

Proteomic analysis of MCF-7 breast cancer cell line exposed to leptin

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Abstract. *Background:* Obesity is a well-known factor risk for breast cancer in postmenopausal women. Circulating leptin levels are increased in obese and it has been suggested to play an important role in mammary tumor formation and progression. To contribute to the understanding of the molecular mechanisms underlying leptin action in breast cancer, our aim was to identify proteins regulated by leptin in MCF-7 human breast cancer cells.

Methods: We used two-dimensional gel electrophoresis (2-DE) and matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) to identify proteins affected by leptin.

Results: Thirty proteins were found differentially expressed in MCF-7 cells after 48 h leptin exposure. Proteins regulated by leptin included proteins previously implicated in breast cancer such as catechol-o-methyltransferase, cathepsin D, hsp27, serine/threonine-protein phosphatase and regulatory proteins of the Ras signaling pathway. Proteins involved in other cellular functions such as stress response, cytoskeleton remodeling and proteins belonging to ubiquitin-proteasome system, were also identified. Furthermore, leptin-treated cells showed a substantial uptake of the serum carrier proteins albumin and alpha-2-HS-glycoprotein.

Conclusions: This screening reveals that leptin influences the levels of key proteins involved in breast cancer which opens new avenues for the study of the molecular mechanisms linking obesity to breast cancer.

Keywords: Leptin, MCF-7, breast cancer, proteomics

1. Introduction

Obesity has been associated to increased risk of breast cancer in postmenopausal women [1–3]. Excessive fat mass has been shown to favor metastasis development, cancer recurrence and a poor prognosis in breast cancer [4]. Although epidemiologic evidence has been recognized for many years, the molecular mechanisms underlying the relationship between obesity and breast cancer is still under debate. It has been proposed that increased exposure of mammary epithelial cells to several growth factors and hormones, such as estrogens, insulin-like growth factor-I (IGF-I) and

leptin might be potential contributing factors to breast cancer pathogenesis in obese women. Although the role of estrogen, insulin and IGF-1 in breast cancer has been extensively studied, the potential role of leptin is just beginning to be characterized [5].

Leptin is a 16 kDa protein produced mainly by adipose tissue and, at a lower extent, by other organs such as stomach, placenta, muscle, immune cells and mammary gland [6, 7]. Leptin concentration in the blood increases as body weight and fat mass increase and regulates energy homeostasis by suppressing food intake and increasing energy expenditure acting directly on hypothalamic nuclei [8–10]. In addition to its central nervous system activities, however, leptin has been shown to influence multiple functions in peripheral tissues including immune response, angiogenesis, hematopoiesis and reproduction [11–13].

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In mammary gland, leptin has been found to be necessary for normal mammary development but also for mammary tumor formation. Mice deficient in leptin (ob/ob) or leptin receptor (db/db) exhibit a significant impairment of postnatal mammary development and a decreased incidence of both spontaneous and oncogene-induced mammary tumors [14, 15]. Supporting evidence to the role of leptin in breast cancer is given by *in vitro* studies showing that leptin stimulates proliferation of normal and cancerous mammary epithelial cells [16, 17]. Furthermore, it has been reported that leptin counteracts apoptosis, increases cell invasion and induces the expression of matrix degrading enzymes in several breast cancer cell lines, which suggests that this adipokine may influence cell cancer behavior through multitude of mechanisms [18–20]. It is worthy to note that leptin, as a cytokine, activates several signaling pathways that are strongly related to cell proliferation and survival, including JAK2/STAT3, Ras/ERK1/2, PI3K/AKT/GSK3, p38 and PKC [21, 22]. Despite the accumulating evidence revealing the influence of leptin on breast cancer, the molecular targets of leptin action in mammary cancer cells are just beginning to be recognized.

Developments in genomics and proteomics have improved the understanding of molecular mechanisms involved in tumor initiation and progression. A recent study based on genomics has provided valuable data on leptin effects on gene expression in breast cancer cells [19]. Although DNA microarray is a powerful tool, the predictive value of mRNA expression is limited with respect to cellular physiology. Consequently, a broader understanding of the leptin effects on breast cancer requires independent analysis of protein expression complementing mRNA expression data. Toward this end, in this study we have combined two-dimensional electrophoresis (2-DE) and mass spectrometry approaches to profile the protein expression pattern induced by leptin in the MCF-7 breast cancer cell line. This large-scale approach allows the simultaneous screening of the expression levels of hundreds of proteins, providing a powerful tool to reveal previously unrecognized links between cancer, leptin and protein expression patterns. Thus, using this proteomic approach, we identified proteins that have already been linked to breast cancer but are, for the first time, linked to leptin action. This study provides valuable insight into the relationship between obesity and breast cancer by identifying novel and previously unexpected targets whose expressions are affected by leptin.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) was from GIBCO (Paisley, UK). Immobilized pH-gradient (IPG) strips, ampholytes and Flamingo™ fluorescent gel stain were purchased from Bio Rad Laboratories (Hercules, CA). Leptin, acrylamide, CHAPS, urea, thiourea, dithiothreitol (DTT), EDTA, iodoacetamide (IAA), colloidal Coomassie blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Benzonase®, propidium iodide and trypsin were from Sigma-Aldrich (St. Louis, MO). Destreak reagent (HED, hydroxyethylidisulphide) was from Amersham biosciences (Uppsala, Sweden). ZipTip C18 microcolumns were from Millipore (Billerica, MA, USA). All solvents used were of high-performance liquid-chromatography (HPLC) grade from Sigma-Aldrich and Panreac (Barcelona, Spain).

2.2. Cell culture and proliferation assay

MCF-7 breast cancer cell line was purchased from ATCC and routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin and streptomycin) at 37°C in 5% CO₂. Cell proliferation was determined by MTT reduction assay. MCF-7 cells were cultured in 96-well culture dishes with 0, 10, 50 and 100 ng/mL leptin for 48 h. After this time, 10 µL/well of a MTT solution (5 mg/mL) was added. MTT is reduced in metabolically active cells to yield an insoluble purple formazan product. One hour later, supernatant was discarded and 100 µL/well of DMSO were added to dissolve the formazan crystals. Optical density (OD) was measured on a microplate autoreader (BIO-TEK Instruments, Winooski, Vermont, USA) at 570 nm against reference wavelength (630 nm).

2.3. Flow cytometry

MCF-7 cells were plated at approximately 10⁵ cells/well in 12-well plates in 2 mL of DMEM containing 10% FBS for 24 hours and then treated with 50 ng/mL leptin or let untreated (control). After 48 hours cells were trypsinized, washed with phosphate buffer saline (PBS) and fixed in methanol overnight at 4°C. After being washed twice with PBS, DNAs were

stained in the dark with 50 $\mu\text{g}/\text{mL}$ propidium iodide in the presence of 50 $\mu\text{g}/\text{mL}$ of RNase A. Samples were analyzed for DNA ploidy using a Coulter Epics XL-MCL Flow Cytometer (Beckman Coulter, Miami, FL, USA).

2.4. 2-DE

For 2-DE, cells were cultured in 100-mm dishes at $5 \cdot 10^5$ cells per dish in DMEM supplemented with 10% FBS for 24 hours and treated with 50 ng/mL leptin or left untreated (control). After 48 hours, medium was removed and the cells were rinsed three times in STE buffer (250 mM sucrose, 5 mM Tris, 2 mM EGTA) and scraped in 800 μl of lysis buffer (8.4 M urea, 2.4 M thiourea, 5% CHAPS, 50 mM DTT, 1% freshly added IPG ampholytes). Crude extracts were homogenized in a polytron homogenizer and sonicated for 30 s to fragment nucleic acids. After sonication, 150 U/ml of Benzonase and 2 mM MgCl_2 were added and the extract incubated for 1 h at room temperature to further degrade nucleic acids. The sample was clarified by centrifugation at $10,000 \times g$ for 1 h, aliquoted and stored at -80°C until use. Protein content of each sample was determined using Bradford method [23]. IPG strips (17 cm; pH 3-10NL) were rehydrated for 20 h in 300 μL of rehydration solution (8 M urea, 2 M thiourea, 2% w/v CHAPS, 0.002% bromophenol blue, 0.5% IPG ampholytes and 100 mM HED) containing 80 μg of protein extract. Focusing started at 200 V, with the voltage gradually increased to 3000 V and then kept constant for a further 35000 Vh (PROTEAN IEF System; Bio Rad). The gel strips were then equilibrated in two steps of 15 min with gentle shaking in 7 mL of equilibration buffer containing 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% v/v glycerol and 2% SDS. DTT (1% w/v) was added to the first step (reduction step), and IAA (5% w/v) was added to the second step (alkylation step). For each sample, the second dimensional separation was performed in 12 and 8% SDS-PAGE. After protein fixation with fixing solution (10% acetic acid, 40% ethanol), gels were stained with FlamingoTM fluorescent gel stain following the manufacturer's instructions (Biorad).

2.5. Image analysis

Fluorescent gel images were generated with the Molecular Imager FX system (Biorad) using 532 nm

excitation and 555 nm longpass emission filters and analyzed using PDQuest software (Bio Rad). Molecular masses were determined by running standard protein markers, covering the range of 10–200 kDa. The pI values used were those given by the supplier of the IPG strips. After background subtraction, spots from each gel were matched to the spots on an artificial reference gel by automatic matching with visual inspection and evaluation of the matches produced. The reference gel was originally based on the image of one of the experimental gels, chosen arbitrarily. Additional protein spots not present on that gel were subsequently added to the reference gel from the other gels. To correct for differences in sample loading or staining intensity among gels "total quantity in valid spots" normalization method was used. Maps corresponding to protein extracts were organized in the corresponding "Replicate Groups", namely control (C) and leptin (L). Significant changes between replicate groups were determined using Student's *t*-test ($p < 0.05$).

2.6. In gel digestion

Differentially expressed spots were automatically excised using ExQuest Spot Cutter (Biorad) from the gels after Coomassie brilliant blue staining. Gel pieces were destained by washing three times with 100 μl aliquots of 25 mM ammonium bicarbonate and further washing step with 50% and 100% acetonitrile. Dried gel pieces were rehydrated with 10 μL of 12.5 ng/ μL TPCK porcine trypsin in 25 mM ammonium bicarbonate solution. Digestions were performed by incubation at 37°C overnight. Peptides were extracted in two steps by sequential addition of 30% acetonitrile followed by 3% trifluoroacetic acid (TFA) for 5 min in a sonication bath. The combined supernatants were concentrated in a SpeedVac Vacuum system (Savant Instruments, Holbrook, NY, USA) for mass spectrometry analysis. When necessary, the tryptic peptide mixture was extracted and purified using a Millipore ZIPTIP C18 column.

2.7. Mass spectrometry and database search

Proteins were identified by the peptide mass fingerprint (PMF) method, based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). One microliter of tryptic

peptides were spotted onto a ready-to-use Prespotted AnchorChip PAC 384 MALDI target. After 3 minutes incubation, the matrix spot was washed with 7 μ l of 10 mM ammonium phosphate 0.1% TFA. Mass spectra were collected on a Bruker Autoflex MALDI-TOF/TOF instrument in the positive ion reflectron mode. The instrument was externally calibrated using the calibrant spots on the prespotted target. Monoisotopic peaks were generated by FlexAnalysis software and proteins were identified by matching the calibrated peptide mass values within Swiss-Prot protein data base for Homo sapiens and mammalian using Mascot Server imbedded in Bruker's Biotoools software. Match variances allowed were a mass tolerance of 50 ppm, one missed trypsin cleavage, fixed modification of carbamidomethyl cysteine, and variable modification of methionine oxidation.

3. Results

3.1. Effects of leptin on MCF7 cell growth

To validate earlier published observations on leptin effects in MCF7 cell growth, cell proliferation and cell cycle analysis were performed in our leptin-treated MCF7 cells. As shown in Fig. 1A, leptin increased cell proliferation in a dose-dependent manner at concentrations deemed to represent physiologic levels from lean to obese humans (10–100 ng/ml). Concentrations of 50 ng/ml of leptin were chosen to represent levels that

would be found in an obese postmenopausal woman [24, 25] and were used for subsequent experiments. Cell cycle state was evaluated by flow cytometry to check that leptin stimulation resulted in cell cycle progression that is characterized by a decrease in cells in G₀/G₁ and an increase in cells in S and G₂/M phases [26].

3.2. Effects of leptin on MCF7 proteome

A two-dimensional electrophoresis/mass spectrometry proteomic approach was used to identify changes in protein expression in MCF7 cells following incubation with leptin (50 ng/ml) for 48 h. In order to analyze a maximal number of proteins, we performed 2D PAGE gels of different acrylamide concentrations. Thus, 8% gels allowed us the study of proteins of molecular weight from 150 to 35 kDa and 12% gels were used for proteins below 35 kDa (Fig. 1). 432 and 960 spots were detected in 8 and 12% gels respectively; of these, 230 spots overlapped, so finally 1162 spots were analyzed. The magnitude and significance of all spots were visualized using a volcano plot (Fig. 2) with cutoffs for spot selection based on fold change (>1.5) and *t*-test (*p* < 0.05). Statistical analysis of the densitometric data revealed significant changes in 71 spots of which 33 were up-regulated and 38 down-regulated by leptin. Only those protein spots that exhibited consistent change in expression over all gels were excised and submitted to digestion, with subsequent protein identification performed using MALDI-TOF

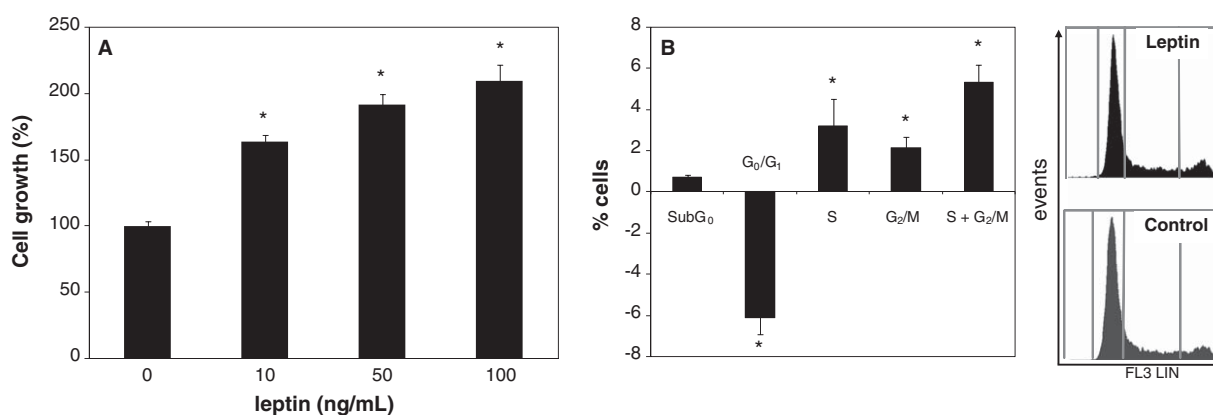


Fig. 1. Effects of leptin on cell proliferation and cell cycle. A) MCF-7 cells were exposed to 0, 10, 50 and 100 ng/ml leptin for 48 h and cell proliferation analyzed by MTT assay as described in Materials and Methods. Results are mean \pm s.e.m. of three experiments and are normalized as percentage of the control value (0 ng/ml leptin). B) Flow cytometry analysis of propidium iodide-stained MCF-7 cells with or without 50 ng/ml leptin treatment for 48 h. Bars represent the mean \pm s.e.m. of the difference between leptin-treated vs. untreated control cells distributed on cell cycle phases and expressed as percentage of total viable cells. Representative histograms are shown (left panels). * *p* < 0.05 Student's *t*-test.

MS. Thirty-nine spots were unambiguously identified, corresponding to 30 different gene products. The molecular weight (Mr) and isoelectric point (pI) of these proteins correspond roughly to their position on the 2-DE gel (Fig. 3). Furthermore, several proteins were identified at multiple spots positions (e.g. albumin), putatively reflecting the occurrence of post-translational modifications. In these cases, however, the variation of the spots, on average, was similar.

3.3. Identification of leptin-regulated proteins

The identities of the proteins and their differential expression ratio are shown in Table 1. Among the 30 proteins identified there are proteins, such as catechol-O-methyl transferase, cathepsin D, alpha-2-HS-glycoprotein and heat shock protein beta-1, that have long been implicated in breast cancer pathogenesis, even being considered or proposed as breast cancer markers. Several other proteins, such as Rho

GDP-dissociation inhibitor 1, Ras GTPase-activating protein-binding protein 1, serine/threonine-protein phosphatase 2A, programmed cell death 6-interacting protein and albumin, have been implicated not only in breast but multitude of other cancer types. Here we also identified proteins not directly related to cancer but whose involvement in cellular processes closely related to cell growth and survival make of them potential contributing factors. Among these are proteins involved in ubiquitin-proteasome system, cytoskeleton remodeling and stress response.

4. Discussion

The purpose of this study was to gain new insights into the mechanisms by which leptin influence breast tumor growth and progression. The application of proteomic approaches provides an important complement to genomics, improving our view of the complex network of molecular pathways underlying the cellular

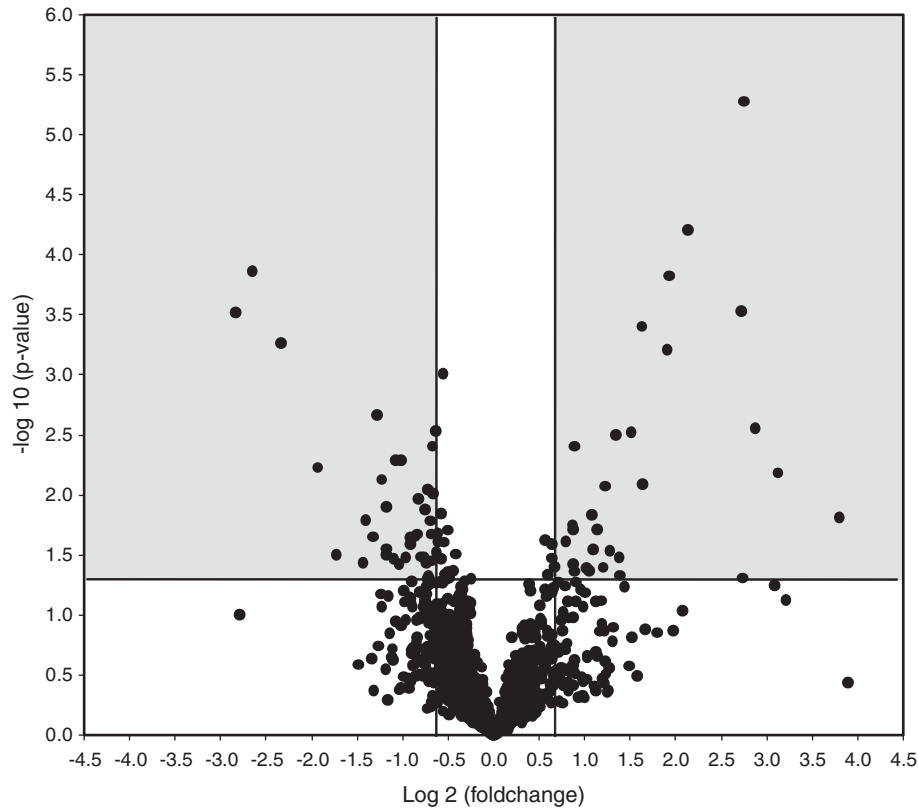


Fig. 2. Selection of leptin-regulated protein spots. Volcano plot showing the selection of the spots based on >1.5 mean fold change (vertical lines) and p -value < 0.05 (horizontal line). Spots that passed the criteria fall within the shaded area.

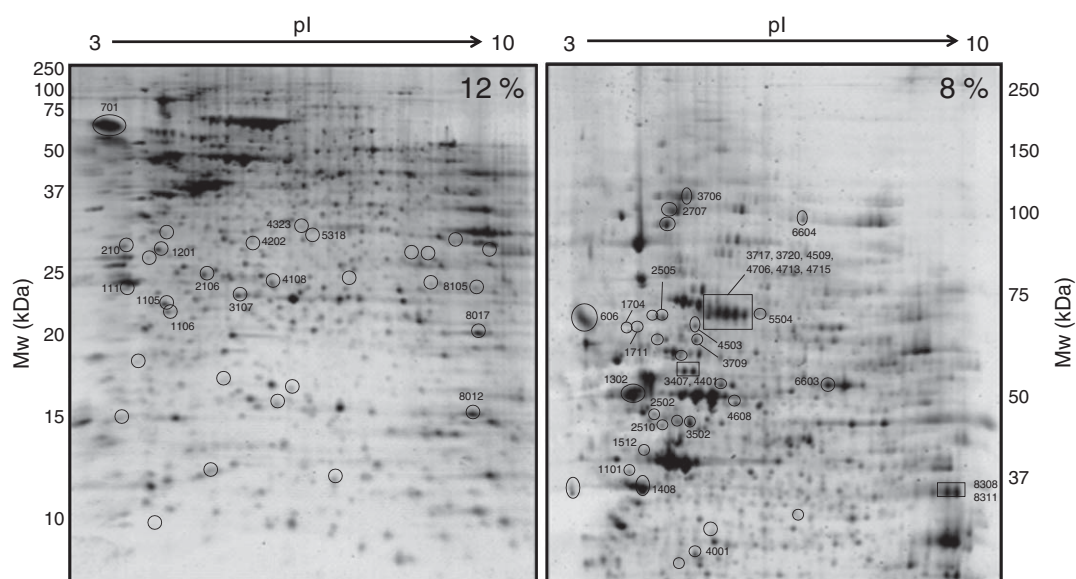


Fig. 3. Representative images of 8 and 12% acrylamide 2-D gels of MCF-7 breast cancer cells. Spots showing significant differences (>1.5 fold change and $p < 0.05$) between leptin-treated and control cells are encircled. Protein spots identified by MALDI-TOF MS are marked by their spot number.

response to hormonal stimuli. To our knowledge this is the first proteomic approach studying changes in whole MCF-7 cell proteome in response to leptin. A previous work by Perera et al studied the secretome of leptin-stimulated MCF-7 cells [20]. These authors showed that leptin could influence mammary tumor cell growth through regulation of autocrine/paracrine signaling. Moreover, these authors studied leptin-regulated gene expression in MCF7 by microarray analysis [19]. This study provided valuable data showing that leptin influences multiple genes affecting cell cycle, apoptosis and extracellular matrix. Our proteomic study, however, showed little overlap with these microarray results. This discrepancy may rely on the fact that 2D-E has limited capacity to detect low abundance proteins, such as regulatory proteins and transcription factors. Proteomics, on the other hand, has the advantage of not being limited to a predefined set of genes, allowing discovering unexplored molecular mechanisms, and therefore, expanding the knowledge provided by other approaches.

Many of the leptin-regulated proteins identified in this study have already been linked to breast cancer incidence and progression. Among these, worth special mention catechol O-methyltransferase (COMT), an enzyme that plays a critical role in the metabolism of estrogen in breast [27, 28]. Estrogens can be metabol-

ically activated to genotoxic metabolites, namely catechol estrogens, by hydroxylation catalyzed by cytochrome P450, CYP1A1 and CYP1B1 [29]. These catechol estrogens are carcinogenic and can be further oxidized to quinones able to form DNA adducts [30]. In order to avoid genetic damage, catechol estrogens are predominantly conjugated through methylation by the enzyme COMT, which plays, therefore, a protective role against estrogen-induced carcinogenesis. This assumption is sustained by several epidemiological evidence showing that: i) expression of the low activity form of this enzyme is associated with increased risk for breast cancer and ii) single nucleotide polymorphisms causing up-regulation of this gene are associated with reduced risk [28, 31, 32]. In our study we show that leptin-exposed MCF7 cells have lower COMT protein levels, which could contribute to explain the higher risk for breast cancer in obese women. Interestingly, it has been observed that obese women carrying a low activity COMT allele are more prone to breast cancer than non obese [33]. Although the increased estrogen levels in obesity may explain this association, future studies should explore whether leptin may potentiate estrogen-induced carcinogenesis by repressing COMT expression in mammary gland.

Another significant protein in breast cancer down-regulated by leptin was the lysosomal aspartic protease

Table 1
Identification of leptin-modulated proteins by MALDI-TOF MS in MCF-7 breast cancer cells

Protein name	abbr	Accession nr	Score	Spot nr	Mr	pI	Pept search	Mass match	Seq cove	Log 2 (fold change)
Breast cancer related proteins										
Cathecol-O-methyltransferase	COMT_HUMAN	P21964	75	1106	30474	5.26	40	7	39	-0.7
Cathepsin D	CATD_HUMAN	P07339	87	2106	45037	6.10	39	10	24	-0.8
Alpha-2-HS-glycoprotein	FETUA_BOVIN	P12763	158	606, 701	39193	5.26	35	12	28	3.3
Albumin bovine	ALBU_BOVIN	P02769	194	3717, 3720, 4509, 4706, 4713, 4715	71244	5.82	65	24	36	2.2
FK506-binding protein 4	FKPB4_HUMAN	Q02790	123	3407, 4401	52057	5.35	42	13	36	-0.6
Ras-GTPase-activating protein-binding protein-1	G3BP1_HUMAN	Q13283	79	4503	52189	5.36	33	8	18	-0.9
Rho GDP-dissociation inhibitor 1	GDIR1_HUMAN	P52565	118	1105	23250	5.02	40	10	41	-0.6
Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	2AAA_HUMAN	P30153	68	1711	66065	5.00	39	9	21	1.3
Heat shock protein beta-1 (Hsp27)	HSPB1_HUMAN	Q9UC36	59	3107	22826	5.98	0.026	13	25	-0.8
Heat shock cognate 71 kDa protein (Hsc70)	HSP7C_HUMAN	P11142	72	1101	71082	5.37	26	8	15	-2.1
Programmed cell death 6-interacting protein	PDC6L_HUMAN	Q8WUM4	63	6604	96590	6.13	31	8	17	-0.6
Structural proteins										
Alpha-actinin-1	ACTN1_HUMAN	P12814	91	2707	103563	5.25	47	15	22	-0.7
Cofilin-1	COF1_HUMAN	P23528	83	8012	18719	8.22	84	7	55	-0.6
Keratin, type II cytoskeletal 1	K2C1_HUMAN	P04264	67	1704	66170	8.15	76	12	25	2.5
Keratin, type II cytoskeletal 8	K2C8_HUMAN	Q6P4C7	234	1512, 3502	53671	5.52	68	25	59	-1.6
Keratin, type I cytoskeletal 19	K1C19_HUMAN	Q9P1Y4	384	1408	44065	5.04	91	36	79	-0.8
Tubulin beta chain	TBB5_HUMAN	P07437	101	1302	50095	4.78	94	14	34	-0.6
Lamin-B1	LMNB1_HUMAN	P20700	185	2505	66653	5.11	46	20	43	-0.6
F-actin-capping protein subunit alpha-1	CAZA1_HUMAN	P52907	31*	4001	33073	5.45				-1.1
Ubiquitin-proteasome system										
Ubiquitin thioesterase OTUB1	OTUB1_HUMAN	Q96FW1	90	210	31592	4.85	23	6	26	-1.2
Ubiquitin-like modifier-activating enzyme 1	UBA1_HUMAN	P22314	145	3706	118858	5.49	54	20	22	-0.9
Proteasome subunit alpha type 5	PSA5_HUMAN	P28066	87	111	26565	4.74	13	6	27	-0.7
Other proteins										
Galactin-3	LEG3_HUMAN	P17931	77	8105	26193	8.57	58	8	31	-0.8
Fructose-biphosphate aldolase A	ALDOA_HUMAN	P04075	186	8308, 8311	39851	8.30	52	17	58	-0.6
Thioredoxin-dependent peroxide reductase, mitochondrial	PRDX3_HUMAN	P30048	68	4108	28017	7.67	35	6	19	-0.6
STIP1 homology and U box-containing protein 1	STUB1_HUMAN	Q9UNE7	90	4202	35290	5.61	38	9	25	-0.7
Protein CDV3 homolog	CDV3_HUMAN	Q9UKY7	62	5318	27318	6.06	17	6	43	-0.9
Uncharacterized protein C1orf198	CA198_HUMAN	Q9H425	56	5504	36438	5.6	77	8	31	1.0
Heterogeneous nuclear ribonucleoprotein F	HNRPF_HUMAN	P52597	75	2510	45985	5.38	39	8	25	-0.8
60 S acidic ribosomal protein P0	RLA0_HUMAN	P05388	75	4323	34423	5.71	21	6	25	-0.8

"Accession number" corresponds to Swiss-Prot database. "Spot nr" refers to spot numbers indicated in Fig. 3. "Seq cov" sequence coverage, peptide matches, theoretical Mr and pI and Mascot scores are given. At least six matching peptides were required for an identity assignment. Expression data are shown as Log2 fold change, with positive values indicating an increase and negative values indicating a decrease in protein expression in the leptin-treated MCF-7 cells. * protein identified by fragmentation.

cathepsin D (CATD). This protease is over-expressed and hyper-secreted by epithelial breast cancer cells, and is considered an independent marker of poor prognosis correlated with the incidence of clinical metastasis [34, 35]. CATD is synthesized as a 52kDa inactive pro-CATD which is converted in the lysosomes to an active intermediate 48 kDa single-chain protein that is subsequently cleaved into a mature two-chain enzyme, consisting of a light (14 kDa) and a heavy (34 kDa) chain [36, 37]. Breast cancer cells over-express and secrete the pro-CATD, which, in turn, can be endocytosed by both cancer cells and fibroblasts and undergo maturation successively into 34+14 kDa mature form [38]. In particular we observed that leptin induced a marked decrease of the 34 kDa chain, the mature CATD, which could involve changes both in protein maturation, secretion or reuptake. Considering that CATD acts as an autocrine growth factor for breast cancer and seems to be involved in cancer invasion and metastasis [39–41], further experiments should address the question of whether CATD is involved in the aggressiveness of breast cancer observed in obese postmenopausal women.

Leptin-treated MCF7 cells showed a marked uptake of two proteins from fetal bovine serum of the culture media, namely alpha-2-HS-glycoprotein (AHSG) and albumin. These proteins are carriers of endogenous water-insoluble plasma substances (hydrophobic hormones, vitamins, minerals) that are bound in a reversible non-covalent manner. Both AHSG and albumin are the most abundant carrier protein in fetal and adult blood, respectively. AHSG has been identified as a tumor antigen increased in urine of breast cancer patients [42]. Autoantibodies against this protein can be selectively detected in blood of breast cancer patients in contrast to healthy women, being proposed as a novel biomarker for breast cancer screening and diagnosis [42, 43]. Leptin was found to increase 8-fold the intensities of spots 606 and 701 corresponding to the exogenous fetal bovine AHSG, which suggest an increased endocytosis of this protein. Similarly, spots identified as bovine serum albumin were increased 4-fold in our leptin-exposed MCF-7 cells. It is known that tumors have a high metabolic demand and actively transport albumin into their cells for anabolic processes [44, 45]. In addition, albumin seems to help endothelial transcytosis of protein-bound and unbound plasma constituents [46]. Since cancer cells show increased uptake of these proteins, there is a growing interest in their use as drug carriers for target tumor [46,

47]. A successful example of such strategy is Abraxane[®], a treatment for metastatic breast cancer based on the use of paclitaxel bound to albumin nanoparticles [48]. Therefore, understanding how leptin promotes the uptake of these carrier proteins may contribute in a future to the development of new combined strategies on breast cancer therapy.

Other key proteins associated to breast cancer and regulated by leptin were Rho GDP-dissociation inhibitor 1 (GDIR1) and Ras-GTPase-activating protein-binding protein-1 (G3BP1), both belonging to the Ras superfamily of GTPases. Ras GTPases are often either overexpressed or hyperactive in breast cancer and are regulators of cell proliferation, apoptosis and gene expression [49, 50]. Thus, by altering the levels of regulatory proteins, leptin might modulate the Ras-GTPase pathway. Similarly, other signal transduction protein potentially affected by leptin was the serine/threonine-protein phosphatase 2A (PP2A). PP2A play a pivotal role in cellular growth control and potentially in the development of cancer [51]. Several studies have suggested that this enzyme might actually exert tumor suppressive function [51, 52]. However, other authors have pointed the requirement for PP2A in cell growth and survival, which is not a characteristic of a tumor suppressor [51, 53]. It has to be taken into account that the holoenzyme PP2A is a heterotrimeric enzyme composed by a catalytic (C) and two regulatory (A and B) subunits that are encoded by a heterogeneous group of genes which give rise to a multitude of diverse PP2A complexes. Regulatory subunits determine the substrate specificity, subcellular localization and catalytic activity of these PP2A holoenzymes [54]. Leptin was found to up-regulate the levels of the 65 kDa regulatory subunit A α isoform (PR65 α), subunit that, together with PR65 β , have been identified as tumor suppressors, with their genes being mutated in several cancers including breast carcinomas [55]. Taken together, these data suggest that leptin might influence breast cancer progression through several signaling pathways altered in cancer.

Another interesting issue in the field of leptin in breast carcinogenesis is the crosstalk between leptin and estrogen signaling. Interaction between leptin and estrogen axis has been extensively reported suggesting that these pathways might cooperate in sustaining the growth of estrogen-dependent breast cancer cells [5, 56]. For instance, leptin promotes estrogen synthesis by up-regulation of aromatase in adipose tissue, enhances transactivation and nuclear localization of

estrogen receptor alpha (ER α) and attenuate the effect of the antiestrogen ICI 182,780 in MCF-7 breast cancer cells [56–58]. Apart from the previously mentioned effects of leptin on the estrogen-metabolism enzyme COMT, here we extend the knowledge on estrogen/leptin crosstalk showing that leptin also modulates the levels of F506-binding protein 4 (FKBP52), a protein that form complexes with unliganded steroid receptors (ERs) regulating their intracellular trafficking and function [59].

Heat shock proteins are overexpressed in a wide range of human cancers and are useful biomarkers for carcinogenesis in some tissues and signal the degree of differentiation and the aggressiveness of some cancers. Among the most implicated with the prognosis and the response to anticancer treatment are hsp27 and hsp70 [60]. In this study we observed that leptin decreased the levels of hsp27 which has been involved in the resistance to chemotherapy in breast cancer [60, 61]. In contrast, hsp27 expression has been inversely correlated with cell proliferation suggesting that this protein may be involved in cell growth arrest and differentiation [62]. Leptin also down-regulated the levels of heat shock cognate 71 kDa protein (Hsc70). Recently, Hsc70 has been found to be down-regulated in MCF-7 cells resistant to 5-fluorouracil [63]. Bakkenist et al. localized Hsc70 to 11q23.3, a region deleted in 40% of sporadic breast and other cancers, and suggested that Hsc70 was a target of somatic mutation and deletion in a fraction of breast carcinomas [64]. It has been observed that decreased expression of Hsc70 is accompanied by a greater over-expression of Hsp70, which has been implicated in resistance to anticancer treatments and, furthermore, is a marker of poor prognosis in breast carcinoma [65, 66]. Altogether, these studies suggest a significant role for Hsc70 in breast cancer initiation and development of drug-resistance.

Ubiquitin-proteasome pathway is essential for many fundamental cellular processes, including the regulation of receptor signaling pathways and the timely degradation of cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors during mitosis. Among the proteins identified in this study, there were three proteins belonging to the ubiquitin-proteasome system (ubiquitin thioesterase, ubiquitin-like modifier-activating enzyme 1, proteasome subunit alpha type 5) and seven proteins involved in cytoskeleton remodeling (alpha-actinin 1, cofilin 1, cytokeratins 8 and 19, lamin-B1, tubulin and F-actin-capping protein α -1). These findings suggest that proteasome activity and

cytoskeleton remodeling, which play part on the complex regulation of cell cycle and cell growth, may be targets of leptin action in mammary cancer cells.

5. Conclusion

Obesity is positively associated with breast cancer incidence and mortality [5, 56]. Moreover, it is well established that obesity favors metastasis development and breast cancer recurrence [4]. Leptin has emerged as a potential link between obesity and breast cancer [68]. Herein, by applying a proteomic approach, we identified MCF7 breast cancer cell line. Our results show that leptin may influence multitude of molecular mechanisms implicated in breast cancer pathogenesis. Among others, leptin modified the levels of key proteins involved in estrogen genotoxicity, metastases, cell growth control, stress response, protein degradation and cytoskeleton remodeling. Another interesting outcome was that leptin acts as a nourishing factor for breast cancer cells by promoting a substantial uptake of serum proteins (albumin and AHSG). The identification of targets of leptin action, some of them previously recognized as breast cancer markers, provide new insight into the understanding of the relationship between obesity and breast cancer.

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