Cellular and molecular crosstalk between leptin receptor and estrogen receptor- α in breast cancer: molecular basis for a novel therapeutic setting

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Abstract

Obesity is associated with an increased risk of breast cancer. A number of adipocytokines are increased in obesity causing low-level chronic inflammation associated with an increased risk of tumors. The adipocytokine leptin shows profound anti-obesity and pro-inflammatory activities. We have hypothesized that in common obesity, high circulating leptin levels might contribute to an increased risk of breast cancer by affecting mammary cell proliferation and survival. Leptin exerts its activity not only through leptin receptor (LepR), but also through crosstalk with other signaling systems implicated in tumorigenesis. In this study, we focused our attention on the relationship between the leptin/LepR axis and the estrogen receptor- α (ER α). To this aim, we utilized two human breast cancer cell lines, one ER α -positive cell line (MCF 7) and the other ER α -negative cell line (MDA-MB 231). We observed that the two cell lines had a different sensitivity to recombinant leptin (rleptin): on MCF 7 cells, rleptin induced a strong phosphorylation of the signal transducer and activator of transcription (STAT) 3 and of the extracellular related kinase 1/2 pathways with an increased cell viability and proliferation associated with an increased expression of ER α receptor. This response was not present in the MDA-MB 231 cells. The effects induced by leptin were lost when LepR was neutralized using either a monoclonal inhibitory antibody to LepR or LepR gene-silencing siRNA. These data suggest that there is a bidirectional communication between LepR and ER α , and that neutralization and/or inactivation of LepR inhibits proliferation and viability of human breast cancer cell lines. This evidence was confirmed by *ex vivo* studies, in which we analyzed 33 patients with breast cancer at different stages of disease, and observed that there was a statistically significant correlation between the expression of LepR and ER α . In conclusion, this study suggests a crosstalk between LepR and ER α , and could envisage novel therapeutic settings aimed at targeting the LepR in breast cancers.

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Introduction

In recent years, obesity has assumed epidemic proportions in industrialized Western countries, and has been linked to increased risk for a series of cancers, in particular those characterized by hormone dependence such as postmenopausal breast and ovarian

cancers (Rodriguez *et al.* 2001, Carroll 2003, Stephenson & Rose 2003, Moore *et al.* 2008, Perera *et al.* 2008). Increased fat mass and body mass index are the typical features of human obesity and are associated with an increased secretion of the adipocyte-derived hormone, leptin. Leptin, a 16 kDa non-glycosylated

peptide, is the product of the obese (*ob*) gene, and is mainly secreted by adipose tissue and, at a lower extent, by other organs such as the stomach, the placenta, the muscle, and immune cells (Zhang *et al.* 1994, Ahima & Flier 2000). Leptin is involved in tuning the energy balance and in the regulation of food intake by providing inhibitory signals to the hypothalamus (Woods & D'Alessio 2008). In addition to its central nervous system activities, leptin regulates multiple processes in peripheral tissues including hematopoiesis, immune responses, puberty, and pregnancy (Chehab *et al.* 1996, Lord *et al.* 1998, Cohen 2006). Leptin activates the JAK/signal transducer and activator of transcription 3 (STAT3) intracellular signaling pathway by binding to its receptor (LepR), which is part of the class I cytokine receptors. In the breast, leptin is required for normal mammary gland development and lactation (Hu *et al.* 2002), but it might also contribute to mammary tumorigenesis (Mauro *et al.* 2007). A strong relationship between breast cancer and obesity has long been recognized for many years. Interestingly, there is a substantial difference in the impact of obesity on carcinogenesis in premenopausal and postmenopausal women. While increased body weight seems to be inversely related to breast cancer risk in premenopausal women, obesity represents a significant risk factor for breast cancer development in postmenopausal women (Garofalo & Surmacz 2006). In postmenopausal obese women, adipose tissue is the only source of estrogen production by aromatization of C19 steroid androstenedione. As there is increased aromatase activity and androstenedione production in obesity, the total pool of estrogen is higher in obese women. The adipose tissue-derived hormone is readily prepared for peripheral conversion to the more biologically potent estradiol. Obesity also affects the binding of plasma estradiol to the sex hormone-binding globulin. Therefore, increased estrogen stimulation in postmenopausal obese women might be the cause of higher breast cancer risk (Henderson *et al.* 1988, Bernstein & Ross 1993, Toniolo *et al.* 1995). Evidence suggests that leptin might be involved in the pathogenesis of different types of human carcinomas, including breast cancer (Stattin *et al.* 2001, Garofalo *et al.* 2006). In particular, several actions of leptin, including the stimulation of tumor cell growth, migration and invasion, and enhancement of angiogenesis, may play a relevant role in breast cancer progression (Saxena *et al.* 2008). Although these findings are suggestive of the role of leptin in the growth of breast cancer, the lack of drugs specifically targeting the leptin system has not allowed to assess the

potential clinical impact of leptin inhibition in breast carcinoma up to now.

The aim of our study was to characterize, at the cellular and molecular levels, the precise relationship between the leptin/LepR axis and the estrogen receptor- α (ER α) expression in the modulation of growth, survival, and progression of human breast cancer cell lines. Our analyses were also corroborated by *ex vivo* analyses of human breast cancers (Fiorio *et al.* 2008, Grossmann *et al.* 2008, Wu *et al.* 2009). Our results might be relevant for understanding the relationship between obesity and increased risk of breast cancer in obesity, and could envisage a therapeutic setting aimed at targeting LepR during tumor growth.

Materials and methods

Reagents

For *in vitro* blocking experiments, human LepR-neutralizing mAb 9F8 (Fazeli *et al.* 2006), kindly provided by Prof. Richard Ross (University of Sheffield, UK), was used at a final concentration of 25 ng/ml. Human recombinant leptin (rleptin; R&D Systems, Minneapolis, MN, USA) was used at different concentrations (1, 10, and 100 ng/ml). For biochemical studies, the following antibodies were used: anti-STAT3 and anti-phospho-STAT3 (Y705; Cell Signaling Technology, Beverly, MA, USA); and anti-LepR, anti-ER α (HC-20), anti-extracellular related kinase 1/2 (ERK1/2), and anti-phospho-ERK2 (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The filters were also probed with a tubulin antibody (Sigma) to normalize the amount of loaded protein. The media that we used were DMEM and X-VIVO medium (BioWhittaker, Walkersville, MD, USA).

Cell lines

The MCF 7 (ER-sensitive cells) and MDA-MB 231 (ER-insensitive cells) cell lines were routinely grown at 37 °C in a 95% humidified atmosphere of 5% v/v CO₂ in DMEM supplemented with 10% v/v fetal bovine serum (FBS), 100 IU penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine.

Western blotting

Cancer cells were grown to subconfluent levels in DMEM in six-well culture plates (10⁶ cells/well). The cells were then washed once with a basal medium, and were starved of growth factors by switching them to the

basal medium for 16 h. Cells were again washed once with the basal medium treated with 100 ng/ml of rleptin in the presence or in the absence of 25 ng/ml of mAb 9F8 for 4 h. Total cell lysates were obtained in 50 mM HEPES (pH 7.5), 250 mM NaCl, 1 mM EDTA, 1.0% Triton X-100, 10 mM sodium fluoride, 1 mM sodium orthovanadate, and 2 µg/ml aprotinin, 2 µg/ml leupeptin, and 2 µg/ml pepstatin. Fifty micrograms of total proteins were subjected to SDS-PAGE under reducing conditions. After electrophoresis, proteins were transferred onto a nitrocellulose filter membrane (GE-Healthcare, New Haven, CT, USA) with a Trans-Blot Cell (Bio-Rad Laboratories) and a transfer buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol. Membranes were placed in 5% non-fat milk in PBS, 0.5% Tween 20 (PBST) at 4 °C for 2 h to block the non-specific binding sites. Filters were incubated with specific antibodies before being washed three times in PBST, and were then incubated with a peroxidase-conjugated secondary antibody (GE-Healthcare). After washing with PBST, peroxidase activity was detected with the ECL system (GE-Healthcare).

Viability assays

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) viability assay was used to determine cell viability induced by rleptin in the presence or in the absence of m9F8 antibody. The cells were cultured in 96-well plates, 2×10^4 cells/well, with 0.05 ml of DMEM supplemented with 10% FBS, 1% L-glutamine, and 1% 100 U/ml penicillin and 100 µg/ml streptomycin (all from Life Technologies Inc.) for 24 h. Before treatment with rleptin or 9F8 mAb, the cells were starved for 24 h in serum-free DMEM or in DMEM containing 2% serum or in X-VIVO medium, and were then incubated with 1, 10, and 100 ng/ml of rleptin in the presence or in the absence of 25 ng/ml of mAb 9F8 for 24 h. MTT was added to each well at a concentration of 0.5 mg/ml. After 4 h of incubation at 37 °C, the supernatant was removed and was added to 100 µl of dimethyl sulfoxide. Optical density at 492 nm was measured using a plate reader (Bio-Rad Laboratories). Each experiment was performed in triplicate, and the values are reported as mean $\pm$ s.d.

Proliferation assays

The cells were cultured in 96-well plates, 2×10^4 cells/well, with 0.05 ml of DMEM supplemented with 10% FBS, 1% L-glutamine, and 1% 100 U/ml penicillin and 100 µg/ml streptomycin (all from Life Technologies Inc.). Before treatment with rleptin or

9F8 mAb, the cells were starved in serum-free DMEM or in DMEM containing 2% serum or in X-VIVO medium overnight. Cells were stimulated for 24 h with 1, 10, and 100 ng/ml of rleptin in the presence or in the absence of 25 ng/ml of mAb 9F8. On the last day, [³H]thymidine (0.5 µCi/well; GE-Healthcare) was added to the cultures, and the cells were harvested after 12 h. Radioactivity was measured with a β -cell-plate scintillation counter (Wallac, Waltham, MA, USA). Each experiment was performed in triplicate, and the values are reported as mean $\pm$ s.d.

Lentiviral infection

MCF 7 and MDA-MB 231 cells were infected with three different clones of LepR siRNA lentiviral particles using polybrene reagent according to the manufacturer's protocol (Sigma). For MCF 7 and MDA-MB 231 infection, 4×10^5 cells were infected with the lentiviral particles (2.5×10^7 TU/ml) for 24 h in the presence of polybrene (3 µg/ml) (Fiorio *et al.* 2008). After this period, the cells were used for the assays as described above.

Selection of patients

Breast cancer samples were obtained from the files of Pathology Department of the National Tumor Institute 'G.Pascale' of Naples, Italy, and the approval from the internal ethical committee was obtained before starting the study. Selection was performed using the high positivity/negativity of breast tumors to ER. In particular, in our case, cancer samples were considered 'highly positive' when more than 75% of the neoplastic cells expressed ER; on the contrary, samples were considered ER negative when none (0%) of the neoplastic cells expressed ER, according to the Allred score (Allred *et al.* 1998). Thus, 33 patients affected by breast cancer were enrolled: 19 with high expression of ER and 14 with the absence of ER. Main clinicopathological data for each patient have been recorded.

Immunohistochemistry

All immunostaining techniques were performed in paraffin-embedded 4-µm tissue sections using a previous step of heat-induced antigen retrieval technique for all the antibodies. Thus, before incubation with the primary antibody, slides were treated in a microwave oven at 650 W for three cycles in a solution of 0.01 mol/l sodium citrate (pH 6). The following antibodies were used: anti-LepR (M-18, Santa Cruz Biotechnology Inc.); anti-ER (ER-1D5, Dako, Glostrup, Denmark); and anti-progesteron

receptor (PgR-636, Dako). The sections were incubated with biotinylated immunoglobulin for 20 min, followed by peroxidase-labeled streptavidin for 20' (Peroxidase Detection System, Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) with diaminobenzidine chromogen as a substrate. Nuclei were counterstained with hematoxylin. In each experiment, positive and negative controls were included. Evaluation was performed considering the percentage of the neoplastic cells expressing each of the examined receptors. Moreover, high expression of LepR was scored when more than 50% of the neoplastic cells expressed it.

Statistical analysis

The Mann–Whitney *U* test was used to perform statistical analysis using Graphpad software. Data were expressed as the mean $\pm$ s.d. of three independent experiments. Fisher's χ^2 test was used, where appropriate, to establish whether there were any relationships between LepR and ER- α expression in clinicopathologic data. A *P* < 0.05 was considered to indicate significant differences.

Results

LepR expression and signaling correlate with the presence of ER α in human breast cancer cell lines

The expression of LepR was examined in human breast cancer cell lines either expressing or not expressing the ER α , such as MCF 7 and MDA-MB 231, respectively. Treatment with 100 ng/ml of human rleptin induced an increase in LepR expression in MCF 7 cells, while no expression was detected in MDA-MB 231 cell line (Fig. 1A), either before and upon leptin stimulation. Molecular pathways downstream LepR activation were also investigated. More specifically, activation of STAT3 and ERK1/2 was analyzed. Both cell lines were treated with 100 ng/ml of rleptin, and the STAT3 and ERK1/2 phosphorylation levels were measured (Fig. 1A). rLeptin induced a robust phosphorylation of STAT3 (Fig. 1A) and ERK1/2 (Fig. 1A) in MCF 7 cells, whereas no effect was revealed in estrogen-insensitive MDA-MB 231 cells. rLeptin was also able to increase ER α expression in MCF 7 cells, whereas the absence of

Figure 1 Leptin induces LepR expression, STAT3 and ERK1/2 phosphorylation, and ER α upregulation in ER-positive human breast cancer cell lines. MCF 7 (ER α ⁺) and MDA-MB 231 (ER α ⁻) human breast cancer cell lines were cultured in a serum-free medium overnight followed by exposure to 100 ng/ml of rleptin. Eighty micrograms of total proteins were equally loaded and resolved by SDS-PAGE and western blot analysis. (A) LepR expression was detected using a specific anti-LepR antibody and chemiluminescent detection. Densitometric value of LepR/tubulin was determined. p-STAT3 expression was detected using a specific anti-STAT3 antibody and chemiluminescent detection. Densitometric value of p-STAT3/STAT3 was determined. Phosphorylated ERK1/2 levels were obtained after incubation with anti-p44/42 MAPK antibody and chemiluminescent detection. Densitometric value of p-ERK1/2/ERK1/2 levels was determined. (B) ER α expression was detected using a specific anti-ER α antibody and chemiluminescent detection. Densitometric value of ER α /tubulin levels was determined. All the above experiments represent one representative experiment of three experiments with similar results.

ER α on the surface of MDA-MB 231 cells was confirmed, and no induction was observed either before or after rleptin treatment (Fig. 1B).

Leptin increases human breast cancer cell line viability and proliferation, and these effects are inhibited by LepR neutralization

An MTT assay (see Materials and methods for details) was performed to evaluate the effect of rleptin on cell viability in human breast cancer. After achieving the basal level of growth by culturing the cells overnight in a medium, an increasing concentration of rleptin was added to the cells utilizing in parallel different sets of either serum-free controlled components (X-VIVO), or DMEM supplemented with or not supplemented with FCS; this approach was utilized to rule out the possible effects of contaminant leptin present in FCS. Treatments with increasing doses of rleptin (0, 1, 10, and 100 ng/ml) resulted in an increase in the viability of MCF 7 cells (Supplementary Figure 1A–C, left, see section on [supplementary data](#) given at the end of this article), whereas no effect was observed in MDA-MB 231 cells (Supplementary Figure 1A–C, right). A similar approach was utilized to analyze the proliferation of human breast cancer cells. A dose-dependent increase in proliferation (measured as [^3H]thymidine incorporation) was observed when the MCF 7 cells were incubated with rleptin (Supplementary Figure 2A–C, left, see section on [supplementary data](#) given at the end of this article), and no effect was revealed in the estrogen-insensitive cell line (Supplementary Figure 2A–C, right). In parallel, we analyzed the effect of the anti-LepR-neutralizing mAb, 9F8, on leptin-induced cell viability and proliferation in all the above experimental conditions. We performed an MTT assay by treating both MCF 7 and MDA-MB 231 cells

with 25 ng/ml of mAb 9F8 in the presence or in the absence of increasing concentrations of rleptin (0, 1, 10, and 100 ng/ml). The mAb 9F8 blocked the effect of rleptin on the viability of MCF 7 cells (Supplementary Figure 1A–C, left), whereas no effect was evident on the viability of MDA-MB 231 cells (Supplementary Figure 1A–C, right). Using the same treatment described for the MTT assay, we evaluated the effect of mAb 9F8 on cell proliferation by [^3H]thymidine incorporation (Supplementary Figure 2A–C). The mAb 9F8 inhibited the proliferation of MCF 7 cell line (Supplementary Figure 2A–C, left), whereas there was no effect in the MDA-MB 231 cells (Supplementary Figure 2A–C, right).

Neutralization of LepR inhibits intracellular signaling of human breast cancer cell lines

Moreover, the effect of mAb 9F8 on the main post-LepR intracellular traducers was evaluated. The phosphorylation of STAT3 and ERK1/2 was studied (Fig. 2A and B respectively). After treating both the cell lines with 25 ng/ml of mAb 9F8 in the presence or in the absence of 100 ng/ml of rleptin, the p-STAT3 and p-ERK1/2 activation levels were analyzed. As shown in Fig. 2, treatment with mAb 9F8 inhibited STAT3 and ERK1/2 phosphorylation induced by rleptin alone in the estrogen-sensitive cell line only, thus further confirming the presence of LepR in the ER α -positive cell lines.

LepR gene silencing inhibits intracellular signaling, ER α expression, viability, and proliferation of human breast cancer cell lines

Both MCF 7 and MDA-MB 231 cell lines were infected with lentiviral particles to silence LepR. We observed that the siRNA constructs targeting

Figure 2 Anti-LepR-neutralizing mAb inhibits STAT3 and ERK1/2 phosphorylation induced by leptin. MCF 7 and MDA-MB 231 human breast cancer cell lines were cultured in a serum-free medium overnight followed by exposure to 100 ng/ml of rleptin in the presence or in the absence of 25 ng/ml of anti-LepR-neutralizing mAb. Eighty micrograms of total proteins were equally loaded and resolved by SDS-PAGE and western blot analysis. (A) p-STAT3 expression was detected using a specific anti-STAT3 antibody and chemiluminescent detection. Densitometric value of p-STAT3/STAT3 was determined. (B) Phosphorylated ERK1/2 levels were obtained after incubation with anti-p44/42 MAPK antibody and chemiluminescent detection. Densitometric value of p-ERK1/2/ERK1/2 levels was determined. All the above experiments represent one representative experiment of three experiments with similar results.

specifically LepR transcript provoked a reduction in the expression of the corresponding protein in estrogen-sensitive cell line (Fig. 3A). Also, the silencing effect of LepR on STAT3 and ERK1/2 phosphorylation was analyzed. A dramatic reduction in the phosphorylation levels of STAT3 (Fig. 3A) and ERK1/2 (Fig. 3A) was observed. In order to analyze the possible relation between LepR and ERα expression, we detected ERα expression after LepR silencing. As shown in Fig. 3B, the LepR silencing inhibited the ERα expression in MCF 7 cell line, whereas this effect was not observed in MDA-MB 231 cell line. Finally, we tested the effect of LepR silencing on the proliferation and viability of both human breast cancer cell lines. To this aim, we performed [³H]thymidine incorporation (Fig. 3C, left) and MTT assays (Fig. 3C, right) respectively. The results obtained indicate that the LepR silencing inhibits cell

proliferation and viability induced by leptin, and that silencing of LepR inhibits ERα expression, thus suggesting a direct interaction between the two pathways.

Expression of LepR in patients with breast cancer correlates with ERα expression

In order to analyze the presence of LepR and its possible correlation with the expression of ERα in human breast cancer, we analyzed 33 breast tumors derived from patients with different tumor gradings (Table 1). Slight LepR expression was observed mainly in the cytoplasm with reinforcement of plasmatic membrane in cells constituting normal ductal-lobular unit of breast (Fig. 4). Observed mean value was 54% of that of LepR-expressing neoplastic cells (range: 0–90%); LepR positivity was significantly associated with the high expression of ERα ($P=0.03$).

Figure 3 Ablation of LepR by siRNA interference impairs its intracellular signaling, reduces ERα expression, and inhibits proliferation and viability of human breast cancer cell lines. MCF 7 and MDA-MB 231 human breast cancer cell lines were infected with three different lentiviral LepR siRNA clones to silence LepR expression. Scrambled siRNA lentiviruses were used as the negative control of infection. The cells were then exposed to 100 ng/ml of leptin. Eighty micrograms of total proteins were equally loaded and resolved by SDS-PAGE and western blot analysis. (A) LepR expression was detected using a specific anti-LepR antibody and chemiluminescent detection. Densitometric value of LepR/tubulin was determined. p-STAT3 expression was detected using a specific anti-STAT3 antibody and chemiluminescent detection. Densitometric value of p-STAT3/STAT3 was determined. Phosphorylated ERK1/2 levels were obtained after incubation with anti-p44/42 MAPK antibody and chemiluminescent detection. Densitometric value of p-ERK1/2/ERK1/2 levels was determined. (B) ERα expression was detected using a specific anti-ERα antibody and chemiluminescent detection. Densitometric value of ERα/tubulin levels was determined. (C) Growth factor-starved MCF 7 and MDA-MB 231 cells were incubated with 100 ng/ml of leptin in DMEM (serum-free). The proliferation index was measured by the incorporation of [³H]thymidine. Values represent the mean + S.E.M. of triplicate samples of three individual experiments (* = $P < 0.01$ versus untreated control). Growth factor-starved MCF 7 and MDA-MB 231 cells were incubated with 100 ng/ml of leptin in DMEM (serum-free). The viability was measured with MTT assay. Values represent the mean + S.E.M. of triplicate samples of three individual experiments (* = $P < 0.01$ versus untreated control).

Table 1 Main clinical and pathological characteristics of breast cancer patients according to the expression of LepR and ER α

	Age	Site	Diagnosis	Grading	Staging	Stage	ER α	LepR
1	37	R	ILC	G1	pT1pN0	I	90%	H (90%)
2	71	R	ILC	G3	pT2pN0	IIA	90%	0
3	49	R	ILC	G3	pT2pN0	IIA	80%	0
4	69	L	IDC	G2	pT2pN0	IIA	90%	0
5	53	R	IDC	G2	pT2pN0	IIA	90%	L (30%)
6	56	R	IDC	G2	pT1pN1	IIA	80%	0
7	44	R	ILC	G3	pT2pN2	IIIA	85%	L (30%)
8	71	L	IDC	G2	pT2pN0	IIA	90%	H (90%)
9	51	L	ILC	G1	pT1pN0	I	80%	H (90%)
10	65	L	IDC	G3	pT2pN3	IIIC	85%	H (90%)
11	57	L	ILC	G2	pT1pN0	I	85%	H (90%)
12	51	L	IDC	G2	pT1pN0	I	90%	L (40%)
13	87	R	IDC	G3	pT2pN1	IIB	85%	H (60%)
14	55	R	IDC	G2	pT1pN0	I	75%	H (90%)
15	26	R	IDC	G3	pT2pN2	IIIA	80%	H (90%)
16	63	R	IDC	G3	pT1pN0	I	85%	L (5%)
17	45	L	ILC	G1	pT1pN0	I	85%	H (80%)
18	73	R	IDC	G3	pT1pN0	I	90%	H (90%)
19	69	R	ILC	G2	pT1pN0	I	90%	H (60%)
20	73	L	IDC	G3	pT2pN1	IIB	0	0
21	86	L	IDC	G3	pT1pN2	IIIA	0	0
22	74	L	IDC	G3	pT2pN2	IIIA	0	H (60%)
23	56	R	IDC	G3	pT2pN0	IIA	0	0
24	43	R	ILC	G3	pT2pN0	IIA	0	L (30%)
25	50	R	IDC	G3	pT1pN1	IIA	0	0
26	41	L	IDC	G2	pT2pN0	IIA	0	L (30%)
27	56	L	Thoracic relapse	G3	M	IV	0	0
28	55	R	IDC	G3	pT1pN0	I	0	0
29	59	L	IDC	G3	pT2pN2	IIIA	0	0
30	62	L	IDC	G2	pT1pN0	I	0	L (40%)
31	55	R	IDC	G3	pT2pN3	IIIC	0	H (60%)
32	70	L	IDC	G2	pT3pN2	IIIA	0	L (5%)
33	70	R	IDC	G3	pT1pN1	IIA	0	L (30%)

R, right; L, left; H, high (more than 50%); L, low (<50%); IDC, infiltrating ductal carcinoma; ILC, infiltrating lobular carcinoma; ER α , estrogen receptor expression; LepR, leptin receptor expression; M, metastasis.

High expression of LepR was revealed only in 2 of 14 cases with absent ER α expression (mean value 18%; range: 0–60%; Table 2 and Fig. 4). Statistical evaluation of LepR expression versus main clinico-pathological data showed a significant association ($P=0.05$) between low–intermediate (G1–G2) grade cancer and LepR (Table 2).

Discussion

Obesity increases the risk of postmenopausal breast cancer by 30–50% (Stephenson & Rose 2003, Garofalo & Surmacz 2006, Wu *et al.* 2009). Excess body weight is associated with poor survival and increased recurrence of cancer regardless of menopausal status after adjustment for stage and treatment (Garofalo *et al.* 2006). Because of the increasing number of obese breast cancer patients, the mechanism of this

phenomenon is currently under investigation. Multiple evidence in cellular and animal breast cancer models indicate that leptin, the major hormone produced by fat tissue, can also be involved in the pathogenesis and maintenance of breast cancer (Carroll 2003, Perera *et al.* 2008).

However, the potential role of leptin/LepR in the pathogenesis of breast cancer has not been fully elucidated, and particularly, the direct relationship between LepR and ER α is still unresolved. In our experimental system, we observed that there is a strong correlation between the expression of LepR and ER α . This interaction is testified by the evidence that the treatment of ER α -positive MCF cell line 7 with rleptin induces an upregulation of both LepR itself and ER α . These effects were not observed when breast cancer cells did not express LepR. Furthermore, we employed a combined approach, either blocking LepR with a

Figure 4 *Ex vivo* analysis of the expression of LepR in human breast cancers correlates with ER α expression. Immunohistochemical staining for LepR and ER α (microscopic magnification 63 $\times$): (A) left, slight cytoplasmic LepR expression in a ductal-lobular unit of breast parenchyma; right, high nuclear ER α expression in the same field. (B) Left, high cytoplasmic LepR expression in an intermediate-grade infiltrating ductal carcinoma; right, high nuclear ER α expression in the same field. (C) Left, absent LepR expression in a high-grade infiltrating ductal carcinoma; right, absent nuclear ER α expression in same field.

mAb or by LepR gene silencing, in order to dissect at the molecular level whether the expression of LepR was responsible for ER α sensitivity. LepR neutralization or LepR gene silencing in ER-positive breast cancer cells caused a significant reduction in the ER α expression, thus suggesting for the first time a direct crosstalk between these two receptors in breast cancer. Moreover, we further confirmed that leptin also influences human cancer cell line viability and proliferation only when tumor cells expressed the ER α . Our *in vitro* data were also corroborated by *ex vivo* analyses on human breast cancers in which the expression of LepR correlated with ER α . We observed robust correlation between ER α positivity and high LepR expression. On the contrary, the majority of ER α -negative patients did not express significant

levels of LepR. This also suggests that the LepR may be considered as a novel marker of the differentiation of breast cancer, despite its potential role in mammary cell transformation.

Our report also shows that LepR neutralization with either LepR mAb or gene silencing can be effective in inhibiting breast cancer cell growth, proliferation, and viability *in vitro*. This evidence supports not only the possibility that strategies aimed at reducing leptin in obesity could be effective as an adjuvant in the therapy of breast cancer, but also the hypothesis that body weight reduction and calorie restriction in healthy subjects could be effective in preventing breast cancer. The notion that calorie restriction increases lifespan and can represent a novel approach in treating cancer has generated great interest in the scientific community

Table 2 Stratification of LepR expression according to the main clinical and pathological data and statistical association (Fisher's χ^2 test)

	High LepR expression 13 cases	Low/absent LepR expression 20 cases	P value
Histotype			
ILC	38.5% (5)	20.0% (4)	NS
IDC	61.5% (8)	80.0% (16)	
Grading			
G1	23.1% (3)	0.0% (0)	0.05
G2	30.8% (4)	35.0% (7)	
G3	46.1% (6)	65.0% (13)	
Stage			
I	54.0% (7)	20.0% (4)	NS
II	7.7% (1)	55.0% (11)	
III	38.5% (5)	20.0% (4)	
IV	0.0% (0)	5.0% (1)	
ER α expression			
High	84.6% (11)	40.0% (8)	0.03
Low/absent	15.4% (2)	60.0% (12)	

LepR, leptin receptor; ER α , estrogen receptor- α ; ILC, infiltrating lobular carcinoma; IDC, infiltrating ductal carcinoma; NS, not significant.

(Fontana 2009). This approach could lead to novel strategies possibly able to impact breast cancer progression, and these are the objects of extensive investigation by our group.

The link between estrogens and leptin has been shown since long time when it was demonstrated that there was a strong sex-related difference in leptin secretion in mice and humans (Ahima & Flier 2000). Indeed, females express higher leptin levels than males after normalizing for body fat mass; these differences were ascribed to estrogens (Ahima & Flier 2000). Similarly to leptin, estrogens can also reduce appetite and adiposity. It has been recently shown that estradiol can induce weight loss and inhibition of food intake through STAT3 activation (Gao *et al.* 2007). These observations support the notion that there is a general crosstalk between leptin and estrogens that show widely overlapping effects in terms of metabolic and molecular actions. In this context, both leptin and extrogens also display strong pro-inflammatory properties well known to contribute to multistep cancer induction and progression (Porta *et al.* 2009). STAT3 may represent the common element in this process in which both estrogens and leptin signal into the cells and induce chronic inflammation, and may lead to an increased risk of tumor in obese subjects.

In summary, our study is the first to decipher the cellular and molecular mechanisms responsible for leptin/LepR-mediated breast cancer cell proliferation,

survival, and ER α expression, thus establishing a direct link between leptin, obesity, and breast carcinoma. Also, our clinicopathological data show that there is a direct association between expression of ER α and LepR in breast cancer samples obtained from women at different stages of the disease. This association may also act as a novel marker in the clinical follow-up of the tumor. Finally, these data also set the cellular and molecular basis for a novel therapeutic approach in breast cancer aimed at targeting LepR.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1677/ERC-09-0340>.

Declaration of interest

The authors declare that there is no conflict of interest; G Matarese is also named on PCT application number EP2004/013043.

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References

Ahima RS & Flier JS 2000 Leptin. *Annual Review of Physiology* **62** 413–437.
Allred DC, Harvey JM, Berardo M & Clark GM 1998 Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Modern Pathology* **11** 155–168.
Bernstein L & Ross RK 1993 Endogenous hormones and breast cancer risk. *Epidemiologic Reviews* **15** 48–65.
Carroll KK 2003 Obesity as a risk factor for certain types of cancer. *Lipids* **11** 1055–1059.
Chehab FF, Lim ME & Lu R 1996 Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. *Nature Genetics* **12** 318–320.

- Cohen MM Jr 2006 Role of leptin in regulating appetite, neuroendocrine function, and bone remodeling. *American Journal of Medical Genetics* **140A** 515–524.
- Fazeli M, Zarkesh-Esfahani H, Wu Z, Maamra M, Bidlingmaier M, Pockley AG, Watson P, Matarese G, Strasburger CJ & Ross RJ 2006 Identification of a monoclonal antibody against the leptin receptor that acts as an antagonist and blocks human monocyte and T cell activation. *Journal of Immunological Methods* **312** 190–200.
- Fiorio E, Mercanti A & Terrasi M 2008 Leptin/HER2 crosstalk in breast cancer: *in vitro* study and preliminary *in vivo* analysis. *BMC Cancer* **8** 305–316.
- Fontana L 2009 The scientific basis of caloric restriction leading to longer life. *Current Opinion in Gastroenterology* **25** 144–150.
- Gao Q, Mezei G, Nie Y, Rao Y, Choi CS, Bechmann I, Leranath C, Toran-Allerand D, Priest CA, Roberts JL *et al.* 2007 Anorectic estrogen mimics leptin's effect on the rewiring of melanocortin cells and Stat3 signaling in obese animals. *Nature Medicine* **13** 89–94.
- Garofalo C & Surmacz E 2006 Leptin and cancer. *Journal of Cell Physiology* **1** 12–22.
- Garofalo C, Koda M, Cascio S, Sulkowska M, Kanczuga-Koda L, Golaszewska J, Russo A, Sulkowski S & Surmacz E 2006 Increased expression of leptin and the leptin receptor as a marker of breast cancer progression: possible role of obesity-related stimuli. *Clinical Cancer Research* **12** 1447–1453.
- Grossmann ME, Ray A, Dogan S, Mizuno NK & Cleary MP 2008 Balance of adiponectin and leptin modulates breast cancer cell growth. *Cell Research* **18** 1154–1156.
- Henderson BE, Ross R & Bernstein L 1988 Estrogens as a cause of human cancer: the Richard and Hinda Rosenthal Foundation award lecture. *Cancer Research* **48** 246–253.
- Hu X, Juneja SC, Maihle NJ & Cleary MP 2002 Leptin a growth factor in normal and malignant breast cells and for normal mammary gland development. *Journal of the National Cancer Institute* **94** 1704–1710.
- Lord GM, Matarese G, Howard JK, Baker RJ, Bloom SR & Lechler RI 1998 Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. *Nature* **394** 897–901.
- Mauro L, Catalano S, Bossi G, Pellegrino M, Barone I, Morales S, Giordano C, Bartella V, Casaburi I & Andò S 2007 Evidences that leptin up-regulates E-cadherin expression in breast cancer: effects on tumor growth and progression. *Cancer Research* **67** 3412–3421.
- Moore SC, Leitzmann MF, Albanes D, Weinstein SJ, Snyder K, Virtamo J, Ahn J, Mayne ST, Yu H, Peters U *et al.* 2008 Adipokine genes and prostate cancer risk. *International Journal of Cancer* **4** 869–876.
- Perera CN, Chin HG, Duru N & Camarillo IG 2008 Leptin-regulated gene expression in MCF-7 breast cancer cells: mechanistic insights into leptin-regulated mammary tumor growth and progression. *Journal of Endocrinology* **2** 221–233.
- Porta C, Larghi P, Rimoldi M, Grazia Totaro M, Allavena P, Mantovani A & Sica A 2009 Cellular and molecular pathways linking inflammation and cancer. *Immunobiology* **214** 761–777.
- Rodriguez C, Patel AV, Calle EE, Jacobs EJ, Chao A & Thun MJ 2001 Body mass index, height, and prostate cancer mortality in two large cohorts of adult men in the United States. *Cancer Epidemiology, Biomarkers & Prevention* **4** 345–353.
- Saxena NK, Taliaferro-Smith L, Knight BB, Merlin D, Anania FA, O'Regan RM & Sharma D 2008 Bidirectional crosstalk between leptin and insulin-like growth factor-I signaling promotes invasion and migration of breast cancer cells via transactivation of epidermal growth factor receptor. *Cancer Research* **68** 9712–9722.
- Stattin P, Söderberg S, Hallmans G, Bylund A, Kaaks R, Stenman UH, Bergh A & Olsson T 2001 Leptin is associated with increased prostate cancer risk: a nested case-referent study. *Journal of Clinical Endocrinology and Metabolism* **86** 1341–1345.
- Stephenson GD & Rose DP 2003 Breast cancer and obesity: an update. *Nutrition and Cancer* **1** 1–16.
- Toniolo PG, Levitz M, Zeleniuch-Jacquotte A, Banerjee S, Koenig KL, Shore RE, Strax P & Pasternack BS 1995 A prospective study of endogenous estrogens and breast cancer in postmenopausal women. *Journal of the National Cancer Institute* **87** 190–197.
- Woods SC & D'Alessio DA 2008 Central control of body weight and appetite. *Journal of Clinical Endocrinology and Metabolism* **93** S37–S50.
- Wu MH, Chou YC, Chou WY, Hsu GC, Chu CH, Yu CP, Yu JC & Sun CA 2009 Circulating levels of leptin, adiposity and breast cancer risk. *British Journal of Cancer* **100** 578–582.
- Zhang Y, Proenca R, Maffei M, Barone M, Leopold L & Friedman JM 1994 Positional cloning of the mouse obese gene and its human homologue. *Nature* **372** 425–432.