Leptin-regulated gene expression in MCF-7 breast cancer cells: mechanistic insights into leptin-regulated mammary tumor growth and progression

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Abstract

Obesity is a recently established risk factor for breast cancer incidence and mortality. A characteristic of obesity is elevated circulating levels of adipocyte-derived hormone leptin. Evidence indicates that leptin plays an important role in mammary tumor formation; however, the mechanisms involved are poorly understood. Toward better defining the role of leptin in breast cancer, we describe the identification of leptin-regulated genes in hormone-responsive Michigan Cancer Foundation-7 (MCF-7) human breast cancer cells using a microarray system. More than 64 leptin-regulated genes were identified including those for growth factors, cell cycle regulators, extracellular matrix (ECM) proteins, and genes associated with metastasis. Cell cycle genes up-regulated by leptin include *cyclins D* and *G*, cyclin-dependent kinase 2, *p21*, *p27*, and *p16*. Leptin suppressed the expression of transforming growth factor-β , a cell cycle suppressor. Determining the significance of this effect, treatment of MCF-7 cells with *TGFB1* abrogated leptin-stimulated proliferation. Leptin up-regulated the expression of connective tissue growth factor, *villin 2*, and *basigin*, factors that are associated with ECM and are known to impact tumor growth. Finally, leptin induced the expression of anti-apoptotic genes *BCL2* and *survivin*, and reduced the expression of apoptotic genes. The effect of leptin on MCF-7 survival was evaluated via TUNEL assay and demonstrated a sixfold reduction in apoptosis in leptin-treated cells, compared with controls. These data suggest leptin promotes mammary tumor growth through multiple mechanisms, including regulating the cell cycle, apoptosis, and by modulating the extracellular environment. The identification of leptin-regulated genes begins to provide mechanistic links into the relationship between obesity and breast cancer incidence and morbidity.

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Introduction

Obesity is a major health problem and is positively associated with breast cancer incidence and mortality (Barnett 2003, Calle & Kaaks 2004, Garofalo & Surmacz 2006, Lorincz & Sukumar 2006). The molecular mechanisms involved in the relationship between obesity and breast cancer have not been delineated. A characteristic of obesity is elevated circulating levels of leptin, an adipocyte-derived hormone that acts at the brain to regulate energy expenditure and food intake, and influences immune and reproductive functioning (Sweeney 2002, Hegyi *et al.* 2004, Fruhbeck 2006). Recent evidence suggests that leptin also plays an important role in normal mammary development and mammary tumor formation. In humans and rodents, serum concentrations of leptin are elevated during late pregnancy, a time of intense increase in mammary epithelial growth and proliferation (Henson & Castracane 2000). It has been reported that in normal mammary tissues, epithelial leptin receptor expression increases during late pregnancy (Laud *et al.* 1999). Furthermore, obese leptin and leptin receptor-deficient mice exhibit a significant impairment of postnatal mammary development and a decreased incidence of mammary tumors (Hu *et al.* 2002, Cleary *et al.* 2003, 2004). Evidence from *in vitro* studies reveals that leptin induces proliferation of normal and cancerous mammary epithelial cells (Hu *et al.* 2002, Somasundar *et al.* 2003, Garofalo & Surmacz 2006). Together, these studies support that leptin is an important regulator of proliferation in normal and malignant breast epithelium. Despite the strong evidence revealing the significance of leptin in breast cancer, the mechanisms by which leptin induces epithelial proliferation are just beginning to be characterized.

In addition to stimulating proliferation in mammary epithelia, leptin has been shown to inhibit apoptosis, promote cell invasion, and modulate the extracellular matrix (ECM) in other cell types (Castellucci *et al.* 2000, Han *et al.* 2001, Saxena *et al.* 2003, 2004, Garofalo & Surmacz 2006, Ogunwobi & Beales 2006). Each of these events are known to play a significant role in tumor growth and progression (Hanahan & Weinberg 2000, Tlsty & Coussens 2006), but the regulation of these processes by leptin in cancer is not well understood. Toward determining the broader influence of leptin on mammary tumor cells, and to gain an insight into the mechanisms involved, this is the first study to

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use a customized gene microarray approach for identifying leptin-regulated genes in human breast cancer. Leptin up-regulated many genes associated with cell cycle and proliferation, DNA synthesis, and ECM in Michigan Cancer Foundation-7 (MCF-7) cells. Expression of genes associated with decreasing proliferation and apoptosis were down-regulated in response to leptin. These results indicate that leptin induces proliferation, modifies ECM, and suppresses apoptosis to promote breast cancer cell growth and survival. The stimulation of MCF-7 cell proliferation by leptin has been demonstrated in other reports (Dieudonne *et al.* 2002, Okumura *et al.* 2002, Somasundar *et al.* 2003, Catalano *et al.* 2004); however, its effect on regulating apoptotic/anti-apoptotic and ECM genes in breast cancer has not been previously described. The robust influence of leptin on ECM genes is of particular interest, given the increased recognition that the microenvironment significantly impacts tumor progression (Eckhardt *et al.* 2005, Tlsty & Coussens 2006). As there is gaining interest in targeting leptin action for novel therapeutic strategies (Garofalo *et al.* 2006, Gonzalez *et al.* 2006, Surmacz 2007), the identification of leptin-regulated genes described here begins to provide valuable insights into the mechanistic links between obesity, breast cancer incidence, and morbidity.

Materials and Methods

Cell culture and hormone treatment

MCF-7 human breast cancer epithelial cells were obtained from American Type Culture Collection (ATCC Manassas, VA, USA). The cells were maintained routinely in Roswell Park Memorial Institute (RPMI) 1640 media (ATCC) supplemented with 10% fetal calf serum (ATCC), 100 U/ml penicillin G, and 0·1 mg/ml streptomycin sulfate at 37 °C in a humidified, 5% CO_2, 95% air atmosphere. Human recombinant leptin was purchased from the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA, USA).

RNA extraction

The MCF-7 cell line was routinely maintained in RPMI 1640 media and supplemented with 10% fetal calf serum for 2 days. The cells were then serum starved for 24 h and treated with media containing 500 or 0 ng/ml leptin (control). This concentration of leptin stimulates MCF-7 cell proliferation and has been used in other *in vitro* signaling studies (Dieudonne *et al.* 2002, Hu *et al.* 2002, Somasundar *et al.* 2003, Fruhbeck 2006, Perera *et al.* 2008). RNA was isolated from these cells after 6 or 24 h of treatment using the RNeasy Micro kit (Qiagen). The integrity of RNA was verified by ethidium bromide staining of agarose gels and by an optical density (OD) absorption ratio of OD 260 nm/OD 280 nm $>1{\cdot}9$. This RNA was used for microarray analysis and real-time PCR.

DualChip microarrays

Total RNA from control- and leptin-treated cells was used to generate cDNA (cMaster labeling kit; Eppendorf, Hamburg, Germany), which was labeled with biotin, according to the recommendations by Eppendorf. cDNA was hybridized to a DualChip human cancer array, containing oligonucleotide probes for 281 cancer-associated genes. After hybridization, detection was carried out by incubating the chips with Cy3-conjugated IgG anti-biotin antibody (Jackson Immuno-Research Laboratories, West Grove, PA, USA), and scanning by ScanArray 5000 scanner (Packard BioChip, Technologies, Billerica, MA, USA) using three different photomultiplier tube (PMT) settings (low, middle, and high gains). To ensure high-quality results, the Eppendorf DualChip human cancer array was equipped with several controls that allow verification of cDNA synthesis efficiency, hybridization efficiency, and signal linearity. In addition, the chips also contained oligonucleotide probes for many housekeeping genes (HKGs) that allowed normalization of generated ratios. Array quantification was done by GenePix 6.0 software (Axon Instruments, Union City, CA, USA). Average signals (median) and background signals (mean) from all three datasets (different PMT settings) were transferred into Eppendorf DualChip evaluation software 2.0. A signal was accepted if the average intensity after background subtraction was at least twice higher than local background. Very bright intensities (saturated signals indicating highly expressed genes) were defined as unquantifiable as they underestimated the intensity ratios. These were excluded from quantitative analysis. A two-step normalization procedure was employed using internal standards and HKGs. Each microarray slide consists of two identical microarrays, printed side by side, to ensure reproducibility. Leptin- and control-treated samples were used side by side in each array slide. Each array consists of three replicate probes per gene. At least three different sets of control (0 ng/ml leptin treated) and 500 ng/ml leptin-treated MCF-7 cells were used for gene analyses for each time point. MCF-7 cells belonging to the same passage number were used in both microarray and real-time PCR experiments.

Identification of leptin-regulated genes

DualChip evaluation software (Eppendorf) was used to identify leptin-regulated genes. The variance of the normalized set of HKGs (except those affected by the tested condition) was used to generate a confidence interval to test the significance of the gene expression ratios (de Longgueville *et al.* 2002). The statistical algorithms of the software are based on a test that was originally developed by Chen *et al.* 1997. This model assumes that intensities are distributed according to Gaussian distribution. Based on these assumptions, a formula was developed for the coefficient of variation (CV) and confidence intervals. The CV is estimated based on the subset of the ratios of HKGs selected for normalization, which are stable between test and reference samples and have

ratios distributed around a value of 1. The significance of the ratios of gene expression is established using the confidence interval computed from this statistical model: ratios outside the 95% (99%) confidence interval were determined to be significantly (highly significantly) different. Acceptable gene expression measurements had signal intensities higher than twice the local background, as well as higher than the mean of the negative hybridization controls plus twice their standard deviation. Different combinations of housekeeping gene sets were evaluated to obtain the optimal CV. From each group of HKGs, the means and standard deviations were calculated and the confidence interval of the Gaussian curve was determined. Subsequently, borderlines/limits were set during the calculation process, where the values are defined as being significant/highly significant relative to these limits. Limits are applied in order to minimize the possibility of obtaining false-positive (e.g., non-regulated genes are called regulated) and false-negative (e.g., regulated genes are called non-regulated) results. The result of these thresholds is that no ratio below 1·49489 is determined to be significant and all ratios above 1·812389 are defined as significant (1/1·49489 and 1/1·812389 for down-regulated genes).

Verification of gene expression data via real-time PCR

Leptin-regulated genes of interest, identified by microarray, were verified using real-time PCR. For real-time PCR, four to five sets of MCF-7 mRNA, which were different from the mRNA employed in microarray, were used. About three to four up- and down-regulated genes identified by microarray analysis was verified for each time point (6 and 24 h) using real-time PCR. One microgram of total RNA was reverse transcribed with MMLV reverse transcriptase using random hexamers (Promega), according to the manufacturer's instructions. Real-time quantitative RT-PCR analysis was performed using 10 ng of reverse-transcribed total RNA with 20 pmol/μl of both sense and antisense primers and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in a final reaction volume of 30 μl. An ABI PRISM 7700 Sequence Detection System Instrument (Applied Biosystems) was used for the amplification. β-Actin was used in each experiment to control for variability in the initial quantities of cDNA. Relative quantification for a gene was expressed as a fold change over the control gene (not treated with leptin). Fold change was calculated using the difference between cycle threshold (C_t) value of the gene and control gene using the formula $2-\Delta C_t A-C_t B$ (comparative CT method/$\Delta\Delta C_t$). PCR was performed using specific primers (described in Table 1). Cycling conditions consisted of an initial denaturation step of 95 °C for 10 min as a 'hot start' followed by 40 cycles of 95 °C for 15 s at the noted annealing temperature for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 10 min. In addition, real-time PCR was used for evaluating the time course (Fig. 2) of the regulation of cell cycle and apoptosis genes identified from microarray analysis.

Cell proliferation assay

To determine the role of transforming growth factor-β (*TGFB1*) in leptin-induced MCF-7 cell proliferation, the cells were initially seeded onto 12-well plates at 6×10^4 cells/well in RPMI 1640 containing 10% FBS. After 24 h, subconfluent cells were synchronized by serum starving for 24 h in RPMI media. The cells were then incubated in the media containing either 10% FBS, 10% FBS plus leptin (500 ng/ml), *TGFB1* (100 pM), or 10% FBS plus leptin and *TGFB1*. A parallel experiment was performed in which synchronized MCF-7 cells were incubated in serum-free media with the same individual or combined hormone treatments. In each study, 24 h after treatment, total viable cells were counted with a hemocytometer. For each treatment, the % change in cell number, relative to its initial

Table 1 Primers used for real-time PCR to confirm selected leptin-regulated genes identified by microarray analysis

Gene name	Forward primer	Reverse primer
CD82	5′-TGG GCT CAG CCT GTA TCA AAG TCA-3′	5′-AGA TGA AAC TGC TCT TGT CGG CCA-3′
FLT4	5′-TAC TGC TTG ACC AAA GAG CCC TCA-3′	5′-AGG TGC TGA AGG GAC ATT GTG AGA-3′
SERPINE1	5′-TGC TGG TGA ATG CCC TCT ACT TCA-3′	5′-AGA GAC AGT GCT GCC GTC TGA TTT-3′
N4BP2	5′-AAT GCA CTC ACC ATG AGC ACC AAC-3′	5′-ACG AGC TGG TAT TTA CTG GGC AGA-3′
GRB2	5′-TCT GCT TCC ATG GCT TCC TGA GAA-3′	5′-TCA CCA TGT TGG CTA GGT TGG TCT-3′
IGFBP2	5′-GCA TGG CCT GTA CAA CCT CAA ACA-3′	5′-AGC CTC CTG CTG CTC ATT GTA GA-3′
PDAP1	5′-AGT GAC ACC TGG TAC TGG CAG TTT-3′	5′-AAA TTG ATC CTG AAG CCC AAC GGC-3′
RRM1	5′-TTG AGT CTC AGA CGG AAA CAG GCA-3′	5′-TTG CTG CAT TTG ATG GTT CCC AGG-3′
BCL2	5′-TTT CTC ATG GCT GTC CTT CAG GGT-3′	5′-AGG TCT GGC TTC ATA CCA CAG GTT-3′
BIRC5	5′-TCA TAG AGC TGC AGG GTG GAT TGT-3′	5′-AGT AGG GTC CAC AGC AGT GTT TGA-3′
Cyclin A2	5′-ATG AGC ATG TCA CCG TTC CTC CTT-3′	5′-TCA GCT GGC TTC TTC TGA GCT TCT-3′
CDK2	5′-AGA TGG ACG GAG CTT GTT ATC GCA-3′	5′-TGG CTT GGT CAC ATC CTG GAA GAA-3′
MT3	5′-TGC AAG TGC GAG GGA TGC AAA T-3′	5′-ACA CAC AGT CCT TGG CAC ACT TCT-3′
LITAF-3′	5′-TTA CTA TGT TGC CCA GGC TGG TGT-3′	5′-TTC AGG CCC AGC ATG GTA GCT TAT-3′
BSG	5′-AAT GAC AAA GGC AAG AAC GTC CGC-3′	5′-ACT TGG AAT CTT GCA AGC ACT GGG-3′
TGFB3	5′-TGG ACT TCG GCC ACA TCA AGA AGA-3′	5′-TGT TGT AAA GGG CCA GGA CCT GAT-3′
CTGF	5′-TCA AGA CCT GTG CCT GCC ATT ACA-3′	5′-ACT CTC TGG CTT CAT GCC ATG TCT-3′

counts, was calculated. In the initial experiments, several concentrations of leptin were tested using cell counting and fluorescence activated cell sorting (FACS) analysis, and it was determined that 500 ng/ml leptin induces proliferation of MCF-7 cells (Perera *et al.* 2008) and, therefore, was used in this study. In accordance with this, various other studies examining the influence of leptin in breast cancer cell proliferation have used 500 ng/ml or a similar concentration. As circulating leptin levels range from 10 to 60 ng/ml, the use of 500 ng/ml leptin to elicit a cellular response is likely a reflection of elevated local hormone concentrations that can occur *in vivo*. In support of this, it has been demonstrated that hormone levels in tissue microenvironments can be five- to tenfold higher, compared with circulating concentrations (Stefanczyk-Krzymowska *et al.* 1998, Dieudonne *et al.* 2002, Hu *et al.* 2002, Okumura *et al.* 2002, Somasundar *et al.* 2003).

Apoptosis assay

To examine the effect of leptin on MCF-7 apoptosis, the cells were grown on coverslips in RPMI 1640 media containing 10% fetal calf serum. Cells at ~60% confluence were growth arrested in serum-free media. After 24 h, the cells were incubated in the media, in the presence (500 ng/ml) or absence of leptin. After leptin treatment for 6 and 24 h, the coverslips were removed and the cells were evaluated for apoptosis via TUNEL assay (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling). TUNEL assay was performed using the TMR red *in situ* cell death detection system (Roche Diagnostics), according to the manufacturer's specifications. Briefly, the cells were washed with 1×PBS and fixed for 20 min in 4% paraformaldehyde at room temperature. The fixed cells were washed twice with 1×PBS+50 mM glycine for 20 min. The cells were then blocked for 2 h in a blocking buffer (PBS, 0·1% Triton X-100, and 1% BSA) and treated with TUNEL reagent and incubated at 37 °C in a humidified environment for 1 h. On completion of incubation, the cells were washed three times with the blocking buffer and evaluated by fluorescence microscopy. To quantify *in situ* TUNEL assay fluorescence, total number of cells and the number of apoptotic cells in each field were counted and the percentage of apoptotic cells calculated. A minimum of 1000 cells per slide were counted in random fields. The cells treated with serum for 24 h or the cells exposed to UV for 2 h (to induce DNA strand breaks) were used as negative and positive apoptosis controls respectively.

Statistical analysis

Values from real-time PCR, MCF-7 proliferation, and apoptosis assays were expressed as means±S.E.M. from at least three different experiments (each with $n=3$–5). Statistical analysis was performed using ANOVA and Student's *t*-test using Microsoft Excel or Statview 5.0 software (SAS Institute Inc., Cary, NC, USA). A value of $P<0{\cdot}05$ was considered statistically significant.

Results

Identification of leptin-regulated genes

The purpose of this study was to gain an insight into the mechanisms by which leptin influences tumor growth. Toward this, MCF-7 hormone-dependent human cancerous mammary epithelial cells were treated with leptin, and leptin-responsive genes were identified by microarray. To examine the time-dependent regulation of gene expression, MCF-7 cells were treated with or without leptin for 6 and 24 h. A fold change in expression was calculated for each gene at each treatment time point. The significance of the ratios for the genes identified by microarray analysis was established using the confidence interval computed from the statistical model, where ratios above the 95% confidence interval are considered as statistically significant. The cut-off criteria for significance included that the fold change was at least 1·5 or greater compared with control (0 ng/ml leptin sample).

Out of the 281 cancer genes analyzed, 217 were found to be non-significant or produced signal intensities too low to be detected. A total of 64 different genes were significantly differentially expressed, where 35 were up-regulated and 29 down-regulated by leptin. A total of 75 genes were regulated at either 6 or 24 h, with 11 of these genes changed at both time points (Table 2). Thus, for the most part, the early, when compared with late, categories represent different sets of genes. Some of the leptin-responsive genes identified here have been reported by others. As described in Table 3, the majority of these previously identified leptin-regulated genes have been in cell types other than breast cells.

Time-course patterns of leptin-regulated genes

Time-course analyses were performed to gain an insight into the general pattern of leptin gene stimulation. Time-course patterns of leptin-regulated gene expression were evaluated by assigning microarray-identified genes to one of three categories: 1) early regulated (6 h), 2) early and late regulated (6 and 24 h), or 3) late regulated (24 h). When grouped in this manner, 25 genes were early regulated, 11 genes were regulated at early and late time points, and the majority of the genes (50) were regulated at the late time point. These expression patterns indicate that leptin has a transient effect on most of the genes it regulates. This effect was verified by subsequent real-time PCR experiments. At the early time point, there were 16 stimulated and 9 inhibited genes (total 25 genes). At the late time point, the number of stimulated and inhibited genes was similar.

Functional categories of novel leptin-regulated genes

The major functional categories of leptin-regulated genes include cell cycle, proliferation, apoptosis, cell adhesion/ECM, structural, growth factors/hormones/cytokines, receptors, signal transduction, transcription, protein

Table 2 Functional categories of leptin-regulated genes

Gene name	6 h	24 h	Acc no.	Symbol
Cell cycle/proliferation				
Metallothionein 3	40·1	0·4	NM 005954	*MT3*
Cyclin-dependent kinase 2	7·5	1·1	NM 001798	*CDK2*
CDC-like kinase 1	2·9	1·0	NM 004071	*CLK1*
Cyclin D1	2·5	1·0	NM 053056	*CCND1*
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	1·9	1·0	U03106	*CDKN1A*
Cyclin G1	1·7	1·1	U53328	*CCNG1*
Mitogen-activated protein kinase kinase 1	1·7	1·6	NM 002755	*MAP2K1*
Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	1·6	0·2	NM 00464	*CDKN1B*
Ubiquitin-conjugating enzyme E2A (RAD6 homolog)	0·6	1·5	M74524	*UBE2A*
PDGFA-associated protein 1	0·2	1·1	NM 014891	*PDAP1*
Cyclin A2	0·9	6·5	NM 001237	*Cyclin A2*
Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	N/D	4·0	L27211	*CDKN2A*
Replication factor C (activator 1) 2, 40 kDa	N/D	1·6	NM 002914	*RFC2*
Kangai 1	N/D	0·4	NM 002231	*CD82*
Protein (peptidyl-prolyl *cis/trans* isomerase) NIMA-interacting 1	N/D	0·4	NM 006221	*UBL5*
Growth factor receptor-bound protein 2	N/D	0·1	NM 002086	*GRB2*
Apoptosis				
B-cell CLL/lymphoma2	10·5	1·1	NM 000633	*BCL2*
Caspase 8, apoptosis-related cysteine peptidase	2·9	1·0	X98172	*CASP8*
Survivin (baculoviral IAP repeat-containing 5)	2·3	1·1	NM 001168	*BIRC5*
BCL2-antagonist/killer 1	N/D	1·6	U16811	*BAK1*
BCL2-related protein A1	N/D	1·5	NM 004049	*BCL2A1*
BCL2-associated antagonist of cell death	N/D	1·5	NM004322	*BAD*
TRAF interacting protein	N/D	0·5	NM 005879	*TRAIP*
IGF1 receptor	N/D	0·4	NM000875	*IGF1R*
TNFRSF1A-associated via death domain	N/D	0·4	NM 003789	*TRADD*
Cell adhesion/extracellular matrix				
Connective tissue growth factor	N/D	8·2	U14750	*CTGF*
Villin 2	N/D	2·8	NM 003379	*EZR*
Basigin (Ok blood group)	N/D	2·7	NM 001728	*BSG*
Vimentin	N/D	1·6	NM 003380	*VIM*
Heparan sulfate proteoglycan 2	N/D	0·5	NM 005529	*HSPG2*
Ninjurin 1	N/D	0·4	U91512	*NINJ1*
Integrin β30·05 binding protein (β3-endonexin)	N/D	0·3	NM 014288	*N4BP2*
Cytoskeleton/structural				
Keratin 8	5·7	2·0	NM 002273	*KRT8*
Keratin 10	0·6	1·1	NM 000421	*KRT10*
Envoplakin	N/D	0·5	NM001988	*EVPL*
Desmin	N/D	0·1	NM 001927	*DES*
Transcription/signal transduction				
Signal transducer and activator of transcription 1, 91 kDa	0·6	1·4	NM 007315	*STAT1*
Lipopolysaccharide-induced TNF factor	0·1	7·7	NM 004862	*LITAF*
Purine-rich element binding protein A	N/D	1·5	NM 005859	*PURA*
v-fos FBJ murine osteosarcoma viral oncogene homolog	N/D	1·5	NM005252	*FOS*
E2F transcription factor 1	0·6	0·6	NM 005225	*E2F1*
Retinoic acid receptor α	N/D	0·6	NM 000964	*RARA*
Early growth response 1	1·1	0·5	NM 001964	*EGR1*
TYRO3 protein tyrosine kinase	N/D	1·5	NM 006293	*TYRO3*
TRAF family member-associated NF-κB activator	N/D	1·5	U59863	*TANK*
DNA repair/synthesis				
Thymidylate synthetase	2·0	1·3	NM001071	*TYMS*
O-6-methylguanine-DNA methyltransferase	1·5	1·0	NM 002412	*MGMT*
Ribonucleotide reductase M1	0·2	0·6	NM 001033	*RRM1*
Polymerase (DNA directed) α 2 (70 kD subunit)	N/D	1·7	NM 002689	*POLA2*
Ubiquitin-conjugating enzyme E2A (RAD6 homolog)	N/D	1·5	M74524	*UBE2A*

(continued)

Table 2 *(Continued)*

	6 h	24 h	Acc no.	Symbol
Growth factors/cytokines				
IGF-binding protein 2, 36 kDa	0·5	0·6	M35410	*IGFBP2*
Amphiregulin (schwannoma-derived growth factor)	N/D	1·7	NM 001657	*AREG*
Transforming growth factor-β3	N/D	0·5	NM 003239	*TGFB3*
Transforming growth factor-β1	N/D	0·3	NM 000660	*TGFB1*
Receptors				
Hyaluronan-mediated motility receptor (RHAMM)	N/D	5·0	U29343	*HMMR*
IGF1 receptor	N/D	0·4	NM 000875	*IGF1R*
Erythropoietin receptor	N/D	0·2	NM 000121	*EPOR*
Angiogenesis				
Vimentin	N/D	1·6	NM003380	*VIM*
Vascular endothelial growth factor receptor 3	N/D	0·4	NM 002020	*FLT4*
Tumor suppressor				
BRCA1-associated RING domain 1	1·9	1·6	NM 000465	*BARDI1*
KiSS-1 metastasis-suppressor	N/D	0·6	NM 002256	*KISS1*
Protein binding/modification				
Plasminogen activator inhibitor, type 1	0·1	0·1	M14083	*SERPINE1*
Protein kinase, DNA-activated, catalytic polypeptide	1·5	0·3	NM 006904	*PRKDC*

Values are fold change at each time point relative to control and are organized by time course of regulation (early to late), with the first time of significant regulation boxed. Genes are grouped according to specific function. N/D represents genes not detected for that time point.

binding/modification, DNA repair/synthesis, and tumor suppressor genes. The majority of these genes have not been previously reported to be regulated by leptin. Out of the 64 leptin-regulated genes identified, 16 were associated with cell cycle or proliferation, 9 with apoptosis, and 7 with cell adhesion/ECM (Table 2). A major trend observed was that leptin regulated cell cycle/proliferation, apoptosis, and DNA repair/synthesis genes mainly at the early time point but cell adhesion/ECM proteins, growth factors/cytokines, and receptor genes were mainly regulated at the late time point. These data suggest that leptin promotes mammary tumor formation initially by inducing cell cycle progression and

Table 3 Leptin-regulated genes identified by microarray when compared with previous reports. Listed in the table above are leptin-regulated genes that were determined by microarray, which have been previously identified. The effect of leptin measured by microarray is compared with the results of prior reports, with the type of cell previously evaluated included.

	Published results (references)			
	Result	Cell/tissue type	Reference	**Our findings**
Gene				
CDK2	↑ by leptin	Human MCF-7 cells	18	↑ by leptin
MAP2K1	↑ by leptin	Human T47-D cells	57	↑ by leptin
STAT1	↑ by leptin	Mouse adipose tissue	58	↑ by leptin
	↑ by leptin	Human pancreatic β cells	59	
FOS	↑ by leptin	Human placental cells	60	↑ by leptin
	↑ by leptin	Human neuron cells	61	
EPOR	↓ by leptin	Rat T-cells	62	↓ by leptin
KISS1	↓ by leptin	Mouse neuron cells	63	↓ by leptin
CTGF	↑ by leptin	Human NRK-49F cells	64	↑ by leptin
Cyclin D1	↑ by leptin	Human MCF-7	18	↑ by leptin
	↑ by leptin	Mouse osteoblast cells human	65	
	↑ by leptin	Hepatic stellate cells	66	
CDKN1B (p27)	↓ by leptin	Pancreatic β cells	67	↑ by leptin
	↓ by leptin	Mouse CD4 T cells	68	
E2FA	↓ by leptin	Pancreatic β cells	69	↓ by leptin
TGFB1	↑ by leptin	Rat glomerular endothelial cells	70	↓ by leptin
	↑ by leptin	Human liver cells	71	
IGF1R	↓ by leptin	Ovaries	72	↓ by leptin

proliferation followed by modulating angiogenesis, cell adhesion, and ECM to promote a more aggressive tumor phenotype.

Leptin regulates cell cycle, proliferation, and apoptosis genes

Reports focusing on individual cell-signaling molecules have demonstrated that leptin stimulates breast cancer cell proliferation (Hu *et al.* 2002, Somasundar *et al.* 2003, Garofalo & Surmacz 2006). However, the underlying mechanisms of this leptin effect have not been studied using a global genomic approach. Here, we find that a substantially greater proportion of genes involved in cell cycle, compared with other categories, were induced by leptin. *Cyclins D1*, *A2*, and *G* and cyclin-dependent kinase 2 (*CDK2*) were increased by leptin, suggesting that leptin is important in promoting cell cycle progression by altering the cyclin expression (Table 2). Leptin also regulated the cell cycle by altering the expression of inhibitors of G1-specific CDK–cyclin complexes including cyclin-dependent kinase inhibitor (*CDKN1A*) (p21), *CDKN1B* (p27), and *CDKN2A* (p16). In addition, leptin-induced *MT3*, a gene associated with poor tumor prognosis, and replication factor C2 (RFC2), important in DNA synthesis. The expression of *MT3*, *CDK2*, *PDAP1*, *cyclin A2*, growth factor receptor bound protein 2 (*GRB2*), and *CD82* were verified using real-time PCR (Fig. 1A and B). Additionally, leptin induced the expression of anti-apoptotic genes *BCL2* and *survivin*, and reduced the expression of many apoptotic genes such as *TRAIP*, *IGF1R*, and *TRADD*.

To gain further insight into the pattern of leptin stimulation on the more robustly affected genes, an expanded PCR time course was performed. This involved determining the expression profiles of cell cycle genes, *cyclin A2* and *CLK1* (Fig. 2A), and apoptotic genes, *BCL2* and *survivin* (Fig. 2B),

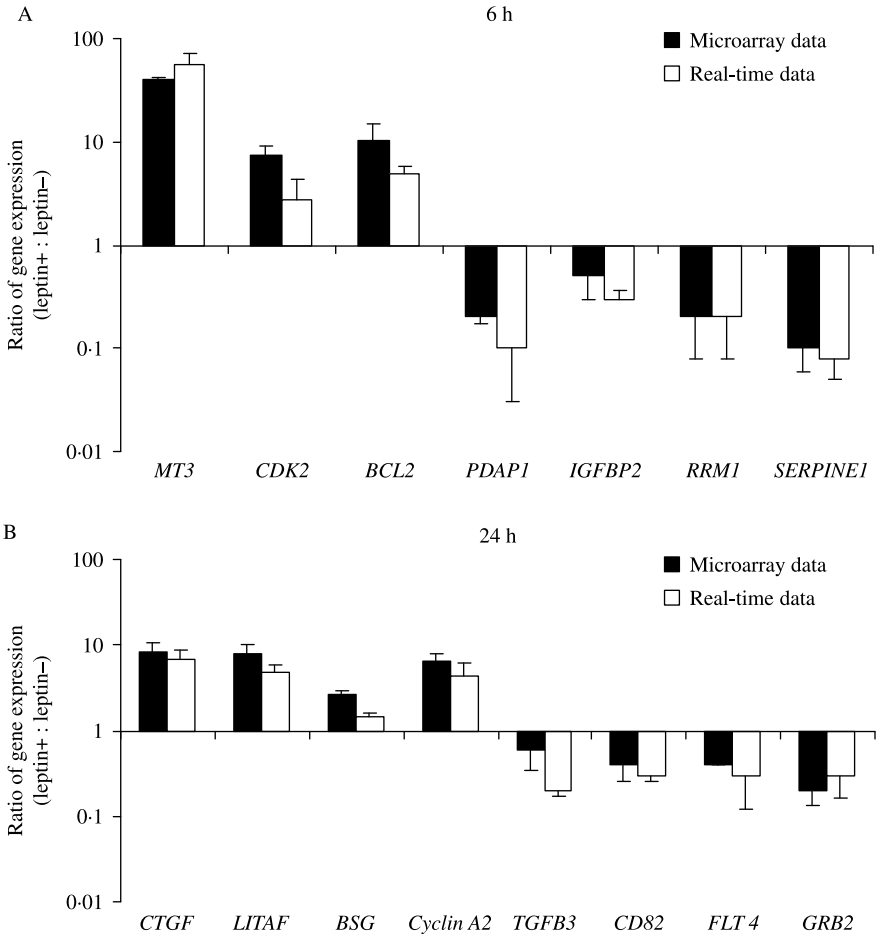

Figure 1 Real-time PCR evaluation of leptin-regulated genes identified by microarray analysis in MCF-7 cells. Expression level of selected leptin-regulated genes identified by microarray was validated using real-time PCR. Comparison of the expression of each gene as measured by microarray and real-time PCR at (A) 6 and (B) 24 h. The fold change in expression for microarray and real-time PCR experiments was calculated relative to control values (no leptin treatment). Real-time PCR data results are from at least three different experiments (each with $n=4$–5) with β actin expression as an internal control.

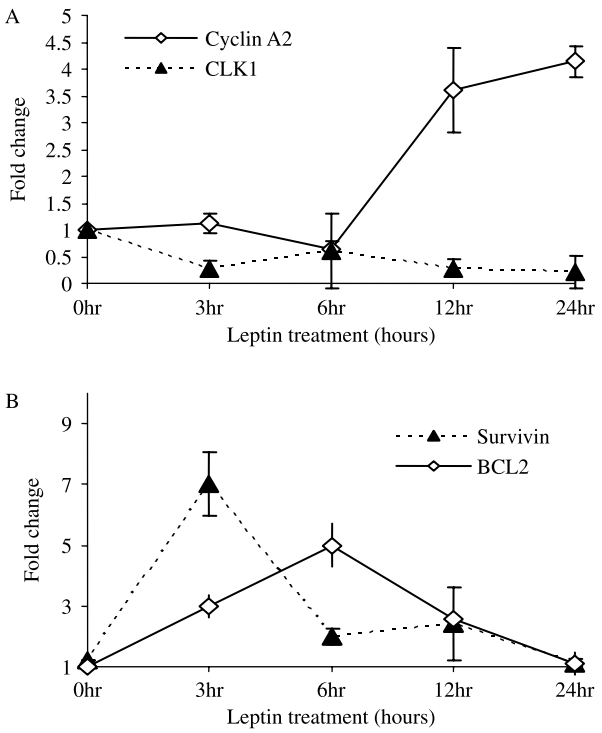

Figure 2 Time course of expression of cell cycle and apoptosis genes in MCF-7 cells treated with leptin. Gene expression profile for (A) cell cycle and (B) apoptotic genes over a 24-h treatment period was determined by real-time PCR. The fold change in expression was calculated relative to control values (without leptin) with β-actin as the internal control. Results are means (n=4–5)±S.E.M. from three different experiments.

at several time points, during 24 h. Supporting microarray observations, these results verify *CLK1*, *survivin*, and *BCL2* as early genes, and *cyclin A2* as a late gene. Interestingly, the time course also revealed a much greater induction of *survivin* at 3 h (sevenfold), when compared with the 6-h time point (2·3-fold) measured by the array. This corroborates a role for *survivin* as a mediator of leptin and supports the significance of genes identified by array, in that they can be more robustly regulated at other time points. Furthermore, time-course data indicate that leptin can simultaneously promote cell cycle progression and suppress apoptosis in breast cancer cells.

Leptin regulates cell adhesion, ECM, and cytoskeleton genes

The effect of leptin on the expression of cell adhesion, ECM, and cytoskeleton genes has not been evaluated in breast cancer cells. Leptin up-regulated the expression of many ECM genes, including connective tissue growth factor (*CTGF*), *villin 2*, and *basigin* (*BSG*; Table 2). Overexpression of these genes is associated with breast cancer progression and metastasis, but has not been correlated with obesity or leptin. The effect of leptin on *BSG* and *CTGF* expression was confirmed using real-time PCR (Fig. 1B). Many of the cell adhesion, ECM and cytoskeleton genes were regulated at the late time point (24 h), in contrast to the early effect of leptin on cell cycle genes.

Leptin regulates genes encoding DNA repair/synthesis and transcription

Herein, we demonstrate that leptin up-regulates genes involved in DNA repair/synthesis, including thymidylate synthetase, *O*-6-methylguanine-DNA methyltransferase (*MGMT*), polymerase α (*POLA2*), and ubiquitin-conjugating enzyme E2 (*UBE2A*). Leptin also regulated the expression of transcription factors lipopolysaccharide-induced TNF factor (*LITAF*), purine rich element binding protein A (*PURA*), and TRAF family member associated NFKB activator (*TANK*) (Table 2). The expression of *LITAF* was initially suppressed (6 h) and then substantially induced at 24 h. Real-time PCR data for *LITAF* expression at 24 h was consistent with microarray analysis (Fig. 1B).

Leptin regulates growth factors, cytokines, and receptors

Leptin regulated the expression of growth factors, cytokines, and receptors mainly at the late time point in MCF-7 cells (Table 2). Interestingly, leptin down-regulated *TGFB2*, *TGFB3*, and *IGFBP2*, factors that are known to suppress mammary epithelial proliferation. Leptin also induced hyaluronan-mediated motility receptor (*HMMR*) and suppressed insulin-like growth factor-I (IGF1) receptor (*IGF1R*) and erythropoietin receptor, genes involved in tumor cell

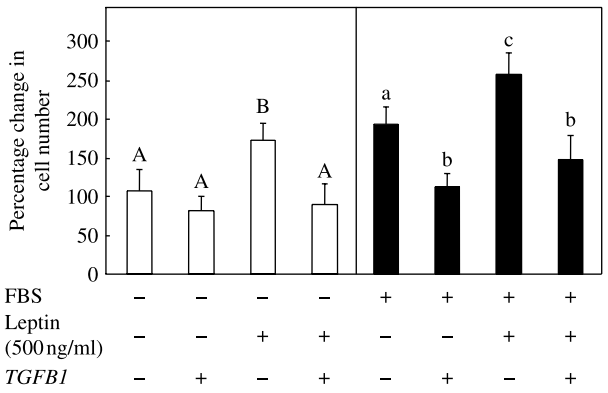

Figure 3 *TGFB1* suppresses leptin-stimulated MCF-7 cell proliferation. As described in the Materials and Methods section, synchronized MCF-7 cells were incubated in serum-free media or in media containing 10% FBS. For each case, the media was not supplemented or supplemented with leptin (500 ng/ml), *TGFB1* (100 pM), or leptin plus *TGFB1*. Twenty-four hours after treatment, total viable cells were counted via hemocytometer. For each treatment, % change in cell number, relative to its initial counts, was calculated. Results are means (n=5)±S.E.M. A one-way ANOVA test and two sample *t*-tests were applied to assess the differences in % change. Statistical analysis was performed to compare treatments within the serum-free or 10% FBS groups; however, samples from these two groups were not cross-compared. Treatment groups having different letters are statistically significant at $P<0·05$.

migration and metastasis. To determine the significance of leptin's suppression of *TGFB1*, the role of *TGFB1* in leptin-stimulated MCF-7 cell proliferation was tested. As described in the Materials and Methods section, synchronized MCF-7 cells were incubated for 24 h in media containing either leptin (500 ng/ml), *TGFB1*, leptin and *TGFB1* combined, or no hormone supplements. These experiments were done in two ways, where synchronized MCF-7 cells were incubated in serum-free media or 10% FBS media with each media containing hormone treatments described. As shown in Fig. 3, leptin induced a significant increase (40–60%), while *TGFB1* caused a reduction (30%) in cell numbers under the serum-free and 10% FBS incubation conditions. When leptin and *TGFB1*were given in combination, *TGFB1* completely abrogated leptin-induced proliferation in both serum-free and FBS conditions. Identification of these leptin-regulated growth factor genes begins to suggest potential mechanisms by which leptin promotes tumor aggressiveness.

Leptin acts as an anti-apoptotic factor

In these microarray studies, several apoptosis-related genes were regulated by leptin. To support the idea that leptin promotes tumor cell viability by suppressing apoptosis, the anti-apoptotic effect of leptin was quantified by TUNEL assay (Roche Diagnostics). Briefly, MCF-7 cells were incubated for 6 and 24 h in media without leptin or containing 500 ng/ml leptin. Via TUNEL, the percentage of labeled apoptotic nuclei was calculated. Treatment with 500 ng/ml leptin for 24 h lead to a significant sixfold reduction in apoptotic cells ($2 \pm 0{\cdot}41\%$), compared with 0 ng/ml leptin treatment ($12 \pm 1{\cdot}5\%$; Fig. 4). Subsequently, a notable correlation is observed between the time courses of the TUNEL assay and the gene expression levels. In real-time PCR verification of leptin-regulated genes (Fig. 2), we measured a sevenfold increase in survivin at 3 h and a tenfold increase in *BCL2* at 6 h. The timing and sizeable induction of these anti-apoptotic genes by leptin place them in the early regulated category. The timing of early increased levels of *survivin* and *BCL2* transcripts correlates well with the TUNEL assay (Fig. 4), where leptin significantly reduces apoptosis at 24 h, but not at 6 h.

Discussion

Obesity is a major health problem and is associated with breast cancer incidence and mortality (Barnett 2003, Calle & Kaaks 2004, Garofalo & Surmacz 2006, Lorincz & Sukumar 2006). The molecular mechanisms underlying the relationship between obesity and breast cancer have not been delineated. A characteristic of obesity is elevated circulating levels of leptin, an adipocyte-derived hormone that regulates energy expenditure and food intake (Sweeney 2002, Hegyi *et al.* 2004, Fruhbeck 2006). Evidence suggests that leptin also plays an important role in mammary tumor formation. Studies reveal that obese mice deficient in leptin or its receptor are

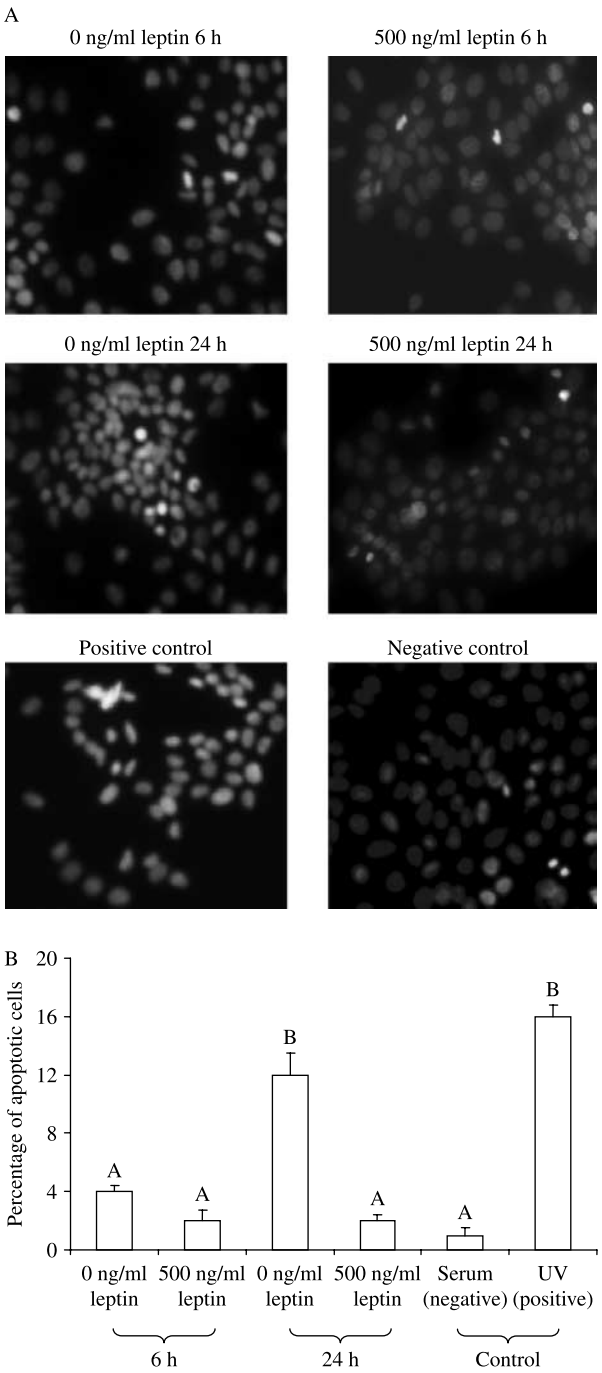

Figure 4 Leptin is an anti-apoptotic factor in MCF-7 cells. (A) Apoptosis was measured at 6- and 24-h time points, via TUNEL assay, in MCF-7 cells treated with 500 ng/ml leptin or without leptin. MCF-7 cells treated with UV or serum were used as positive and negative controls respectively. (B) The percentage of labeled apoptotic nuclei, calculated as described in the Materials and Methods section, in MCF-7 cells incubated with or without leptin. Results are means ($n = 3$–4) $\pm$ S.E.M. from three experiments. Treatment groups having different letters are statistically significant at $P < 0.05$.

resistant to mammary tumorigenesis (Hu *et al.* 2002, Cleary *et al.* 2003, 2004), and that leptin induces proliferation of malignant mammary epithelial cells *in vitro* (Dieudonne *et al.* 2002, Hu *et al.* 2002, Okumura *et al.* 2002, Somasundar *et al.* 2003, Garofalo & Surmacz 2006). These reports support the notion that leptin action may be a valuable clinical target and they emphasize a need for better understanding of leptin mechanisms in breast cancer.

In the studies described here, a novel microarray system was exploited to determine the networks of genes regulated by leptin in human breast cancer cells. We show that leptin influences numerous cancer-associated genes, the majority of which have not been identified as leptin regulated, and some of which have been previously reported. In general, we found that leptin regulates the greatest number of genes in the cell cycle/proliferation, apoptosis, and cell adhesion/ECM categories. We also demonstrate that leptin has a transient effect on most of the genes it regulates, a result verified via PCR time-course experiments. Potential mechanisms for the genes regulated at early-only time points may be a rapid increase in protein production leading to quick negative feedback on transcription. For late-only regulated genes, this may result from the downstream effects of leptin. In support of this, our recent report using proteomics approaches reveals that leptin regulates the levels of other cytokine growth factors in MCF-7 cells (Perera *et al.* 2008). At present, the mechanisms of leptin's transient regulatory effects are not known; however, the influence of leptin on proliferation and cell survival is becoming evident. Thus, further studies defining leptin's transcription regulatory processes should yield valuable clues into its role in tumorigenesis.

Leptin regulates expression of cell cycle and apoptosis genes

Prior *in vitro* studies show that leptin induces proliferation of cancerous mammary epithelial cells, including MCF-7 cells, and have focused on individual signaling molecules as mediators of leptin action (Dieudonne *et al.* 2002, Hu *et al.* 2002, Okumura *et al.* 2002, Somasundar *et al.* 2003). The present microarray work indicates that leptin impacts proliferation by affecting many genes, in particular through up-regulating cell cycle progression and suppressing cell cycle inhibitor genes. Cell cycle progression genes up-regulated by leptin included various cyclins. Expression of CDKNI, which are mediators of G1-specific CDK–cyclin complexes, was also regulated by leptin. *CDKN1A* (p21), which has anti-apoptotic properties and contributes to cell cycle progression, was induced by leptin (Weiss *et al.* 2003). Leptin substantially decreased the expression of *CDKN1B* (p27), a molecule well recognized as a tumor suppressor and whose degradation plays a pivotal role in cell cycle progression (Sherr & Roberts 1999). Leptin also up-regulated *CDKN2A* (p16) expression, a molecule that accumulates as cells age and whose overexpression is associated with more aggressive breast tumors (Milde-Langosch *et al.* 2001). Interestingly, leptin stimulated a 40-fold induction in metallothionein 3 (*MT3*) gene expression. Metallothioneins are low-molecular-weight metal-binding proteins associated with proliferation, are overexpressed during breast cancer, and serve as potential biomarkers for poor cancer prognosis (Sens *et al.* 2001, Bay *et al.* 2006).

Included as one of the hallmarks of cancer (Hanahan & Weinberg 2000), uncontrolled proliferation is achieved from an imbalance between cell cycle progression and apoptosis. This array analysis provides evidence on the involvement of leptin in promoting tumor cell viability by regulating genes that suppress apoptosis. More specifically, leptin induced the expression of anti-apoptotic gene *BCL2* (Martinez-Arribas *et al.* 2007) and reduced the expression of other apoptotic genes (TNF receptor associated factor (TRAF) interacting protein, *IGF1R*, and TNFRSF1A associated via death domain (*TRADD*)) involved in the tumor necrosis factor (TNF)-induced apoptotic pathway. Binding of TNF to its receptor leads to the recruitment of an intracellular death-inducing signaling complex (DISC), consisting of at least six different members that include *TRADD*, *TRAF1*, and *TRAF2* to cause apoptosis (Wajant *et al.* 2003). Additionally, leptin-stimulated expression of *survivin*, an inhibitor of apoptosis, is linked with radiation- and drug-resistant cancers (Xia *et al.* 2006, Zhang *et al.* 2006). These data suggest that, along with effecting expression of cell cycle components, leptin inhibits apoptosis and promotes cell survival through regulating several apoptosis-associated genes. This inhibitory effect of leptin in breast cancer cell apoptosis has not been previously reported and was verified via TUNEL assay. The early and sizeable induction of the anti-apoptotic genes, *survivin* and *BCL2*, which we verified by PCR, correlates very well with our TUNEL data where we demonstrate leptin significantly reduces apoptosis at 24 h but not at 6 h. This delay in leptin's anti-apoptotic effect likely stems from the time necessary for translational processes to produce sufficient protein levels for altering signaling dynamics. Taken together, the timing of increased *survivin* and *BCL2* levels with the effect of leptin on MCF-7 apoptosis suggests that these molecules are important mediators of leptin-induced cell survival.

Leptin regulates genes encoding DNA repair/synthesis and transcription

Although prior reports demonstrate leptin stimulates DNA synthesis and transcription in MCF-7 cells (Dieudonne *et al.* 2002, Okumura *et al.* 2002), the mechanisms involved in this process are not well understood. It has been shown that leptin activates the cell-signaling molecules STAT3, MAPK3/1, protein kinase B (Akt), glycogen synthase kinase 3 (GSK-3), PKC, and the transcription factor JUN during mammary epithelial cell proliferation (Dieudonne *et al.* 2002, Hu *et al.* 2002, Okumura *et al.* 2002, Catalano *et al.* 2003, 2004, Fruhbeck 2006). In accordance with these studies, some leptin-regulated signaling genes previously identified were also found via microarray. In addition, we reveal several other novel leptin-regulated genes related to DNA repair/synthesis and cell-

signaling molecules, including *LITAF*, *PURA*, and *TANK*. Identification of these genes begins to define additional signaling pathways for leptin-induced breast cancer cell proliferation.

Leptin regulates ECM and cytoskeleton genes

Overexpression of ECM is associated with cancer cell-invasive potential and metastatic tumors (Eckhardt *et al.* 2005, Tlsty & Coussens 2006). Leptin increases the expression of the ECM gene collagen in liver, mesangial, and trophoblast cells (Castellucci *et al.* 2000, Han *et al.* 2001, Saxena *et al.* 2003). Here, we observe that leptin up-regulates the expression of the ECM genes, *CTGF*, *villin 2*, and *basigin* in MCF-7 cells. *CTGF* is linked to breast cancer mortality and can contribute to tumor aggressiveness by serving as an angiogenic factor, stimulating ECM deposition and promoting breast cancer metastasis to bone (Kondo *et al.* 2002, Minn *et al.* 2005). *Villin 2*, also known as ezrin, is involved in cell adhesion, motility, and survival (Elliott *et al.* 2005). *Basigin*, an inducer of ECM metalloproteinase, promotes tumor growth, invasion, and breast cancer metastasis (Yang *et al.* 2006). Hence, the up-regulation of *CTGF*, *villin 2*, and *basigin* by leptin may represent mechanisms central to breast cancer progression and morbidity in obese patients. Favoring the idea that leptin leads to elevated ECM in breast cancer, our group has recently described that mammary tumors in obese rats have higher levels of collagen 1, when compared with tumors of lean rats. Collagen 1 content, quantified by CARS advanced imaging, was correlated with tumor aggressiveness, the predominant tumor phenotype in obese rats (Le *et al.* 2007). We have also shown that in MCF-7 cells leptin induces a substantial increase in collagens at the protein and mRNA levels (Perera *et al.* 2008 and unpublished data). A related study demonstrates that in mice bearing MCF-7 xenograft tumors, administration of leptin promotes tumor growth and stimulated E-cadherin, an adhesion molecule implicated in cell proliferation and survival (Mauro *et al.* 2007). Collectively, these *in vitro* and *in vivo* data suggest that leptin influences various tumor cell behaviors including adhesion, migration, and metastases through modulating the microenvironment and more importantly begins to uncover the mediators of these actions. Interestingly, our time course of microarray gene expression profile categories suggests that leptin promotes mammary tumor formation by initially inducing cell cycle/proliferation genes followed by regulating angiogenesis, cell adhesion, and the ECM.

Leptin regulates growth factor, cytokine, and receptor genes

Even though leptin itself is a cytokine, little is known regarding its influence on the production of other autocrine/paracrine factors. These studies show that in tumor cells leptin regulates the genes for other growth factors and receptors at the late time points evaluated. Leptin reduced the expression of *TGFB1* and *IGFBP2* genes, peptides known for their growth-inhibitory effects in mammary epithelial cells (Nam *et al.* 1997, Kaaks 2001, Pereira *et al.* 2004, Kibbey *et al.* 2006, Buck & Knabbe 2006, Frommer *et al.* 2006). Initial studies assigned dual and opposing roles for *TGFB1* in tumor cells, both as a growth suppressor and as a component of the cell invasion signal cascade. Toward clarifying this ambiguity, recent work conveys that the transition of *TGFB1* from tumor inhibitor to enhancer is estrogen receptor (ER) dependent, with *TGFB1* impeding growth in ER-positive tumor cells, such as MCF-7 (Buck & Knabbe 2006). Herein, we determine the functional significance of leptin's effect on *TGFB1* suppression by demonstrating that exogenous *TGFB1* completely abrogates leptin-induced MCF-7 cell proliferation. We also show that leptin significantly suppresses MCF-7 cell apoptosis. Based on previous studies showing that *TGFB1* promotes apoptosis in various mammary tumor cell lines (Tobin *et al.* 2001, Li *et al.* 2005, Souchelnytskyi 2005), it is highly likely that *TGFB1* regulation is an important mechanism in leptin's anti-apoptotic effects as well. Overall, these are the first studies to describe a mechanistic relationship between leptin, *TGFB1*, and breast cancer. The ability of leptin to reduce *IGFBP2* expression may contribute to breast tumorigenesis by two mechanisms. First, extracellular *IGFBP2* competes with cell surface IGF receptors for binding to IGF1, a potent mitogen in tumor cells (Kaaks 2001, Kibbey *et al.* 2006). Thus, lowered levels of antagonistic *IGFBP2*, which have been observed in obese subjects (Nam *et al.* 1997), may lead to greater availability of IGF1 to its receptor resulting in tumor cell proliferation. Second, *IGFBP2* suppression by leptin can support tumor progression, given that *IGFBP2* induces apoptosis and inhibits migration of breast cancer cells in a ligand-independent manner (Pereira *et al.* 2004, Frommer *et al.* 2006).

Here, we also demonstrate that leptin regulates expression of receptors and induces the *HMMR* gene and reduced erythropoietin receptor (*EPOR*). The presence of EPO receptors in breast cancer cells has only recently been established (Lester *et al.* 2005). Initial reports indicate that *EPOR* mediates an anti-apoptotic effect of EPO (Hardee *et al.* 2006); therefore, the reduction in *EPOR* expression by leptin was unexpected. Increased HMMR expression by leptin can contribute greatly to migratory potential, as the binding of hyaluronan (HA) to *HMMR* has been shown to be important for metastasis of breast cancer cells to lymph nodes (Bose & Masellis 2005). These data suggest that leptin promotes breast cancer progression through exploiting pathways of other growth factors and receptors.

Conclusion

Obesity is an epidemic and an established risk factor for breast cancer incidence and morbidity. As obesity is also associated with advanced breast cancer aggressiveness at diagnosis and tumor drug resistance (Carmichael 2006), a better understanding of the molecular links between obesity and cancer is essential. Leptin is emerging as an important link between

obesity and breast cancer (Surmacz 2007). Serum leptin levels are elevated in obese subjects and breast tumor leptin levels are associated with tumor grade (Garofalo & Surmacz 2006, Lorincz & Sukumar 2006). Herein, we show that leptin may influence various mammary tumor cell behaviors through a multitude of potential mechanisms. Initial studies of leptin in cancer focused on the stimulation of the tumor cell cycle and proliferation (Dieudonne *et al*. 2002, Hu *et al*. 2002, Okumura *et al*. 2002, Catalano *et al*. 2003, 2004, Somasundar *et al*. 2003). This work supports that leptin also contributes to other hallmarks of cancer (apoptosis, migration, and metastasis) by affecting the expression of genes for cell adhesion, ECM, cytoskeleton, other growth factors, and cytokine receptors. The identification of leptin-regulated genes begins to provide mechanistic links between leptin and tumor progression. This work gives new insight into understanding the relationship between obesity and breast cancer incidence and morbidity and substantiates that further studies of identified genes are warranted, as they represent valuable contributors to leptin action. Ultimately, the *in vitro* and *in vivo* molecular characterization of tumors exposed to elevated leptin will be important toward identifying novel potential targets and improving cancer therapy in the obese patient population.

Declaration of interest

Authors have no conflict of interest that would prejudice its impartiality.

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