Identification of Proteins Secreted from Leptin Stimulated MCF-7 Breast Cancer Cells: A Dual Proteomic Approach

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Leptin is an adipocyte-derived hormone that regulates energy expenditure and food intake. A significant role for leptin in breast cancer has also been indicated by the resistance of leptin knockout mice in development of mammary tumors. *In vitro*, leptin induces proliferation of MCF-7 cells by activating cellular signaling pathways (1, 11, 12, 16, 17, 56). As leptin is emerging as an important factor for tumor growth, and hormones can exert their actions via autocrine/paracrine mechanisms, we hypothesized leptin may act by regulating epithelial-derived proteins. To test this hypothesis, leptin-regulated proteins secreted from MCF-7 mammary tumor cells were identified using proteomics techniques. Treatment of MCF-7 cells with 500 ng/ml leptin for 24 hours resulted in a 40% increase in cell number and a 5-fold increase in protein secretion as compared to controls. Establishing the significance of leptin-induced secreted factors, the addition of conditioned media from leptin-treated MCF-7 cells to synchronized MCF-7 cells resulted in 40% increase in cell number. Identification of leptin-regulated secreted proteins was done by 2D gel electrophoresis coupled with MALDI-TOF mass spectrometry. Proteins identified using Pro Found software and NCBI database included KF10 Collagen Precursor, Serologically Defined Breast Cancer Antigen NY-BR-62 and Cortactin Isoform a. A Human Cytokine Antibody Array system was used to identify low abundant proteins in the media of control and 500 ng/ml leptin-stimulated MCF-7 cells. In leptin treated cells, levels of FGF-9 were increased while IGFBP-3 and TGF-β3 levels were decreased. Many previous studies have focused on the regulation of distinct cellular proteins by leptin during mammary tumor cell proliferation. However, ours is the first study to identify leptin-regulated secreted proteins, many of which are known to play important roles in cancer. Our data support that leptin can influence mammary tumor growth and progression through regulation of autocrine/paracrine factors and by modulating the extracellular matrix composition. Exp Biol Med 233:708–720, 2008

Key words: leptin; breast cancer; MCF-7; 2D gel electrophoresis; MALDI-TOF mass spectrometry; human cytokine array

Introduction

Obesity has become an epidemic in the United States and its impact on cardiovascular disease and diabetes is well recognized (1–2). More recently, obesity has become an established risk factor for postmenopausal breast cancer and is positively associated with breast cancer mortality (2–4). In rodents, high body weight is associated with increased incidence of spontaneous and chemically induced tumors (5). The underlying mechanisms involved in the relationship between obesity and breast cancer have not been delineated. A characteristic of obesity is increased circulating levels of the adipocyte-derived hormone leptin. Previous studies have revealed leptin is involved in regulation of body weight, energy expenditure, and has effects on the immune, cardiovascular and reproductive systems (2). Recent evidence indicates leptin also plays a significant role in normal mammary development and mammary tumor formation in mice (5, 9).

Initial studies suggested a role for leptin in mammary development by demonstrating serum leptin, in humans and rodents, which is elevated during late pregnancy, a time of intense mammary epithelial growth and proliferation (6). Subsequently, the expression of leptin and its receptor were detected in normal mammary tissue and in breast tumors (7, 8). The functional significance of these molecules was illustrated by the notable impairment of postnatal mammary gland development in leptin and leptin receptor deficient mice (5). Interestingly, in each of these models devoid of

This work was supported by a Showalter Trust Award 1320046936 and Purdue Cancer Center grant ACS IRG 58-006-47.

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Received October 13, 2007.
Accepted January 29, 2008.

DOI: 10.3181/0710-RM-281
1535-3702/08/2336-0708$15.00

leptin signaling, the incidence of spontaneous and oncogene-induced mammary tumors is significantly decreased (5, 9). More recent studies have shown a positive correlation between breast cancer progression and the expression of leptin and its receptor in tumor tissue (7, 8). Together, these evidences provide a strong foundation for the importance of leptin towards normal and cancerous mammary epithelial proliferation *in vivo*.

Various *in vitro* reports have demonstrated leptin regulates the proliferation of tumor cell lines from the prostate, pancreas, colon and ovary (1, 10, 15). These studies have begun to identify cellular signaling pathways mediating the actions of leptin in cancer. Towards determining the action of leptin on mammary tumor cells, several *in vitro* reports have shown leptin induces proliferation of the malignant human mammary epithelial cell lines MCF-7 and T47D (5, 10–12, 16, 17). In general, leptin, a 16 kDa protein, primarily acts on target cells by binding to its specific single transmembrane receptor ObR1 (13–15). Leptin activates a variety of signaling pathway molecules such as signal transducers and activators of transcription 3 (STAT3), extracellular regulated kinase 1/2 (ERK1/2), protein kinase B (Akt), glycogen synthase kinase 3 (GSK-3), protein kinase C (PKC) and the transcription factor AP-1 in MCF-7 and T47D cells (5, 13–17). Leptin also upregulates the expression of the cell cycle regulators, cyclin D1 and cyclin dependent kinase 2 (cdk2) in these cell lines (12). These initial observations have provided a valuable foundation of insights into the cellular signaling events of leptin-induced mammary tumor cell proliferation.

Thus, many studies on leptin and breast cancer have focused on cellular mitogenic signaling events. However, in addition to proliferation, leptin has been linked to invasion, angiogenesis and inhibition of apoptosis in other types of cells (1, 25, 32–34, 55). Each of these events contribute to tumor growth (35) and the mechanisms by which leptin regulates these processes in breast cancer have not been defined. As leptin is emerging as an important local growth factor in mammary tumor progression (1–4, 36), the goal of this study was to better understand the autocrine/paracrine influences of leptin towards mammary tumor growth. Towards this end, two proteomics techniques were employed to identify secreted proteins regulated by leptin in MCF-7 cells. In brief, we found leptin regulates the secretion of collagen precursors and various other growth factors that have important roles in tumor growth. These results suggest that, in addition to activating mitogenic cell signaling, leptin promotes mammary tumor growth by modifying the extracellular matrix and regulating epithelia-derived autocrine/paracrine growth factors. This provides new insight into the role of leptin in mammary tumor progression and identifies novel potential mechanisms involved in the relationship between obesity and breast cancer morbidity.

Materials and Methods

Cell Culture, Hormone Treatment and Proliferation Assay. MCF-7 human breast cancer epithelial cells used in this study were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained routinely in RPMI 1640 media (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum (ATCC, Manassas, VA), 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 & Peptide Program (Harbor-UCLA Medical Center, CA). To examine the effect of leptin on MCF-7 cell proliferation, cells were initially seeded on 24-well plates at approximately 4×10^4 cells/well in 1 ml of RPMI 1640 containing 10% fetal bovine serum. After 48 hours, subconfluent cells were synchronized by serum starving for 24 hours in RPMI media. Cells were then incubated in media, in the absence or presence of varying concentrations of leptin at 10 ng/ml, 100 ng/ml and 500 ng/ml. Twenty-four hours after leptin treatment, media was collected for secreted protein assays and total viable cell counts were determined using the hemocytometer. The number of cells present in the control (0 ng/ml leptin) was designated 100% and the changes in cell number in leptin treated samples were expressed as percentages relative to control. To maintain cell viability, 0.1% bovine serum albumin (BSA) (Bio-Rad Laboratories, CA) was added to media of cells treated with or without leptin.

Flow Cytometry. MCF-7 cells were grown in RPMI media containing 10% fetal bovine serum for 48 hours, synchronized using serum starvation in RPMI media for 24 hours and treated with different leptin concentrations (0, 10, 100 and 500 ng/ml). After 24 hours cells were trypsinized, washed with phosphate buffer saline (PBS) and fixed in 70% ethanol overnight at 4°C. Fixed cells were washed twice with PBS and stained with 500 µl of propidium iodide (50 µg/ml). Samples were analyzed for DNA ploidy using a Coulter Epics XL-MCL Cytometer Fluorescence Activated Cell Sorter (FACS) (Beckman Coulter, Inc.).

Treatment of MCF-7 Cells with Conditioned Media. Cells were seeded on 24-well plates at approximately 4×10^4 cells/well in 1 ml of RPMI media containing 10% fetal bovine serum for 48 hours, serum starved 24 hours in RPMI media to synchronize cells and then treated with control (0 ng/ml leptin) or 500 ng/ml leptin. Conditioned media from MCF-7 cells treated with or without leptin for 24 hours was collected. The collected media was transferred to different sets of cells (4×10^4 cells/well) grown in a 24-well plate for 48 hours and synchronized. After 24 hours, proliferation was measured in cells incubated with conditioned media. In a separate experiment, conditioned media from leptin treated and control cells was collected after 24 hours and incubated with excess amounts (2000 ng/ml) of leptin antibody (Bio Vision, Mountain View, CA), to inhibit the action of any

residual amounts of leptin present in the media. The conditioned media containing neutralized leptin was transferred to a second set of serum starved MCF-7 cells and cell counts were determined after 24 hours. To confirm the inhibition of action of residual leptin present in the media by anti leptin antibody, media containing pre-incubated conjugate of anti leptin antibody (2000 ug/ml) and 500 ng/ml leptin was transferred to synchronized MCF-7 cells and cell counts were determined after 24 hours. For each treatment the number of cells in the control sample was designated as 100% and the change in cell numbers was expressed as a percent relative to control.

Isolation of Secreted Proteins from MCF-7 Cells. MCF-7 cells were grown, synchronized by serum starvation and treated with different concentrations of leptin as described above. Twenty-four hours after leptin treatment, media was collected and filtered through a Nalgene 0.45 μm pore size syringe filter to remove debris and dead cells. Collected media was then dialyzed using a Slide-A-Lyzer 3.5K Dialysis Cassette (Pierce, Rockford, IL) and lyophilized using a Labconco Free Zone Plus 2.5 freeze dryer (Kansas City, MO). Resulting protein crystals were suspended in solubilization buffer containing 8 M urea, 4% CHAPS, 40 mM Tris and 0.2% Bio-Lyte 3–10 (Bio-Rad Laboratories, CA). The total protein concentration in media from each sample was measured using the BCA protein assay Kit (Pierce, Rockford, IL). Amount of protein secreted per cell was calculated by dividing the measured total protein concentration by the corresponding cell number (from proliferation assay). When calculating protein concentrations, we accounted for the amount of leptin added and BSA proteins present in RPMI-1640 media.

2D Gel Electrophoresis of Secreted Proteins. Equal amount of secreted proteins (150 μg) from control (without leptin) and 500 ng/ml leptin treated samples were rehydrated in 185 μl of rehydration buffer overnight (Bio-Rad Laboratories, CA). Samples were loaded on first dimension strips (11 cm, pI 3–10 NL IPG). The first dimension of isoelectric focusing was performed using a Protein IEF cell (Bio-Rad Laboratories, CA). After running, first dimension gels were equilibrated for 10 min in equilibration buffer I (6 M Urea, 0.375 M Tris pH 8.8, 2% SDS, 20% glycerol, 2% (W/V) DTT) followed by 10 min in equilibration buffer II (6 M urea, 0.375 M Tris pH 8.8, 2% SDS, 20% glycerol, 2.5% (W/V) iodoacetamide). Samples were then separated by second dimension on precast 4–16% SDS-PAGE gels (Bio-Rad Laboratories, CA). Gels were stained with SYPRO Ruby protein stain and images were acquired and analyzed using PDQuest software (Bio-Rad Laboratories, CA). The control (without leptin) and 500 ng/ml leptin treated protein sample were passed through an albumin column (Vivascience, Hannover, Germany) to remove albumin and to concentrate low abundant protein spots. This step was essential for obtaining sufficient amounts of protein for mass spectrometry analysis.

"In Gel" Digestion of Proteins. Selected spots from the gel were excised using a Proteome Works TM Spot Cutter (Bio-Rad Laboratories, CA) and transferred to a 96-well plate. The proteins were enzymatically digested and the tryptic peptides ZipTip purified. After ZipTip purification, the tryptic peptides were eluted from the ZipTip with 2 mg/ml cyano-4-hydroxycinnamic acid (CHCA) solution in 60% ACN/0.2% formic acid and spotted directly onto wax-coated matrix-assisted laser desorption/ionization (MALDI) target plates. Digestion and spotting steps were performed automatically by the ProPrep Investigator (Genomics Solutions, Ann Arbor, MI).

Mass Spectrometry & Database Search. The tryptic peptides on the MALDI target plate were analyzed with a Voyager-DE Pro MALDI-time of flight mass spectrometer (Applied Biosystems, Framingham, MA). Mass spectra were recorded in the positive-ion, delayed-extraction (DE) mode. All spectra were acquired with 20 kV accelerating voltage, 72% grid voltage, 1.12 mirror voltage ratio, 0.005% guide wire, 120 nsec delayed extraction time, and 500 Da low-mass gate. All spectra were checked for accurate masses, trypsin autodigestion peptides and matrix peaks. If not, the spectra were internally mass-calibrated with the protonated molecular ions $(M + H)+$ of trypsin autodigestion peptides (m/z 515.33, 842.51, and 2211.10), and matrix peak (m/z 568.12). Matching of the experimental tryptic peptide mass with the in-silico derived tryptic peptide masses was performed using the Pro Found search engine (Version 4.10.5, The Rockefeller University Edition). The NCBI database was searched within a mass tolerance of $\pm$ 100 ppm for the appropriate species proteins; with one missed cleavage allowed. Alkylation of a cysteine residue and the oxidation of methionine are considered modifications. Proteins were evaluated by considering the number of matched tryptic peptides, the percentage coverage of the entire protein sequence, the apparent MW, and the pI of the protein. The probability that a candidate in a database search is the protein analyzed is expressed by a Z score in the Pro Found search engine. Z score corresponds to the percentile of the search in the random match population. Z score of 1.65 for a search indicates the search is in the 95th percentile. The following is a list for Z scores and corresponding percentiles: Z of $1.282 = 90.0$ percentile, Z of $1.645 = 95.0$ percentile, Z of $2.326 = 99.0$ percentile and a Z of $3.090 = 99.9$ percentile. The secreted proteins identified by this method had a Z score of at least 1.3.

Human Cytokine Arrays. MCF-7 cells were grown in a 24-well plate for 48 hours, synchronized by serum starvation for 24 hours and treated with 0 ng/ml leptin (control) or 500 ng/ml leptin. After 24 hours, media was collected and analyzed for multiple secreted cytokines using human cytokine arrays, RayBioTM Cytokine Antibody Arrays V, purchased from RayBiotech, Inc. (Atlanta, GA). Each array was incubated with 1 ml of leptin treated or control conditioned media at 4°C overnight and bound cytokines were detected by enhanced chemiluminesence

Table 1. Identification of Leptin Induced MCF-7 Proteins by MALDI-TOF Mass Spectroscopy[a]

Spot symbol	Protein name	MW (KDa)	PI	Accession number	Z score (profound)	Number of matched peptides	% of sequence coverage
a	Collagen precursor (KF10-human protein KIAA1510)	141.1	7.9	Q9P218	2.43	7	7
b	Albumin	71.3	5.8	AAN17824.1	2.29	14	22
c	Cortactin isoform a; oncogene EMS1 (mammary tumor & cell carcinoma associated protein)	61.7	5.2	NP005222	2.43	12	11
d	Truncated Cdc42 interaction protein 4 [Homo sapiens]	52.8	6.4	AAL89588.1	1.49	8	22
e	Serologically defined breast cancer antigen NY-BR-62	36.9	5.5	AAG48261.1	1.32	27	10
f	Protein-tyrosine kinase FLT3 (fms homolog)–human (fragment)	18.7	6.3	NP004110	2.06	7	29

[a] Proteins secreted from leptin treated MCF-7 cells were visualized by 2 D gel electrophoresis. Detected protein spots a–h (Fig. 5B) were cut from the gel, trypsin digested and analyzed by MALDI-TOF mass spectrometry. For identification, the trypsin digest profile, isoelectric point, and molecular weight of each protein were used in conjunction with Pro Found software and NCBI databases.

according to the manufacturer's instructions. The relative signal intensity of cytokines in leptin treated and control samples were quantified using a GS-800 densitometer (Bio-Rad Laboratories, CA). This experiment was repeated three times and average cytokine spot intensities were calculated with signal intensity of internal positive controls used to normalize values between membranes. Fold differences of cytokine levels are expressed as a ratio of leptin treated; control treated spot intensities. Only spots having a 2-fold difference or greater are shown in Table 2.

Real Time PCR. Leptin regulated molecules of interest, identified using proteomic techniques, were further investigated for the change in gene expression using real time PCR. As described previously, RNA was isolated from MCF-7 cells that were synchronized using serum starvation and treated with 0 ng/ml (control) or 500 ng/ml leptin for 24 hours. One microgram of total RNA was reverse transcribed with M-MLV-reverse transcriptase using random hexamers (Promega, Madison WI) 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) in a final reaction volume of 30 μl. An ABI PRISM 7700 Sequence Detection System Instrument (Applied Biosystems) was used for 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 sample (not treated with leptin). Fold change was calculated using the difference between cycle threshold (Ct) value of the gene and control gene (β-actin) using the formula $2 - \Delta CtA - CtB$ (Comparative CT Method/ΔΔCT). PCR was performed using specific primers. For collagen IV, 5′-TAC ATT GGC CTA CAT CCT TGC CCT was used as forward primer and 5′-AAT TGG CAT CTC CGC CTA CAT CCT was used as reverse primer. For TGF-β3, 5′-TGT TGT AAA GGG CCA GGA CCT GAT was used as the forward primer and 5′-TGG ACT TCG GCC ACA TCA AGA AGA as the reverse primer. For FGF-9, 5′-TGG AGG CTT GGA TGG GAA TAT GCT was used as the forward primer and 5′-ACA CAC ACA CAC ACA CAC ACA CAC was used as the reverse primer. 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

Table 2. Fold Difference of Leptin Regulated Cytokines Identified from Cytokine Array[a]

Protein name	Symbol	Position in the chip	Fold difference
Fibroblast growth factor 9	FGF-9	D-6	5.60
Tumor necrosis factor-β	TNF-β	H-4	3.04
Macrophage colony stimulating factor	MCSF	H-3	2.27
Insulin-like growth factor binding protein-3	IGFBP-3	A-7	0.34
Transforming growth factor-β 3	TGF-β 3	F-8	0.12

[a] Leptin regulates various cytokines secreted from MCF-7 cells. The relative levels of secreted cytokines from control (0 ng/ml) and 500 ng/ml leptin treated MCF-7 cells was analyzed by cytokine array. The detected spots were quantitated using a GS-800 laser densitometer. Experiments were repeated three times and average spot intensities were calculated. Fold differences are expressed as a ratio of leptin treated; control treated spot intensities ± SEM. Only spots having a 2-fold difference or greater are shown.

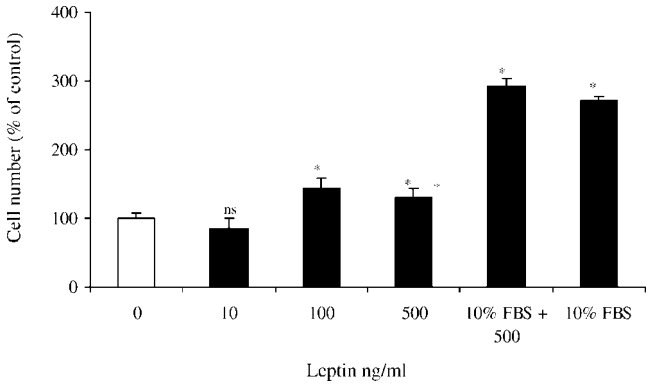

Figure 1. Effects of leptin on MCF-7 cell proliferation. MCF-7 cells were grown in a 24 well plate with media containing 10% fetal bovine serum (FBS) for 2 days and synchronized for 24 hours in serum free media. Cells were then incubated with 0, 10, 100, 500 ng/ml of leptin in serum free media or 500 ng/ml leptin in 10% FBS or 10% FBS only in media (controls) for 24 hours and counted by hemocytometer. Results are means ($n = 5$–6) ± SEM from three experiments and are normalized as percentages of the control values (without leptin). * = $P < 0.05$, ns = non significant.

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.

Statistics. Values from MCF-7 proliferation, protein secretion, conditioned media/proliferation, cytokine assays and real time PCR were expressed as means ± SEM from at least 3 different experiments (each with $n = 3$–6). Statistical analysis was performed using ANOVA and Student's t test using Microsoft Excel or Stat-view 5.0 software (SAS Institute Inc., Cary, NC). A value of $P < 0.05$ was considered statistically significant.

Results

Leptin Stimulates MCF-7 Cell Proliferation and Cell Cycle. MCF-7 proliferation was evaluated using physiologically relevant concentrations of leptin. Low leptin concentrations representing lean individuals (10 ng/ml) and high leptin concentrations representing obese or pregnant individuals (100–500 ng/ml) were used in the study (5, 6, 10, 13). As shown in Figure 1, leptin, *in vitro*, induced a significant increase in cell number at higher concentrations. Treatment of MCF-7 cells with 100 ng/ml or 500 ng/ml leptin for 24 hours induced an approximate 40% increase in cell numbers, compared to controls. Interestingly, we also found that at 10 ng/ml, leptin had an inhibitory effect on MCF-7 proliferation. To confirm cell count observations and determine the effect of leptin on cell cycle, MCF-7 cells treated with leptin for 24 hours were analyzed by FACS (Fig. 2). This analysis demonstrated treatment of MCF-7 cells with 500 ng/ml leptin resulted in a greater proportion of cells (29%) entering the S phase of the cell cycle, as compared to controls (16%) not treated with leptin. These two experiments established leptin induces MCF-7 cell proliferation. Based on this data and conditions employed, 500 ng/ml leptin was used to stimulate MCF-7 cells in subsequent conditioned media, 2D gel and protein array experiments.

Leptin Induces Protein Secretion in MCF-7 Cells. To explore the possibility that leptin acts through autocrine/paracrine mechanisms to influence tumor cell growth, we sought to identify secreted proteins regulated by leptin in MCF-7 cells. Towards this, the effect of leptin on total protein secretion in MCF-7 cells was measured. The range of leptin concentrations employed in Figures 1–2 were also used in these experiments. As shown in Figure 3, the amount of total proteins secreted per cell increased with higher concentrations of leptin. Treatment of MCF-7 cells with 100 ng/ml or 500 ng/ml leptin for 24 hours induced a significant 2-fold and 5-fold increase in protein secretion ($P < 0.0001$), as compared to controls (0 ng/ml leptin), respectively.

Leptin-Regulated Secreted Proteins Induce MCF-7 Proliferation. Having shown leptin induces proliferation and protein secretion in MCF-7 cells, we next determined the functional significance of these leptin-induced secreted factors (10–12). To do this, the conditioned media from leptin and control treated MCF-7 cells was collected and transferred to subsequent sets of synchronized MCF-7 cells. In these experiments, 500 ng/ml leptin was used, a concentration demonstrated to stimulate proliferation and protein secretion in Figures 1–3. After 24 hours cells incubated with conditioned media were counted. As shown in Figure 4, treatment of MCF-7 cells with 500 ng/ml leptin induces a 40% increase in cell number, as compared to non-leptin treated cells (similar to the effect demonstrated in Fig. 1). As an additional control, MCF-7 cells were incubated with media containing pre-incubated conjugate of function-blocking anti-leptin antibody plus 500 ng/ml leptin. This control indicates the antibody employed was able to completely block the proliferative action of leptin. Incubation of synchronized MCF-7 cells with conditioned media from 500 ng/ml leptin treated cells induced a 53% increase in cell number, compared to conditioned media control. Parallel with this experiment, the possible proliferative influence of residual leptin, present in conditioned media, towards proliferation was tested. To determine this, synchronized MCF-7 cell were incubated with conditioned media (from cells treated with 0 or 500 ng/ml leptin) that contained anti-leptin antibody and cell counts were measured (described in Materials and Methods). Incubation of synchronized cells with conditioned media from leptin treated cells containing anti-leptin antibody lead to a 50% increase in cell number (Fig. 4). As we demonstrated the leptin blocking capacity of the antibody used, this data supports that leptin-regulated secreted factors can act in an autocrine/paracrine manner and that they may serve as valuable mediators of leptin stimulated tumor cell growth.

Identification of MCF-7 Secreted Proteins Using 2D Gels and Mass Spectroscopy. To identify leptin-regulated secreted proteins, the conditioned media from

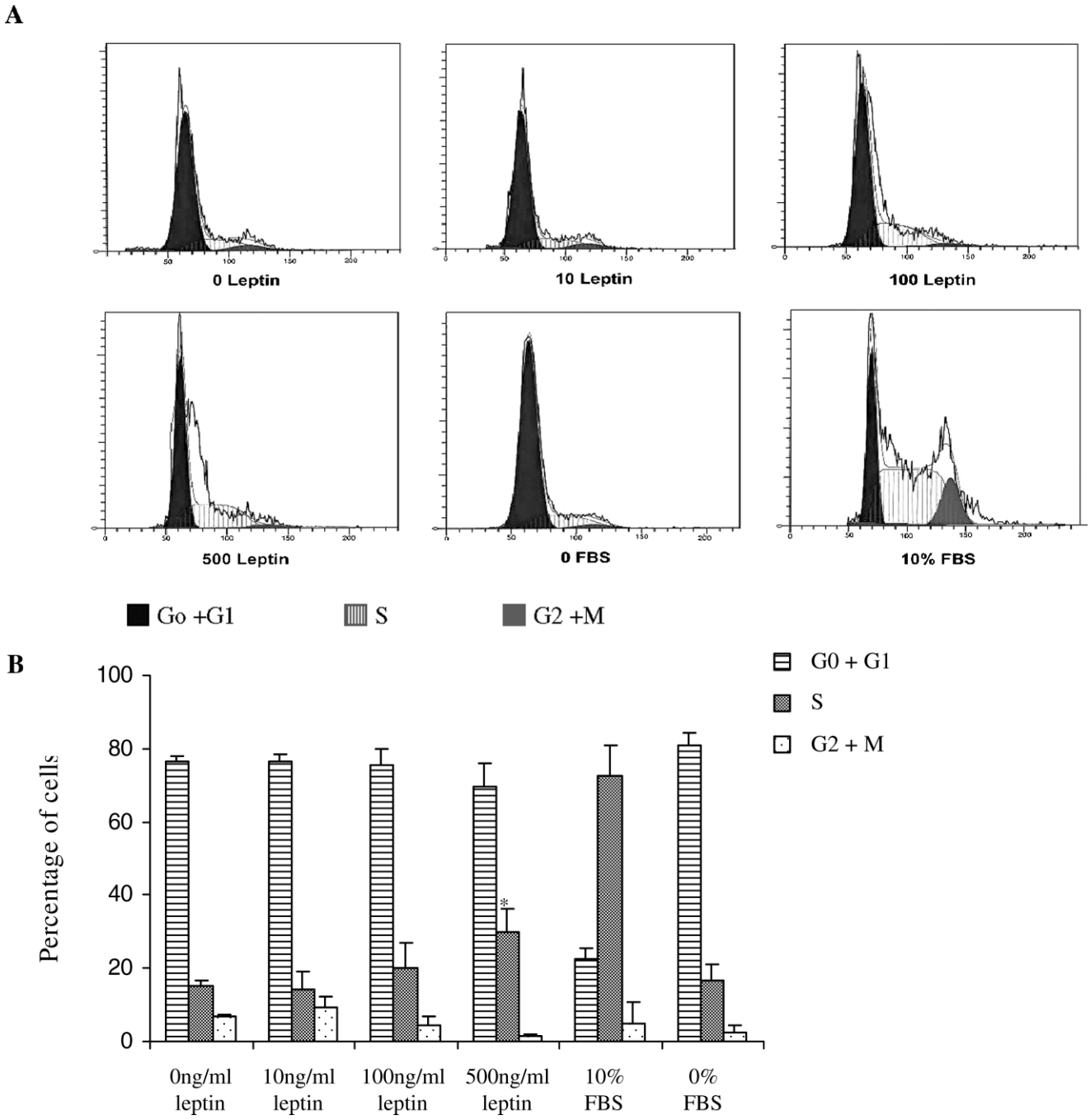

Figure 2. Effect of leptin on the cell cycle of MCF-7 cells determined by Fluorescence Activated Cell Sorting (FACS) Analysis. (A) MCF-7 cells were grown in 10% fetal bovine serum in RPMI media for 2 days in a 75-cm^2 flask. Cells were then synchronized by serum starvation for 24 hours and treated with increasing concentrations of leptin in serum free media or in the presence or absence of 10% fetal bovine serum (FBS) for 24 hours. FBS control treated cells did not contain leptin. Cells were collected, fixed and stained with propidium iodide and subjected to FACS. Results are means ($n = 3$) ± SEM from three experiments. (B) Transformed data indicate the percentage of cells in each phase of the cell cycle. The number of cells in the S phase was significantly different compared to the controls (* = $P < 0.05$).

MCF-7 cells was analyzed via 2-dimensional gel electrophoresis. As maximum protein secretion was observed in cells treated with 500 ng/ml leptin, secreted proteins from this treatment were compared with non-leptin treated control samples. Prior to 2D gel analysis, media from control or leptin-stimulated cells was lyophilized, resuspended in solubilization buffer and quantified by BCA assay. Secreted proteins (150 μg) were separated with 2D gels, stained with SYPRO Ruby protein and images were analyzed using PDQuest software (Bio-Rad Laboratories, CA). As shown in Figure 5B, leptin regulated the secretion of more than 20 different proteins from MCF-7 cells.

To identify leptin-regulated proteins, the most highly abundant spots were cut from gels containing secreted

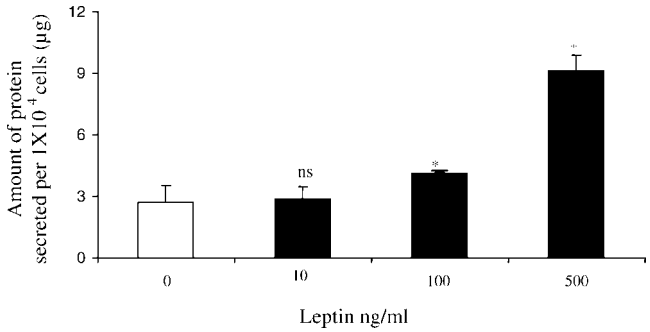

Figure 3. Effect of leptin on MCF-7 cell protein secretion. 4×10^4 cells per well were grown in a 24 well plate with media containing 10% fetal bovine serum for 2 days, synchronized using serum free media for 24 hours and treated with 0, 10, 100, or 500 ng/ml of leptin. After 24 hours, media was collected, dialyzed, lyophilized and quantified by BCA protein assay. Total numbers of cells were counted by hemocytometer. Results are means ($n = 5$–6) ± SEM from three experiments * = $P < 0.001$, ns = non significant.

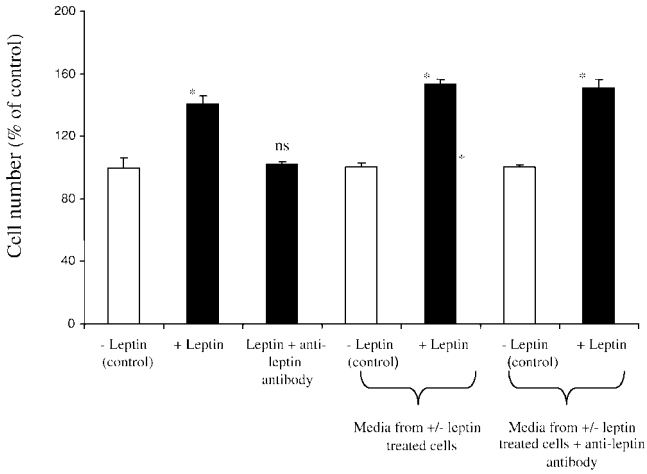

Figure 4. Leptin-induced secreted factors induce MCF-7 cell proliferation. MCF-7 cells were grown in a 24 well plate and synchronized by serum starvation for 24 hours. Demonstrating the effect of leptin, synchronized cells were incubated with either 0 ng/ml Leptin (control), 500 ng/ml leptin or pre-incubated conjugate of 500 ng/ml leptin + anti-leptin antibody (2000 µg/ml). Determining the significance of secreted proteins on proliferation, synchronized MCF-7 cells were incubated with conditioned media from MCF-7 cells treated with 0 ng/ml leptin or 500 ng/ml leptin. As a control for residual leptin, synchronized MCF-7 cells were incubated with conditioned media (containing anti-leptin antibody) from cells treated with 0 ng/ml leptin or 500 ng/ml leptin. In this case conditioned media from control and leptin treated cells was collected after 24 hours, incubated with anti-leptin antibody (2000 µg/ml) and then used to treat synchronized cells. For all experiments above, cell counts were determined after 24 hours of treatment. The number of cells in the control sample was designated as 100% and change in cell number is expressed as a percent relative to control. Results are mean ($n =$ 3–5) ± SEM of three experiments. * = $P < 0.05$.

proteins of leptin treated cells (Fig. 5B, spots a–h). Spots were trypsin digested and analyzed by MALDI-TOF mass spectrometry. For identification, the trypsin digest profile, isoelectric point, and molecular weight of each protein were used in conjunction with Pro Found software and NCBI databases. Table 1 summarizes mass spectrometry data and includes the name, accession number and Z score for spots a–h. For each protein identified, the Z score was at least 1.3, meaning the search in the random match population is at least in the 90th percentile. Briefly, leptin was found to regulate the secretion of the following proteins from MCF-7 cells: KF10-Human Protein KIAA1510 (a collagen precursor), Cortactin isoform a (involved in cell adhesion), protein-tyrosine kinase FLT3 and serologically defined breast cancer antigen NY-BR-62.

Identification of Low Abundant MCF-7 Secreted Proteins Using Cytokine Arrays. One drawback of using 2D gel methods is dye sensitivity that limits detection of low abundant proteins. To circumvent this problem, and to identify additional leptin-regulated secreted MCF-7 proteins, media from control (0 ng/ml leptin) and 500 ng/ml leptin treated cells was analyzed using an antibody based cytokine membrane array (described in Materials and Methods). Figure 6A provides the template, representing the position of each antibody, for the membrane array that collectively can detect more than 80 different cytokines. Antibody-antigen interactions were visualized by enhanced chemiluminesence (Fig. 6B). For each membrane array, relative signal intensities were quantified using laser densitometry. The fold difference values presented in Table 2 were calculated based on the ratio of signal intensities from corresponding spots on the 500 ng/ml leptin to 0 ng/ml leptin control membranes. These experiments demonstrate that levels of secreted fibroblast growth factor-9 (FGF-9), macrophage colony stimulating factor (MCSF) and tumor necrosis factor-beta (TNF-β) were all increased from MCF-7 cells treated with 500 ng/ml leptin, as compared to controls. Conversely, treatment of MCF-7 cells with leptin lead to a decrease in the media levels of transforming growth factor-beta 3 (TGF-β3) and insulin-like growth factor binding protein-3 (IGFBP-3), compared to controls.

Real Time PCR Analysis of Leptin Regulated Collagen IV, TGF-β3 and FGF-9 Expression. The identification of collagen precursor via 2D gel and mass spectroscopy prompted us to study the effect of leptin on collagen IV expression, a collagen associated with mammary tumor progression (26, 38). Protein chip array data revealed leptin increases levels of secreted FGF-9 (5.6-fold) and decreased that of TGF-β3 (0.12-fold) in MCF-7 cells. To support these data the effect of leptin on expression of these genes was evaluated by real time PCR. Leptin induced a 4-fold increase in Collagen IV and 6-fold increase in FGF-9 mRNA levels, compared to control (Fig. 7A and 7B, respectively). Leptin decreased by approximately 5-fold (0.2-fold less) the mRNA levels of TGF-β3, a proliferation inhibitor, compared to control (Fig. 7C). These mRNA data are in strong accordance with the corresponding protein data from mass spectrometry and cytokine membrane array studies.

Discussion

Recent epidemiological evidences have established obesity as a risk factor for breast cancer incidence

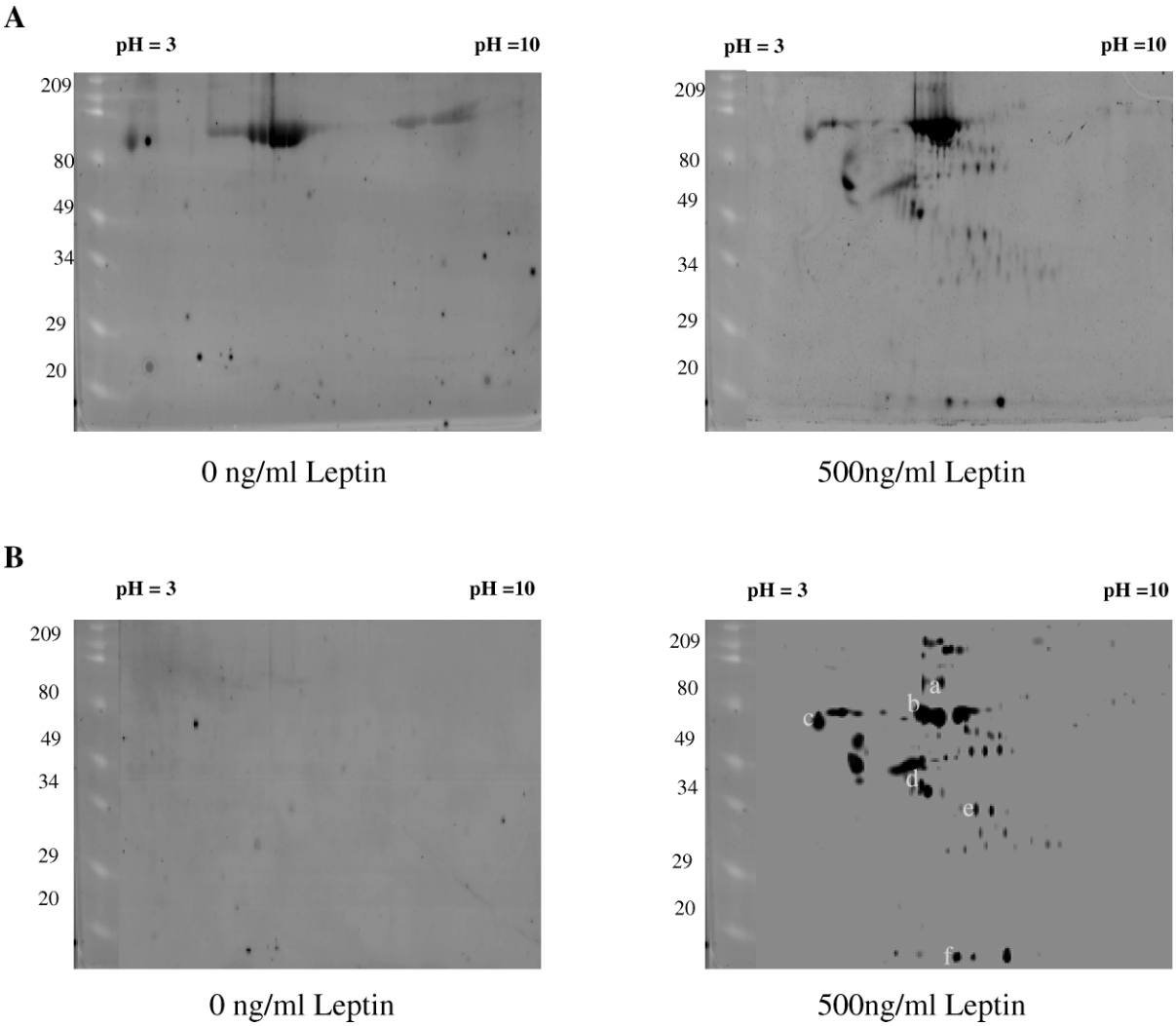

Figure 5. Analysis of leptin induced MCF-7 secreted proteins using 2D gel electrophoresis. (A) 2D gel profile for leptin-induced secreted proteins before albumin removal. Secreted proteins (200 μg) from MCF-7 cells, treated without leptin (control) and 500 ng/ml leptin were rehydrated onto 11 cm, pH 3–10 NL IPG strips. The first dimension of isoelectric focusing was performed using a Protein IEF cell (Bio-Rad). Samples were then separated on precast SDS-PAGE 4–16% gels, and stained with SYPRO Ruby protein stain. Images were acquired and analyzed using PDQuest software (Bio-Rad). (B) 2D gel profile of leptin treated samples after passage through an albumin column. A fraction from the samples used in panel A above were passed through an albumin column and requantified using BCA assay. Equal amounts of proteins (150 μg) were run on 2D gels. Letters a–f represent spots picked for MS. Gels are representative of three separate experiments. Molecular weight (M wt) markers are shown on left side of each gel.

(postmenopausal) and morbidity (1–4, 22). Towards defining mechanistic links in this relationship, there is considerable interest in the role of the adipocyte hormone leptin (37). Circulating levels of leptin are elevated during obesity (1–4). Additionally, leptin and its receptor are present in normal mammary tissue and are upregulated in breast tumors (7, 8, 36). Obese leptin knockout mice exhibit an impairment of postnatal mammary development and their incidence of spontaneous and oncogene-induced mammary tumors is significantly decreased (5, 9). In support of these *in vivo* studies, leptin has been shown to stimulate proliferation of normal and cancerous mammary epithelial cells *in vitro*. Collectively, this body of work suggests leptin is an important regulator of mammary tumor growth.

Considering the potential importance of leptin as a local proliferative growth factor, and the influence of leptin on cell migration and apoptosis in other cell types (1, 2, 25, 32, 33), the focus of this study was to identify autocrine/paracrine factors that can mediate the effects of leptin during breast tumor growth and progression. Towards this end, our first experiment (Fig. 1) demonstrates leptin, at concentrations reflecting elevated physiological levels, stimulates MCF-7 cell proliferation with a degree of effect similar to several other studies (10–12, 17). Supporting this initial data, in Figure 2 we employ FACS analysis to show high leptin levels stimulate MCF-7 cells to enter the DNA synthesis phase, an indicator of cell cycle progression. Together, these 2 experiments establish the concentrations of leptin that stimulate tumor proliferation, providing a foundation for our subsequent studies. In Figure 3, using

A

	A	B	C	D	E	F	G	H	I	J	K
1	Pos	Pos	Pos	Pos	Neg	Neg	ENA-78	GCSF	GM-CSF	GRO	GRO-
2	I-309	IL-1	IL-1ß	IL-2	IL-3	I1-4	IL-5	I1-6	IL-7	I1-8	IL-10
3	I1-12	I1-13	IL-15	IFN-	MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1 ß
4	MIP-1	RANTES	SCF	SDF-1	TARC	TGF-ß1	TNF-	TNF-ß	EGF	IGF-1	Ang
5	OSM	TPO	VEGF	PDGF-ß	Leptin	BDNF	BLC	Ck ß 8-1	Eotaxin	Eotaxin-2	Eotaxin-3
6	FGF-4	FGF-6	FGF-7	FGF-9	FLt-3 ligand	Fractalkine	GCP-2	GDNF	HGF	IGFBP-1	IGFBP-2
7	IGFBP-3	IGFBP-4	IL-16	IP-10	LIF	LIGHT	MCP-4	MIF	MIP-3	NAP-2	NT-3
8	NT-4	Osteo-protegerin	PARC	PIGF	TGF-ß2	TGF-ß3	TIMP-1	TIMP-2	Neg	Pos	Pos

B

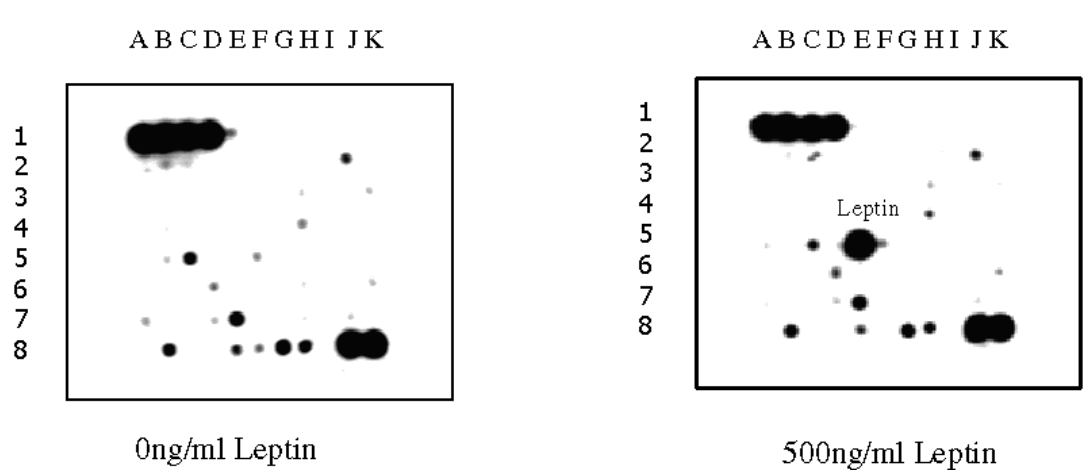

Figure 6. Use of cytokine arrays to identify leptin-regulated low abundant proteins secreted from MCF-7 cells. (A) An array template indicating positions of antibody probes for detectable cytokines. (B) Analysis of conditioned media from leptin and control treated cells using Human Cytokine arrays (RayBiotech, Inc). Briefly, MCF-7 cells were grown in media containing 10% fetal bovine serum for 48 hours, serum starved for 24 hours and incubated with media containing 0 and 500 ng/ml leptin. After 24 hours, media (1 ml) from each treatment was incubated with a cytokine array membrane. Differentially regulated cytokines were detected according to the manufacturer's instructions. Briefly the relative levels of secreted cytokines from control (0 ng/ml) and 500 ng/ml leptin treated MCF-7 cells were quantitated using a GS-800 laser densitometer and average spot intensities were calculated. Fold differences were expressed as a ratio of leptin treated; control treated spot intensities (Table 2). Experiments were repeated three times and only spots having a 2-fold difference or greater are shown.

leptin concentrations that induce proliferation, we reveal for the first time leptin stimulates a substantial amount of protein secretion from MCF-7 cells. These observations support our hypothesis that leptin may stimulate tumor cell proliferation by regulating secretion of autocrine/paracrine factors. In Figure 4 we establish the functional significance of leptin-regulated secreted proteins by demonstrating that conditioned media from leptin stimulated MCF-7 cells can induce proliferation of growth arrested MCF-7s. The concentration of leptin used in conditioned media study (500 ng/ml) was based on Figures 1–3 data and was used for all subsequent experiments. Upon establishing the functional significance of leptin-regulated secreted proteins, our next goal was to identify these factors using two proteomics techniques: 2D gel coupled with mass spectrometry and antibody-based protein arrays. To date there has not been an effort to determine leptin-regulated tumor cell secreted factors using a global approach.

Using 2D gel and mass spectrometry methods several highly abundant leptin regulated secreted proteins were identified. Many proteins identified here are known to be secreted and included a collagen precursor, cortactin isoform a and serologically defined breast cancer antigen NY-BR-62. Interestingly, some proteins identified by these

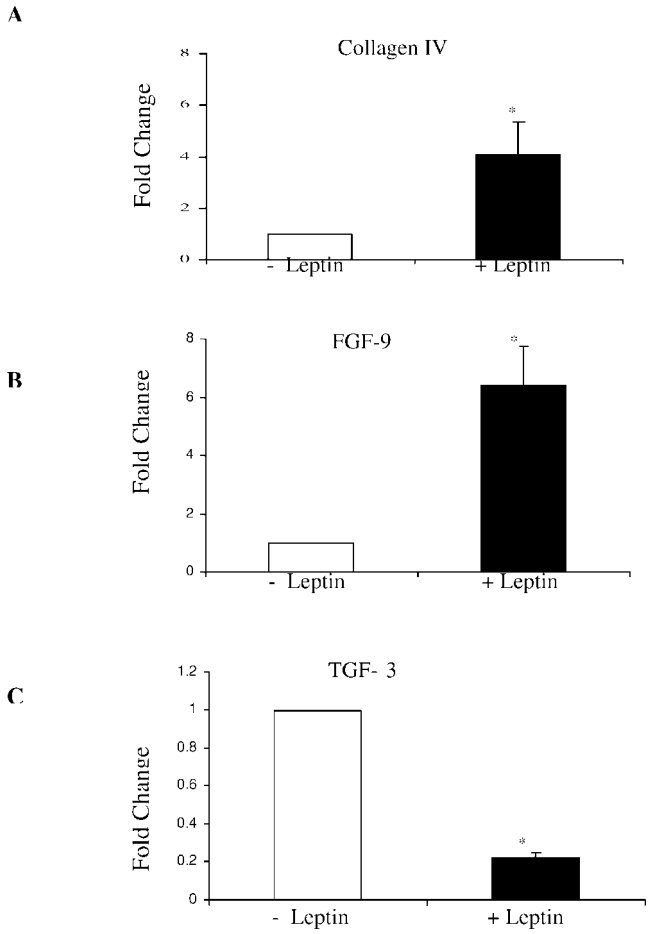

Figure 7. Leptin regulates the mRNA expression of collagen IV, TGF-β3 and FGF-9 in MCF-7 cells**.** Expression levels of leptin regulated proteins (A) Collagen IV, (B) FGF-9 and (C) TGF-β3, identified by proteomic analysis, were validated using real time PCR. The expression level of each gene was measured with β-actin as an internal control. For each gene, expression levels under 0 ng/ml leptin treatment or "– Leptin" served as a baseline. The fold change in mRNA levels resulting from 500 ng/ml leptin or "+ Leptin" were calculated relative to non-leptin treated baseline samples. Data are results from at least three different experiments, each with $n = 3$–5, ± SEM. * = $P < 0.05$.

methods are commonly found intracellularly and membrane-associated, such as FLT3 Kinase. These proteins detected could represent alternate secretable isoforms of these molecules. Alternatively, detection of intracellular proteins outside tumor cells, which is not unusual, could result from the protein's increased plasma membrane localization coupled with sub-optimal membrane integrity and a substantial elevation in the rate of protein secretion. In general, tumors can have high secretory activity leading to the presence of intracellular proteins in the extracellular tumor environment. This was certainly seen in our studies where leptin treatment increased total protein secretion by 5-fold.

The functions of these molecules represent diverse processes by which leptin may influence mammary tumor growth (18–21, 52–53). For example, collagen precursor (KF10-Human Protein KIAA150), found in the extracellular matrix, can act as a ligand for discoidin domain receptors (DDR), a subfamily of tyrosine kinase receptors involved in hyperproliferation and abnormal branching of mammary ducts (18). Cortactin isoform a is a src substrate localized mainly in the cytoplasum or cell-substratum and is involved in cell migration, organization of cell adhesion and is overexpressed in breast cancer (19, 20, 53). Serologically defined breast cancer antigen NY-BR-62, identified by immunoscreening serum from breast cancer patients, is overexpressed in 60–90% of breast cancers (21). Thus, in obese cancer patients elevated leptin may enhance tumor cell proliferation, via increased collagen precursor secretion and promote mammary tumor cell migration and adhesion through regulation of Cortactin a. Furthermore, increased secretion of antigen NY-BR-62 by leptin may indicate this molecule is relevant etiologically in obesity-associated cancer. In general, the identification of these proteins suggests leptin influences mammary tumor microenvironment, growth and tumor progression and more importantly begins to reveal potential mediators of these actions.

Secretion of the collagen precursor from leptin treated MCF-7 cells suggests leptin alters the extracellular matrix (ECM). Collagen precursors are usually secreted into the extracellular space, cleaved of their pro-domains and can go on to synthesize of various collagens including Types I, III, IV. The regulation of collagen by leptin is of interest as increasing evidence indicates ECM composition plays an integral role in tumor progression and invasiveness potential (38). A recent study demonstrates overexpression of genes for ECM proteins, including several collagens, is associated with highly metastatic mammary tumors (26). Accordingly, leptin has been shown to increase collagen gene expression in liver, mesangial and in trophoblast cells during invasion (23–25, 54). Favoring the idea leptin induces elevated ECM in breast cancer, our group has recently described that mammary tumors in obese rats have higher levels of collagen 1, as compared to tumors of lean rats. Collagen 1 content, quantified by CARS advanced imaging, was correlated with tumor aggressiveness, the predominant tumor phenotype in obese rats (39). In a related leptin study, our group has also shown leptin stimulates a large increase in connective tissue growth factor (CTGF) mRNA in MCF-7 cells (40). CTGF is linked to breast cancer mortality and can contribute to tumor aggressiveness by stimulating ECM deposition and promoting breast cancer metastasis to bone (41, 42). In this study the effect of leptin on collagen, observed by mass spectrometry, was confirmed by measuring collagen IV mRNA via real time PCR. In further support of this data we have shown at the protein level, via western and immunofluorescence, that leptin induces a significant increase of various collagens in MCF-7 cells (43, unpublished data). Together, these studies propose a main role of leptin is modulation of tumor ECM composition. The regulation of ECM by leptin can affect tumor cell behaviors such as adhesion, migration and

metastases and thus may be an important mechanism for promoting the aggressive tumor phenotype associated with obesity (4, 36).

Two-dimensional gels coupled with mass spectrometry (MS) is one of the most commonly used proteomic techniques, however, drawbacks of this method are dye sensitivity, limiting detection of low abundant proteins and the amount of isolated protein required for accurate MS analysis. To circumvent this problem and identify low abundance leptin-regulated secreted growth factors of MCF-7 cells, a commercially available antibody based cytokine membrane array was used. In brief, via membrane array we demonstrated that leptin can increase levels of secreted fibroblast growth factor-9 (FGF-9) and decrease the levels of transforming growth factor beta-3 (TGFb3) and insulin-like growth factor binding-protein-3 (IGFBP-3). The effect of leptin towards increasing FGF-9 and decreasing TGF-β3 in MCF-7 cells was verified by measuring the mRNA of these proteins via real time PCR.

The influence of leptin to stimulate FGF-9 and suppress TGF-β3 and IGFPB-3 levels may affect tumor cells by numerous mechanisms to promote growth. The FGF family of growth factors are potent regulators of proliferation in many cell types (44) and have been implicated in the progression of prostate cancer (45). In particular, FGF-9 induces proliferation in lymphocytes, myocytes, glioma cells and endometrium stromal cells, but has not been previously identified in cancerous mammary epithelial cells (27, 28). Members of the TGF-β family of proteins also have a strong influence on mammary tumorigenesis (29, 46). Initial studies assigned dual and opposing roles for TGF-β in tumor cells, both as cell cycle suppressors and as a component of the cell invasion signal cascade. In clarifying this ambiguity, recent work indicates the transition of TGF-β from tumor inhibitor to enhancer is estrogen receptor (ER) dependent, with TGF-β impeding growth in ER positive tumor cells, such as MCF-7 (46). Finally, the reduction of IGFBP-3 by leptin may act in two ways to promote tumor cell growth. First, extracellular IGFBP-3 is known to compete with cell surface insulin-like growth factor (IGF) receptors for binding to IGF-1, a potent mitogen in tumor cells (47, 48). Thus, lowered levels of antagonistic IGFBP-3, which have been observed in obese subjects (49), may lead to greater availability of local IGF-1 for its receptor, resulting in tumor cell proliferation. Second, previous studies demonstrate IGFBP-3 is anti-proliferative and induces apoptosis in breast cancer cells in a ligand-independent manner (30, 31, 50), therefore lowered IGFBP-3 levels may be essential to prevent these direct negative actions and permit tumor progression. Consequently, the regulation of IGFBP-3 can enhance IGF dependent proliferation and simultaneously limit autocrine IGFBP-3 mediated apoptosis and cell cycle suppression. Collectively, this protein array supports leptin can regulate the secretion of binding proteins and other growth factors that are known to play important roles in breast tumorigenesis.

In conclusion, as obesity is associated with breast cancer incidence, advanced breast tumor aggressiveness, and drug resistance (51), 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 (37). Serum leptin is elevated in obese subjects and breast tumor leptin levels are associated with tumor grade (1, 2, 36, 51). Initial studies of leptin and cancer focused on leptin's stimulation of the tumor cell cycle via cytoplasmic signaling molecules (5, 10–12, 16, 17). Herein, our proteomic experiments suggest leptin can contribute to several hallmarks of cancer (including apoptosis, angiogenesis and migration) through many potential mechanisms. This work supports leptin affects these behaviors by regulating the secretion of autocrine/paracrine growth factors and by modulating the extracellular matrix composition (Fig. 8). In this study, we identify novel leptin-regulated growth factors and demonstrate for the first time the significant effect of leptin on the extracellular environment of cancerous mammary epithelial cells. The identification of leptin-regulated secreted factors begins to provide mechanistic links between leptin and tumor progression and gives new insight towards understanding the relationship between obesity and breast cancer incidence and morbidity. Accordingly, the *in vitro* and *in vivo* molecular characterization of tumors exposed to elevated

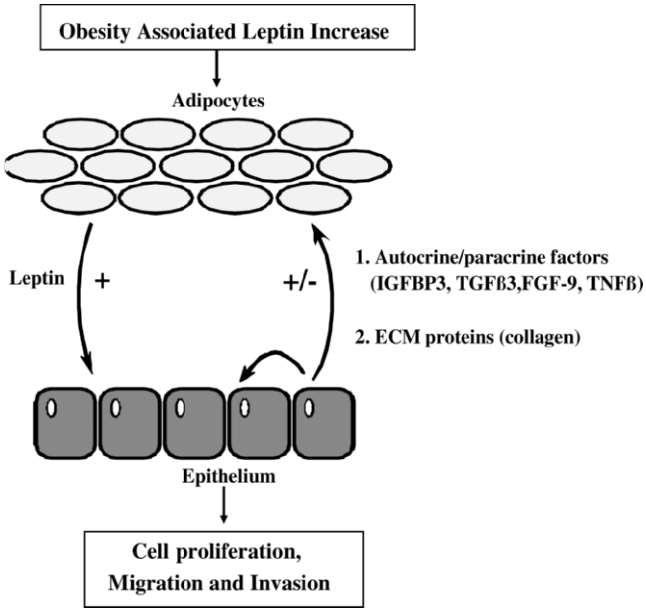

Figure 8. Leptin as a mediator of epithelial-adipocyte interactions to promote tumor growth and progression. The above diagram is a model indicating that leptin, from the circulation or produced by mammary adiposites, can act directly on mammary epithelial cells to regulate the release of extracellular matrix (ECM) proteins and many growth factors. The growth factors identified (insulin-like growth factor binding protein 3, transforming growth factor beta 3, fibroblast growth factor 9, and tumor necrosis factor beta) may act in an autocrine paracrine manner to influence tumor cell proliferation, migration and invasion. Modification of the extracellular environment through leptin induction of epithelial collagen production can also significantly influence tumor growth and progression.

leptin will be important towards identifying novel potential targets and improving cancer therapy in the obese patient population.

The authors acknowledge Sean Spence for his technical support.

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