-473 in TRAMP-C1 cells when treated with leptin for 10 min. JNK inhibition thoroughly blocked Akt phosphorylation (Fig. 9C). Thus, JNK mediates leptin-stimulated STAT3 and Akt activation in not only DU145 but also TRAMP-C1 cells, suggesting that leptin-induced interaction of JNK, STAT3 and Akt may be a common signaling event that mediates AIPC cell proliferation.

4. Discussion

AIPC is an advanced, lethal form of prostate cancer. Obesity is associated with advanced prostate cancer [1–5]. Our recent study has demonstrated that conditioned medium from human primary adipocyte culture augments cell proliferation in human AIPC DU145 and PC-3 cells, indicating that adipose factors mediate AIPC cell proliferation [9]. Leptin is a major adipose cytokine whose production is significantly increased in obesity [13]. We have reported that added leptin stimulates androgen-independent cell proliferation in DU145 and PC-3 cells [9]. These facts together lead to a hypothesis: adipose leptin increased in obesity facilitates AIPC cell growth.

In this study, we found that like DU145 and PC-3 cells mouse prostate cancer TRAMP-C1 cells were androgen-independent and proliferated in response to leptin (Fig. 1B). Taking advantage of this finding, we developed the in vitro reconstitution system involving TRAMP-C1 cells and epididymal fat extracts from obese and lean mice to test the effect of adipose factors, including leptin, on AIPC cell growth. This system is advantageous in that: (1) cytokines produced by mouse adipose tissue are compatible with TRAMP-C1 cells so that interaction of adipose cytokines with tumor cells can be investigated; (2) the effect of experimentally induced obesity on adipose factor-prostate cancer cell interaction can be evaluated. Using this system in combination with high-and low-fat diet-fed C57BL/6J mice and leptin-deficient *ob*/*ob* mice, we showed that adipose leptin played a pivotal role in obesity-stimulated AIPC cell growth (Table 1 and Fig. 1C).

Adipose factors could act on cancer cells in both endocrine and paracrine manners: cancer cells would be exposed to paracrine adipose factors when prostate tumors invade into retropubic fat pads or metastasize to bone marrow. It should be noted that concentrations of exogenous leptin stimulatory to AIPC cell proliferation (100 ng/ml–12.5 μg/ml in DU145 and PC-3 cells, 2–12.5 μg/ml in TRAMP-C1 cells) [9] (Fig. 1B) are significantly higher than leptin levels in circulation in men (5–100 ng/ml) [13,51–53] or mouse fat extracts (10–15 ng/ml) (Table 1). Our data show that other adipose cytokines such as IGF-I and IL-6 sensitize AIPC cells to leptin [9]. Therefore, effective leptin concentrations in vivo should be much lower than those required for leptin alone-stimulated AIPC cell proliferation in vitro. This is supported by a report showing that 4–40 ng/ml leptin stimulates DU145 and PC-3 cell proliferation in the presence of 10 % fetal bovine serum [21]. In this study, we dared to use a high concentration of leptin (12.5 μg/ml) in the absence of serum in order to analyze its pure effect on signal transduction implicated in AIPC cell proliferation.

JNK is required for cell proliferation and survival in androgen-independent PC-3 cells in vitro [32] and growth of PC-3 xenografts in athymic mice [33]. STAT3 is crucial in AIPC cell proliferation and survival [39]. Furthermore, the PI3K-Akt pathway is implicated in the development and progression of AIPC in mice [42–44]. We have reported that JNK is necessary for leptin-stimulated AIPC cell proliferation [9]. In the present study we showed that JNK mediated

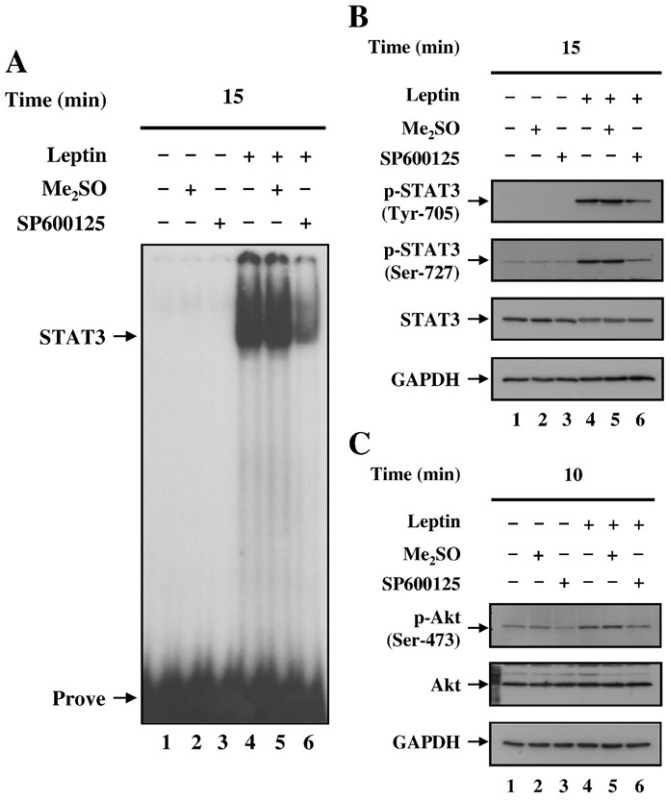

Fig. 9. JNK mediates leptin-stimulated STAT3 DNA binding activity and phosphorylation, as well as Akt phosphorylation, in androgen-independent TRAMP-C1 prostate cancer cells. Following 24-h serum deprivation, TRAMP-C1 cells were treated with 12.5 μg/ml leptin for 15 min with and without pretreatment with Me_2SO (vehicle control) or 10 μM SP600125 (pharmacological JNK inhibitor) for 30 min. Cells without any treatment (lane 1) and treated with Me_2SO alone (lane 2) were included as controls. (A) To assess STAT3 DNA binding activity, cell lysates (10 μg protein) were subjected to EMSA with ^{32}P-end-labeled M67-SIE as a probe. *STAT3*, STAT3–DNA complex; NS, non-specific. (B) Cell lysates (50 μg protein) were analyzed by Western blotting. STAT3 phosphorylation at Tyr-705 and Ser-727 was determined using anti-phospho-STAT3 (Tyr-705) and (Ser-727) antibodies (*p-STAT3 (Tyr-705)* and *p-STAT3 (Ser-727)*). To measure total STAT3 protein levels, membranes hybridized with these antibodies were stripped and re-hybridized with anti-STAT3 antibody that recognizes both phosphorylated and non-phosphorylated forms of STAT3 (*STAT3*). The same membranes were stripped again and re-hybridized with anti-GAPDH antibody to normalize variations in sample loading (*GAPDH*). (C) TRAMP-C1 cells were deprived of serum for 24 h and treated with 12.5 μg/ml leptin for 10 min with and without pretreatment with Me_2SO or 10 μM SP600125 for 30 min. Cells without any treatment (lane 1) and treated with Me_2SO alone (lane 2) were included as controls. Ser-473-phosphorylated Akt was immunoprecipitated in cell lysates (1 mg protein) with immobilized anti-Akt (1G1) antibody and detected by Western blot analysis using anti-phospho-Akt (Ser-473) antibody (*p-Akt (Ser-473)*). To determine total levels of Akt protein, cell lysates (50 μg protein) were subjected to Western blot analysis using anti-Akt antibody that recognizes both phosphorylated and non-phosphorylated forms of Akt (*Akt*). Membranes probed with this antibody were stripped and re-probed with anti-GAPDH antibody to normalize variations in sample loading (*GAPDH*).

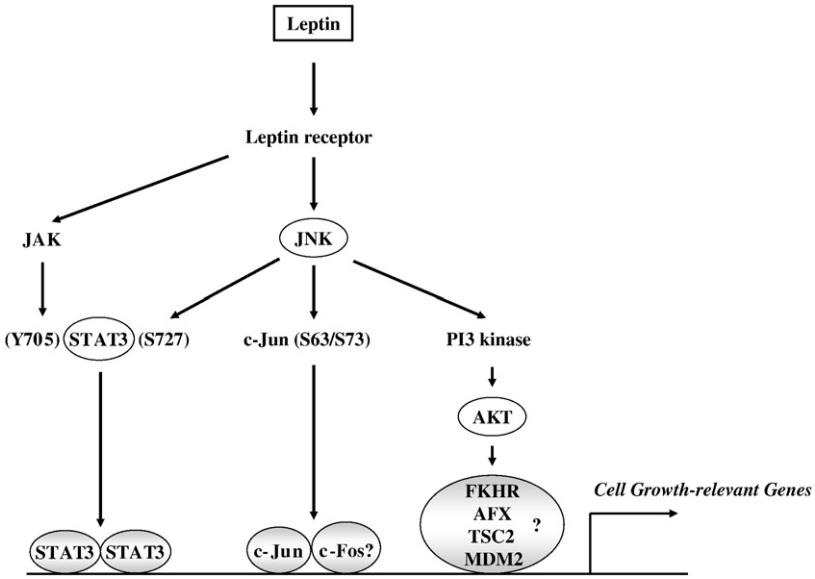

Fig. 10. Model of leptin signaling network that mediates AIPC cell proliferation. Leptin activates JNK and JAK upon binding to the leptin receptor. After STAT3 activation is triggered by JAK-mediated Tyr-705 phosphorylation, JNK further stimulates and stabilizes STAT3 activation via Ser-727 phosphorylation. JNK also activates c-Jun through Ser-63 and -73 phosphorylation. Moreover, JNK mediates Akt activation through PI3K. Activation of these signaling pathways leads to expression of cell growth-relevant genes, resulting in androgen-independent cell proliferation. Transcription factor(s) that form a complex with c-Jun are unknown in leptin-stimulated AIPC cells. Signaling molecules downstream of Akt also need to be identified.

leptin-stimulated AIPC cell proliferation through activation of STAT3 and Akt (Fig. 9). To the best of our knowledge, this is the first evidence for interaction of these three oncogenic molecules in AIPC. Of interest, JNK [54], STAT3 [55–57] and PI3K-Akt [58–60] all play a role in obesity, too. Therefore, these molecules, as well as leptin, are mediators between AIPC and obesity.

Constitutive STAT3 activation is critical in pathogenesis of many types of cancer including AIPC [34,39,61]. Ser-727 phosphorylation is involved in persistent activation of STAT3 [62]. Here we showed that leptin augmented constitutive STAT3 activation through JNK-mediated Ser-727 phosphorylation during AIPC cell proliferation (Fig. 5). Our previous study has demonstrated that adipose cytokine adiponectin, which decreases in obesity and competes with leptin to suppress AIPC cell growth [23], inhibits constitutive STAT3 activation [49]. These findings together suggest that STAT3 is a key signaling determinant in adipose cytokine regulation of AIPC cell growth. Furthermore, it has been reported that STAT3 activation via JNK-mediated Ser-727 phosphorylation is required for Src oncoprotein-induced malignant transformation [37]. Therefore, JNK-mediated STAT3 activation appears to play a crucial role in both malignant transformation and progression in cancer.

We demonstrated that JNK was indispensable for leptin-stimulated rapid activation of Akt during AIPC cell proliferation in DU145 cells (Fig. 7). This is the first evidence for the requirement of JNK for Akt activation in cancer cells. Pharmacological inhibition of PI3K suppressed leptin-stimulated Akt activation in DU145 cells (Fig. 6A), indicating that PI3K is upstream of Akt in the leptin signaling. In addition, PI3K inhibition did not suppress leptin-stimulated JNK activation (data not shown). Therefore, JNK is most likely to be upstream of PI3K to mediate leptin-stimulated Akt activation in DU145 cells (Fig. 10). DU145 cells express wild-type PTEN [21]. Intriguingly, constitutively activated PI3K stimulates JNK activity in PTEN-deficient cells [63]. Thus, the JNK and PI3K pathways could regulate each other; and PTEN status may determine which pathway dictates the other. In vitro evidence for signaling interaction between JNK and Akt agrees with positive correlation between phospho-c-Jun and phospho-Akt in immunohistochemical staining of human prostate cancer tissue [63].

We showed that leptin stimulated activation of JNK-c-Jun, STAT3 and Akt in TRAMP-C1, as well as DU145, cells (Fig. 8). Furthermore, JNK mediated leptin-stimulated STAT3 and Akt activation in both cell lines (Fig. 9). These results suggest that leptin-induced activation/interaction of these molecules is not a phenomenon specifically observed in a certain cell line but a generally occurring event that mediates AIPC cell growth. Therefore, co-activation states of JNK, STAT3 and Akt in tumor cells may well predict and diagnose androgen-independent progression of prostate cancer; and disruption of their signaling interaction could be therapeutic for AIPC.

In addition to the leptin signaling network demonstrated in this study (Fig. 10), some other mechanisms may be involved in leptin regulation of AIPC progression. Leptin stimulates PC-3 cell migration through ERK activation [22]. Thus, ERK may mediate leptin-stimulated AIPC cell growth as well. Leptin is also known to up-regulate genes encoding such growth factors as vascular endothelial growth factor, transforming growth factor-β1 and basic fibroblast growth factor [22]. Therefore, leptin may promote AIPC progression including tumor growth, metastasis and angiogenesis via regulation of such genes. Moreover, it is interesting to note that leptin stimulates expression of the suppressor of cytokine signaling 3 (SOCS3) gene in AIPC cells [21], as well as some other cell types and tissues [64–68]. SOCS3 inhibits leptin signaling through acting on JAK [69,70] so that it may work as a negative feedback mechanism for leptin-mediated AIPC progression. Further investigation is needed to clarify the entire leptin signaling network in AIPC.

In summary, adipose leptin mediates AIPC cell growth accelerated by obesity. Leptin and its downstream signaling molecules JNK, STAT3 and Akt are molecular mediators between obesity and prostate cancer and could be prognostic, diagnostic, therapeutic targets for AIPC. Further studies are ongoing in our laboratory to confirm this hypothesis using mouse models and human subjects.

Acknowledgements

We thank Thomas L. Rummel for technical assistance in Western blot analysis and thymidine incorporation assays. This study was supported by Department of Defense grant DAMD17-02-1-0161, and

grants from the Gustavus and Louise Pfeiffer Research Foundation, the Lady Riders Breast Cancer Research Foundation, the Fotsch Foundation, the Wisconsin Breast Cancer Showhouse, and the MCW Cancer Center.

References

[1] C.L. Amling, C.J. Kane, R.H. Riffenburgh, J.F. Ward, J.L. Roberts, R.S. Lance, P.A. Friedrichs, J.W. Moul, Relationship between obesity and race in predicting adverse pathologic variables in patients undergoing radical prostatectomy, Urology 58 (2001) 723–728.

[2] Y. Furuya, S. Akimoto, K. Akakura, H. Ito, Smoking and obesity in relation to the etiology and disease progression of prostate cancer in Japan, Int. J. Urol. 5 (1998) 134–137.

[3] C. Rodriguez, A.V. Patel, E.E. Calle, E.J. Jacobs, A. Chao, M.J. Thun, Body mass index, height, and prostate cancer mortality in two large cohorts of adult men in the United States, Cancer. Epidemiol. Biomark. Prev. 10 (2001) 345–353.

[4] M.E. Wright, S.C. Chang, A. Schatzkin, D. Albanes, V. Kipnis, T. Mouw, P. Hurwitz, A. Hollenbeck, M.F. Leitzmann, Prospective study of adiposity and weight change in relation to prostate cancer incidence and mortality, Cancer 109 (2007) 675–684.

[5] Z. Gong, M.L. Neuhouser, P.J. Goodman, D. Albanes, C. Chi, A.W. Hsing, S.M. Lippman, E.A. Platz, M.N. Pollak, I.M. Thompson, A.R. Kristal, Obesity, diabetes, and risk of prostate cancer: results from the prostate cancer prevention trial, Cancer Epidemiol. Biomark. Prev. 15 (2006) 1977–1983.

[6] M.P. Porter, J.L. Stanford, Obesity and the risk of prostate cancer, Prostate 62 (2005) 316–321.

[7] A.W. Hsing, J. Deng, I.A. Sesterhenn, F.K. Mostofi, F.Z. Stanczyk, J. Benichou, T. Xie, Y.T. Gao, Body size and prostate cancer: a population-based case-control study in China, Cancer. Epidemiol. Biomark. Prev. 9 (2000) 1335–1341.

[8] G. Fruhbeck, J. Gomez-Ambrosi, F.J. Muruzabal, M.A. Burrell, The adipocyte: a model for integration of endocrine and metabolic signaling in energy metabolism regulation, Am. J. Physiol. Endocrinol. Metab. 280 (2001) E827–847.

[9] M. Onuma, J.D. Bub, T.L. Rummel, Y. Iwamoto, Prostate cancer cell-adipocyte interaction: leptin mediates androgen-independent prostate cancer cell proliferation through c-Jun NH2-terminal kinase, J. Biol. Chem. 278 (2003) 42660–42667.

[10] M.A. Pelleymounter, M.J. Cullen, M.B. Baker, R. Hecht, D. Winters, T. Boone, F. Collins, Effects of the obese gene product on body weight regulation in ob/ob mice, Science 269 (1995) 540–543.

[11] L.A. Campfield, F.J. Smith, Y. Guisez, R. Devos, P. Burn, Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks, Science 269 (1995) 546–549.

[12] Y. Zhang, R. Proenca, M. Maffei, M. Barone, L. Leopold, J.M. Friedman, Positional cloning of the mouse obese gene and its human homologue, Nature 372 (1994) 425–432.

[13] M. Maffei, J. Halaas, E. Ravussin, R.E. Pratley, G.H. Lee, Y. Zhang, H. Fei, S. Kim, R. Lallone, S. Ranganathan, et al., Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects, Nat. Med. 1 (1995) 1155–1161.

[14] R. Ribeiro, A. Vasconcelos, S. Costa, D. Pinto, A. Morais, J. Oliveira, F. Lobo, C. Lopes, R. Medeiros, Overexpressing leptin genetic polymorphism (-2548 G/A) is associated with susceptibility to prostate cancer and risk of advanced disease, Prostate 59 (2004) 268–274.

[15] S. Chang, S.D. Hursting, J.H. Contois, S.S. Strom, Y. Yamamura, R.J. Babaian, P. Troncoso, P.S. Scardino, T.M. Wheeler, C.I. Amos, M.R. Spitz, Leptin and prostate cancer, Prostate 46 (2001) 62–67.

[16] P. Stattin, S. Soderberg, G. Hallmans, A. Bylund, R. Kaaks, U.H. Stenman, A. Bergh, T. Olsson, Leptin is associated with increased prostate cancer risk: a nested case-referent study, J. Clin. Endocrinol. Metab. 86 (2001) 1341–1345.

[17] R. Gade-Andavolu, L.A. Cone, S. Shu, A. Morrow, B. Kowshik, M.V. Andavolu, Molecular interactions of leptin and prostate cancer, Cancer J. 12 (2006) 201–206.

[18] A.W. Hsing, S. Chua, Jr., Y.T. Gao, E. Gentzschein, L. Chang, J. Deng, F.Z. Stanczyk, Prostate cancer risk and serum levels of insulin and leptin: a population-based study, J. Natl. Cancer Inst. 93 (2001) 783–789.

[19] P. Lagiou, L.B. Signorello, D. Trichopoulos, A. Tzonou, A. Trichopoulou, C.S. Mantzoros, Leptin in relation to prostate cancer and benign prostatic hyperplasia, Int. J. Cancer. 76 (1998) 25–28.

[20] P. Stattin, R. Kaaks, R. Johansson, R. Gislefoss, S. Soderberg, H. Alfthan, U.H. Stenman, E. Jellum, T. Olsson, Plasma leptin is not associated with prostate cancer risk, Cancer Epidemiol. Biomark. Prev. 12 (2003) 474–475.

[21] P. Somasundar, K.A. Frankenberry, H. Skinner, G. Vedula, D.W. McFadden, D. Riggs, B. Jackson, R. Vangilder, S.M. Hileman, L.C. Vona-Davis, Prostate cancer cell proliferation is influenced by leptin, J. Surg. Res. 118 (2004) 71–82.

[22] K.A. Frankenberry, P. Somasundar, D.W. McFadden, L.C. Vona-Davis, Leptin induces cell migration and the expression of growth factors in human prostate cancer cells, Am. J. Surg. 188 (2004) 560–565.

[23] J.D. Bub, T. Miyazaki, Y. Iwamoto, Adiponectin as a growth inhibitor in prostate cancer cells, Biochem. Biophys. Res. Commun. 340 (2006) 1158–1166.

[24] J.M. Friedman, J.L. Halaas, Leptin and the regulation of body weight in mammals, Nature 395 (1998) 763–770.

[25] J.A. Cioffi, A.W. Shafer, T.J. Zupancic, J. Smith-Gbur, A. Mikhail, D. Platika, H.R. Snodgrass, Novel B219/OB receptor isoforms: possible role of leptin in hematopoiesis and reproduction, Nat. Med. 2 (1996) 585–589.

[26] C. Bjorbaek, S. Uotani, B. da Silva, J.S. Flier, Divergent signaling capacities of the long and short isoforms of the leptin receptor, J. Biol. Chem. 272 (1997) 32686–32695.

[27] H. Baumann, K.K. Morella, D.W. White, M. Dembski, P.S. Bailon, H. Kim, C.F. Lai, L.A. Tartaglia, The full-length leptin receptor has signaling capabilities of interleukin 6-type cytokine receptors, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 8374–8378.

[28] D.W. White, K.K. Kuropatwinski, R. Devos, H. Baumann, L.A. Tartaglia, Leptin receptor (OB-R) signaling. Cytoplasmic domain mutational analysis and evidence for receptor homo-oligomerization, J. Biol. Chem. 272 (1997) 4065–4071.

[29] B. Cohen, D. Novick, M. Rubinstein, Modulation of insulin activities by leptin, Science 274 (1996) 1185–1188.

[30] A. Bouloumie, T. Marumo, M. Lafontan, R. Busse, Leptin induces oxidative stress in human endothelial cells, FASEB J. 13 (1999) 1231–1238.

[31] R.J. Davis, Signal transduction by the JNK group of MAP kinases, Cell 103 (2000) 239–252.

[32] O. Potapova, S.V. Anisimov, M. Gorospe, R.H. Dougherty, W.A. Gaarde, K.R. Boheler, N.J. Holbrook, Targets of c-Jun NH(2)-terminal kinase 2-mediated tumor growth regulation revealed by serial analysis of gene expression, Cancer Res. 62 (2002) 3257–3263.

[33] Y.M. Yang, F. Bost, W. Charbono, N. Dean, R. McKay, J.S. Rhim, C. Depatie, D. Mercola, C-Jun NH(2)-terminal kinase mediates proliferation and tumor growth of human prostate carcinoma, Clin. Cancer Res. 9 (2003) 391–401.

[34] E.B. Haura, J. Turkson, R. Jove, Mechanisms of disease: Insights into the emerging role of signal transducers and activators of transcription in cancer, Nat. Clin. Pract. Oncol. 2 (2005) 315–324.

[35] D.S. Aaronson, C.M. Horvath, A road map for those who don't know JAK-STAT, Science 296 (2002) 1653–1655.

[36] C.P. Lim, X. Cao, Serine phosphorylation and negative regulation of Stat3 by JNK, J. Biol. Chem. 274 (1999) 31055–31061.

[37] J. Turkson, T. Bowman, J. Adnane, Y. Zhang, J.Y. Djeu, M. Sekharam, D.A. Frank, L.B. Holzman, J. Wu, S. Sebti, R. Jove, Requirement for Ras/Rac1-mediated p38 and c-Jun N-terminal kinase signaling in Stat3 transcriptional activity induced by the Src oncoprotein, Mol. Cell. Biol. 19 (1999) 7519–7528.

[38] C.L. Campbell, Z. Jiang, D.M. Savarese, T.M. Savarese, Increased expression of the interleukin-11 receptor and evidence of STAT3 activation in prostate carcinoma, Am. J. Pathol. 158 (2001) 25–32.

[39] L.B. Mora, R. Buettner, J. Seigne, J. Diaz, N. Ahmad, R. Garcia, T. Bowman, R. Falcone, R. Fairclough, A. Cantor, C. Muro-Cacho, S. Livingston, J. Karras, J. Pow-Sang, R. Jove, Constitutive activation of Stat3 in human prostate tumors and cell lines: direct inhibition of Stat3 signaling induces apoptosis of prostate cancer cells, Cancer Res. 62 (2002) 6659–6666.

[40] A.G. Bader, S. Kang, L. Zhao, P.K. Vogt, Oncogenic PI3K deregulates transcription and translation, Nat. Rev. Cancer 5 (2005) 921–929.

[41] P.K. Majumder, W.R. Sellers, Akt-regulated pathways in prostate cancer, Oncogene 24 (2005) 7465–7474.

[42] S. Wang, J. Gao, Q. Lei, N. Rozengurt, C. Pritchard, J. Jiao, G.V. Thomas, G. Li, P. Roy-Burman, P.S. Nelson, X. Liu, H. Wu, Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer, Cancer Cell 4 (2003) 209–221.

[43] H. Gao, X. Ouyang, W.A. Banach-Petrosky, W.L. Gerald, M.M. Shen, C. Abate-Shen, Combinatorial activities of Akt and B-Raf/Erk signaling in a mouse model of androgen-independent prostate cancer, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 14477–14482.

[44] L. Xin, M.A. Teitell, D.A. Lawson, A. Kwon, I.K. Mellinghoff, O.N. Witte, Progression of prostate cancer by synergy of AKT with genotropic and nongenotropic actions of the androgen receptor, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 7789–7794.

[45] R.D. Klein, S.M. Fischer, Black tea polyphenols inhibit IGF-I-induced signaling through Akt in normal prostate epithelial cells and Du145 prostate carcinoma cells, Carcinogenesis 23 (2002) 217–221.

[46] B.A. Foster, J.R. Gingrich, E.D. Kwon, C. Madias, N.M. Greenberg, Characterization of prostatic epithelial cell lines derived from transgenic adenocarcinoma of the mouse prostate (TRAMP) model, Cancer Res. 57 (1997) 3325–3330.

[47] N. Hoggard, J.G. Mercer, D.V. Rayner, K. Moar, P. Trayhurn, L.M. Williams, Localization of leptin receptor mRNA splice variants in murine peripheral tissues by RT-PCR and in situ hybridization, Biochem. Biophys. Res. Commun. 232 (1997) 383–387.

[48] N. Hoggard, L. Hunter, J.S. Duncan, L.M. Williams, P. Trayhurn, J.G. Mercer, Leptin and leptin receptor mRNA and protein expression in the murine fetus and placenta, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 11073–11078.

[49] T. Miyazaki, J.D. Bub, M. Uzuki, Y. Iwamoto, Adiponectin activates c-Jun NH2-terminal kinase and inhibits signal transducer and activator of transcription 3, Biochem. Biophys. Res. Commun. 333 (2005) 79–87.

[50] Y. Iwamoto, Y.E. Chin, X. Peng, X.Y. Fu, Identification of a membrane-associated inhibitor(s) of epidermal growth factor-induced signal transducer and activator of transcription activation, J. Biol. Chem. 273 (1998) 18198–18204.

[51] S.M. Haffner, R.L. Gingerich, H. Miettinen, M.P. Stern, Leptin concentrations in relation to overall adiposity and regional body fat distribution in Mexican Americans, Int. J. Obes. Relat. Metab. Disord. 20 (1996) 904–908.

[52] R.E. Ostlund, Jr., J.W. Yang, S. Klein, R. Gingerich, Relation between plasma leptin concentration and body fat, gender, diet, age, and metabolic covariates, J. Clin. Endocrinol. Metab. 81 (1996) 3909–3913.

[53] R.V. Considine, M.K. Sinha, M.L. Heiman, A. Kriauciunas, T.W. Stephens, M.R. Nyce, J.P. Ohannesian, C.C. Marco, L.J. McKee, T.L. Bauer, et al., Serum immunoreactive-leptin concentrations in normal-weight and obese humans, N. Engl. J. Med. 334 (1996) 292–295.

[54] J. Hirosumi, G. Tuncman, L. Chang, C.Z. Gorgun, K.T. Uysal, K. Maeda, M. Karin, G.S. Hotamisligil, A central role for JNK in obesity and insulin resistance, Nature 420 (2002) 333–336.

[55] Z. Ni, W. Lou, E.S. Leman, A.C. Gao, Inhibition of constitutively activated Stat3 signaling pathway suppresses growth of prostate cancer cells, Cancer Res. 60 (2000) 1225–1228.
[56] Q. Gao, M.J. Wolfgang, S. Neschen, K. Morino, T.L. Horvath, G.I. Shulman, X.Y. Fu, Disruption of neural signal transducer and activator of transcription 3 causes obesity, diabetes, infertility, and thermal dysregulation, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 4661–4666.
[57] S. Gorogawa, Y. Fujitani, H. Kaneto, Y. Hazama, H. Watada, Y. Miyamoto, K. Takeda, S. Akira, M.A. Magnuson, Y. Yamasaki, Y. Kajimoto, M. Hori, Insulin secretory defects and impaired islet architecture in pancreatic beta-cell-specific STAT3 knockout mice, Biochem. Biophys. Res. Commun. 319 (2004) 1159–1170.
[58] P.M. Ghosh, S. Malik, R. Bedolla, J.I. Kreisberg, Akt in prostate cancer: possible role in androgen-independence, Curr. Drug Metab. 4 (2003) 487–496.
[59] J.R. Graff, Emerging targets in the AKT pathway for treatment of androgen-independent prostatic adenocarcinoma, Expert. Opin. Ther. Targets. 6 (2002) 103–113.
[60] B.D. Manning, Balancing Akt with S6K: implications for both metabolic diseases and tumorigenesis, J. Cell. Biol. 167 (2004) 399–403.
[61] T. Bowman, R. Garcia, J. Turkson, R. Jove, STATs in oncogenesis, Oncogene 19 (2000) 2474–2488.
[62] Z. Wen, Z. Zhong, J.E. Darnell, Jr., Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation, Cell 82 (1995) 241–250.
[63] I. Vivanco, N. Palaskas, C. Tran, S.P. Finn, G. Getz, N.J. Kennedy, J. Jiao, J. Rose, W. Xie, M. Loda, T. Golub, I.K. Mellinghoff, R.J. Davis, H. Wu, C.L. Sawyers, Identification of the JNK signaling pathway as a functional target of the tumor suppressor PTEN, Cancer Cell 11 (2007) 555–569.
[64] C. Bjorbaek, K. El-Haschimi, J.D. Frantz, J.S. Flier, The role of SOCS-3 in leptin signaling and leptin resistance, J. Biol. Chem. 274 (1999) 30059–30065.
[65] I. Tsumanuma, L. Jin, S. Zhang, J.M. Bayliss, B.W. Scheithauer, R.V. Lloyd, Leptin signal transduction in the HP75 human pituitary cell line, Pituitary 3 (2000) 211–220.
[66] C. Bjorbaek, J.K. Elmquist, J.D. Frantz, S.E. Shoelson, J.S. Flier, Identification of SOCS-3 as a potential mediator of central leptin resistance, Mol. Cell 1 (1998) 619–625.
[67] V. Emilsson, J.R. Arch, R.P. de Groot, C.A. Lister, M.A. Cawthorne, Leptin treatment increases suppressors of cytokine signaling in central and peripheral tissues, FEBS Lett. 455 (1999) 170–174.
[68] M. Ziotopoulou, D.M. Erani, S.M. Hileman, C. Bjorbaek, C.S. Mantzoros, Unlike leptin, ciliary neurotrophic factor does not reverse the starvation-induced changes of serum corticosterone and hypothalamic neuropeptide levels but induces expression of hypothalamic inhibitors of leptin signaling, Diabetes 49 (2000) 1890–1896.
[69] C. Bjorbak, H.J. Lavery, S.H. Bates, R.K. Olson, S.M. Davis, J.S. Flier, M.G. Myers, Jr., SOCS3 mediates feedback inhibition of the leptin receptor via Tyr985, J. Biol. Chem. 275 (2000) 40649–40657.
[70] A.S. Banks, S.M. Davis, S.H. Bates, M.G. Myers, Jr., Activation of downstream signals by the long form of the leptin receptor, J. Biol. Chem. 275 (2000) 14563–14572.