Survivin upregulation, dependent on leptin–EGFR–Notch1 axis, is essential for leptin-induced migration of breast carcinoma cells

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Abstract

Obese breast cancer patients exhibit a higher risk for larger tumor burden and an increased likelihood of metastasis. The molecular effects of obesity on carcinogenesis are mediated by the autocrine and paracrine effects of the adipocytokine leptin. Leptin participates in the tumor progression and metastasis of human breast. We show that leptin induces clonogenicity and increases the migration potential of breast cancer cells. We found that survivin expression is induced in response to leptin. In this study, we examine the role and leptin-mediated regulation of survivin. Leptin treatment leads to survivin upregulation, due in part to the activation of Notch1 and the release of a transcriptionally active Notch1 intracellular domain (NICD). Chromatin immunoprecipitation analysis shows that NICD gets recruited to the survivin promoter at the CSL (CBF1/RBP-Jk, Su(H), Lag-1) binding site in response to leptin treatment. Inhibition of Notch1 activity inhibits leptin-induced survivin upregulation. Leptin-induced transactivation of epidermal growth factor receptor (EGFR) is involved in leptin-mediated Notch1 and survivin upregulation, demonstrating a novel upstream role of leptin–EGFR–Notch1 axis. We further show that leptin-induced migration of breast cancer cells requires survivin, as overexpression of survivin further increases, whereas silencing survivin abrogates leptin-induced migration. Using a pharmacological approach to inhibit survivin, we show that 3-hydroxy-3-methylglutaryl-coenzyme-A-reductase inhibitors, such as lovastatin, can effectively inhibit leptin-induced survivin expression and migration. Importantly, leptin increased breast tumor growth in nude mice. These data show a novel role for survivin in leptin-induced migration and put forth pharmacological survivin inhibition as a potential novel therapeutic strategy. This conclusion is supported by in vivo data showing the overexpression of leptin and survivin in epithelial cells of high-grade ductal carcinomas in situ and in high-grade invasive carcinomas.

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Introduction

Leptin is a multifunctional adipocytokine with a broad range of biological activities ranging from regulating appetite and energy expenditure to modulation of various processes such as reproduction, lactation, hematopoiesis, cell differentiation, and importantly carcinogenesis (Huang & Li 2000). Leptin exerts its actions through its specific transmembrane receptors (OB-R) present in a variety of tissues (Ahima & Osei 2004) by activation of various downstream effector molecules. Several epidemiological studies have linked high levels of plasma leptin with increased risk for breast carcinogenesis, but a direct link between leptin signaling and breast cancer was first established...
in a clinical study showing that leptin receptors were not detectable in normal mammary epithelial cells by immunohistochemistry, whereas in 83% of cases, carcinoma cells showed positive staining for the leptin receptor (Ishikawa et al. 2004). Importantly, overexpression of leptin was observed in 92% of examined breast tumors but in none of the normal breast epithelium cases (Ishikawa et al. 2004). Another noteworthy study showed a positive relationship between blood leptin levels and breast cancer risk. In addition, the degree of leptin mRNA expression in the peritumoral adipose tissue was significantly higher in the breast cancer patients than the control women (Tessitore et al. 2000). A recent study also showed that leptin and leptin receptor are overexpressed in primary and metastatic invasive ductal breast carcinoma compared with non-cancer mammary tissue (Garofalo et al. 2006). These studies suggest the importance of paracrine and autocrine effects of leptin and the importance of leptin receptor in breast carcinogenesis. In recent years, many laboratories including ours have shown that leptin increases proliferation of breast, endometrial, hepato-cellular, and many other cancer cells via multiple signaling pathways including Stat3/ERK/Akt signaling (Housa et al. 2006, Sharma et al. 2006b, Saxena et al. 2007a,b, 2008). Leptin signaling can induce various molecules implicated in cell growth, adhesion, inflammation, and angiogenesis, such as β3 integrin (Gonzalez & Leavis 2001), interleukin 1 (IL1), IL1 receptor (Pinteaux et al. 2007), vascular endothelial growth factor, and its receptor type 2 (Gonzalez et al. 2006). Leptin regulates the cell cycle by inducing cyclin D1 expression modulating its local chromatin structure (Saxena et al. 2007b). Leptin increases E-cadherin expression via enhancing CREB-DNA and Sp1-DNA binding activity to E-cadherin promoter and modulates homotypic tumor cell adhesion and proliferation (Mauro et al. 2007). Thus, uncontrolled leptin-induced signaling could contribute to various aspects of tumorigenesis and metastasis by modulating key involved genes.

Survivin, a member of the inhibitor of apoptosis protein (IAP) family, mainly functions to suppress apoptosis and regulate cell division (Altiieri 2004). Although not expressed in normal adult differentiated tissue, it is present in large amounts in fetal tissue and most cancers, regardless of lineage, differentiation, and histological type (Ambrosini et al. 1997). Survivin expression correlates with increased aggressiveness, higher recurrence rate, increased metastasis, unfavorable disease outcome, and abbreviated overall survival in colorectal, gastric, breast, ovarian, and hepatocellular carcinomas, and in neuroblastomas (Kawasaki et al. 1998, Adida et al. 2000, Satoh et al. 2001, Altiieri 2004). The elucidation of the cancer-specific transcription of the survivin promoter showed convergence of several oncogenic pathways to upregulate survivin gene expression in transformed cells. Growth factor receptor signaling, Stat activation, phosphoinositide 3-kinase (PI3K)/Akt signaling, oncogene (Ras) expression, and loss of the tumor suppressor molecules, p53, APC, and PML (O’Connor et al. 2000, Zhang et al. 2001, Hoffman et al. 2002, Mirza et al. 2002, Aoki et al. 2003, Kim et al. 2003, Sommer et al. 2003, Dan et al. 2004, Xu et al. 2004) are a few of the oncogenic pathways implicated in survivin regulation in cancer cells. Some downstream effector molecules of leptin signaling (such as Stat3 and Akt) participate in the regulation of survivin expression. Increased survivin expression has been associated with more aggressive tumor behavior and parameters of poor prognosis in breast cancer. Survivin is an important IAP owing to its cancer-specific overexpression and its importance in inhibiting cell death and regulating cell division. Overexpression of leptin was observed in 92% of examined breast tumors (Ishikawa et al. 2004). We hypothesized that elevated expression of leptin in breast tumors may upregulate survivin expression. We found that leptin increases the expression of survivin mRNA and protein.

In this study, we specifically investigated the effect of leptin-induced survivin on migration of breast cancer cells and examined the underlying molecular mechanisms by which leptin upregulates survivin expression. Intriguingly, we discovered the involvement of a novel upstream leptin–epidermal growth factor receptor (EGFR)–Notch1 axis in survivin regulation, in breast cancer cells treated with leptin. We also found that survivin is required for the leptin-mediated migration of breast cancer cells and that pharmacological inhibition of survivin can inhibit this early event of malignant progression.

Materials and methods
Antibodies
Antibodies for survivin and leptin (Ob) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for XIAP, EGFR, pTyr, Notch1, and Notch1 intracellular domain (NICD) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies for β-actin were purchased from Sigma–Aldrich.
Cell culture, reagents, and treatments

The human breast cancer cell lines, MCF7 and MDA-MB-231, were maintained in DMEM supplemented with 10% fetal bovine serum (FBS; Gemini Bio-products, Woodland, CA, USA) and 2 μM L-glutamine (Invitrogen; Saxena et al. 2008). For treatment, cells were seeded at a density of 1×10⁶/100 mm tissue culture dish. For leptin treatments, cells were incubated in serum-free media for 24 h followed by treatment with human recombinant leptin (Sigma–Aldrich) at 100 ng/ml (Saxena et al. 2008) for indicated durations. In other sets of experiments, cells were treated with EGFR inhibitor erlotinib at 2.5 μM alone and in combination with leptin. In some experiments, cells were treated with 20–40 μM lovastatin (Sigma–Aldrich). G-Secretase inhibitor LY411,575 was kindly provided by Dr Clodia Osipo (Loyola University). For electric cell-substrate impedance sensing (ECIS) migration assay, ECIS cell culture ware was purchased from Applied Biophysics (Troy, NY, USA).

Clonogenicity assay

Colony formation assay was performed following our previously published protocol (Taliaferro-Smith et al. 2009). MCF7 and MDA-MB-231 breast cancer cells (single-cell suspension) were plated in 12-well plates at a density of 250 cells per well overnight. The following day, cells were treated with 100 ng/ml human recombinant full-length leptin and the medium was replaced with fresh medium containing leptin every 3 days. After a 10-day treatment period, the medium was removed and cell colonies were stained with crystal violet (0.1% in 20% methanol). Colony numbers were assessed visually and colonies containing >50 normal-appearing cells were counted. Pictures were taken using a digital camera. All experiments were performed at least three times in triplicate.

Anchorage-independent growth assay

Anchorage-independent growth of MCF7 and MDA-MB-231 cells was assayed by colony formation on soft agar. Briefly, equal volumes of agar (1.2%) and complete medium were mixed to make 0.6% agar growth medium solution in 6-well tissue culture plates. Cells (2×10³ cells/well) were suspended in media with or without treatment followed by mixing with equal volume of agar (0.6%). Cell suspension-agar mix (2 ml) was then added to each well. Plates were incubated at 37 °C with 5% CO₂ in humidified incubator for 3 weeks, and media with or without treatment were added every 3 days. Colonies were stained with 0.005% crystal violet in PBS for 1 h at room temperature and observed using Olympus IX50 inverted microscope. Colonies were counted in six randomly selected fields at 10× magnification. Results are expressed as number of colonies counted. All experiments were performed three times in triplicate.

Migration assay

Migration assays were performed following previously published protocol (Saxena et al. 2007a). Cells were plated into 24-well cell culture plate and pre-coated with human fibronectin (5 μg/cm², Sigma). Cells were allowed to grow in 10% FBS containing DMEM medium to confluence and then were washed with serum-free medium and serum starved for 16 h. A 1 mm wide scratch was made across the cell layer using a sterile pipette tip. After washing with serum-free medium twice, DMEM medium containing 10 μg/ml human fibronectin was added to replace matrix depleted with the cells. Plates were photographed immediately after scratching. Cells were treated with human recombinant leptin at 100 ng/ml. Plates were photographed after 24 and 48 h at the identical location of the initial image. All experiments were performed at least three times.

Invasion assay

Matrigel invasion assay was performed using a Matrigel invasion chamber from BD Biocoat Cellware (San Jose, CA, USA). Cells were seeded at a density of 1×10⁵ cells per insert and cultured overnight. After 16 h of serum starvation, the culture media were changed to serum-free media containing treatments as indicated. Triplicate wells were used for each treatment. Cells were treated with leptin as indicated. After 24 h of incubation, cells remaining above the insert membrane were removed by gentle scraping with a sterile cotton swab. Cells that had invaded through the Matrigel to the bottom of the insert were fixed in methanol for 10 min. After washing with PBS, the cells were stained with hematoxylin–eosin. The insert was subsequently washed with PBS and briefly air-dried and mounted. The slides were coded to prevent counting bias, and the number of invaded cells on representative sections of each membrane was counted under light microscope. The number of invaded cells for each experimental sample represents the average of triplicate wells. All experiments were performed at least three times.
RNA isolation and RT-PCR
RT-PCR analysis was performed following previously published protocol (Sharma et al. 2006a), using specific primers for survivin, actin, Notch1, Notch4, and Hey1. Total cellular RNA was extracted using the TRIZOL Reagent kit (Life Technologies, Inc.) and quantified by u.v. absorption. RT-PCR was carried out using specific sense and anti-sense PCR primers for amplification. PCR products were resolved by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The primers used were survivin-wt sense, 5′-GGA CCA CGG CAT CTC TAC AT-3′ and anti-sense, 5′-GAC AGA AAG GAA AGC GCA AC-3′; β-actin sense, 5′-ACC ATG GAT GAT ATC GC-3′ and anti-sense, 5′-ACA TGG CTG GGG TGT TGA AG-3′; Notch1 sense, 5′-GCA ACA GCT CCT TCC ACT TC-3′ and anti-sense, 5′-GCC TCA GAC TCT AGT AGT TG-3′; Hey1 sense, 5′-AGC TCC TCG GAC AGC GAG CTG-3′ and anti-sense, 5′-TAC CAG CCT TCT CAG TAC AGA CA-3′.

Western blot
Whole cell lysate was prepared following previously described method (Sharma et al. 2006b). Whole cell lysates were prepared by scraping cells in 250 μl of ice-cold modified RIPA buffer (50 mM Tris–Cl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% Na-deoxycholate, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM Na3VO4, and 1 mM NaF). The lysate was rotated 360° for 1 h at 4 °C followed by centrifugation at 12 000 g for 10 min at 4 °C to clear the cellular debris. Proteins were quantified using the Bradford protein assay kit (Bio-Rad). Equal amounts of proteins were resolved on SDS-polyacrylamide gels and transferred to nitrocellulose membranes, and western blot analyses were performed using the previously described antibodies. Immunodetection was performed by blocking the membranes for 1 h in TBS buffer (20 mM Tris–Cl (pH 7.5), 137 mM NaCl, and 0.05% Tween-20) containing 5% powdered nonfat milk followed by addition of the primary antibody (as indicated) in TBS for 2 h at room temperature. Specifically bound primary antibodies were detected with peroxidase-coupled secondary antibodies and developed by enhanced chemiluminescence (ECL system, Amersham Pharmacia Biotech, Inc.) according to the manufacturer’s instructions. All experiments were performed at least three to five times using independent biological replicates.

Immunohistochemistry
These studies are approved by Institutional Review Board. Archived specimens of paraffin-embedded normal breast tissue (n=4), ductal carcinoma in situ (DCIS) (n=26), and associated invasion ductal carcinoma (IDC) (n=26) were obtained from the Emory University Hospital Systems Breast Tumor Bank. Sections were deparaffinized, subjected to antigen retrieval, and incubated with primary antibody. The survivin antibody was from Cell Signaling Technology. The leptin antibody (Ob, A-20) was from Santa Cruz Biotechnology, Inc. Immunocomplexes were detected by the labeled polymer method (EnVision Dual-Link-System-HRP, DAKO, Carpinteria, CA, USA). Sections were counterstained with hematoxylin.

Chromatin immuno precipitation
Chromatin samples were sonicated on ice three times for 10 s each (i.e. until the average length of sheared genomic DNA was 1–1.5 kb) followed by centrifugation for 10 min. The immunoprecipitated DNA was ethanol precipitated and resuspended in 25 μl H2O. Total input samples were resuspended in 100 μl H2O and diluted 1:100 before PCR analysis. Initially, PCR was performed with different numbers of cycles and/or dilutions of input DNA to determine the linear range of amplification; all results show fall within this range. Following 28–30 cycles of amplification, PCR products were run on 1% agarose gel and analyzed by ethidium bromide staining. All chromatin immunoprecipitation (ChIP) assays were performed at least thrice with similar results.

Immunofluorescence and confocal imaging
Breast cancer cells (5×105 cells/well) were plated in 4-well chamber slides (Nunc, Rochester, NY, USA) followed by treatment with 100 ng/ml human recombinant full-length leptin for 2 h. Cells were washed three times with 1× PBS and fixed using freshly prepared fixative containing 3.7% formaldehyde, 0.05% glutaraldehyde, and 0.4% Triton-X-100 in PHEMO buffer (0.068 mol/l PIPES, 0.025 mol/l HEPES, 0.015 mol/l EGTA, 0.003 mol/l MgCl2, and 10%v/v DMSO) for 10 min at room temperature. Primary antibodies (as indicated) were used at 1:500 with an overnight incubation at 4 °C followed by an anti-rabbit IgG Alexa Flour 488 secondary antibody used at 1:500 for 1 h at room temperature. The nucleus was stained with 4′,6-diamidino-2-phenylindole (Sigma–Aldrich) using 300 nmol/l at room temperature for 10 min before mounting in Gel
Mount mounting medium (Biomeda, Foster City, CA, USA). Fixed and immunofluorescently stained cells were imaged using a Zeiss LSM510 Meta (Zeiss, Thornwood, NY, USA) laser scanning confocal system configured to a Zeiss Axioplan 2 upright microscope with a 63XO (NA 1.4) plan-apochromat objective. All experiments were performed multiple times using independent biological replicates.

**Immunoprecipitation of EGFR**

Whole cell lysates from breast cancer cells were incubated with specific antibodies for EGFR and the immunoprecipitation was performed following previously described method (Saxena et al. 2008). Whole cell lysate from breast cancer cells was incubated with specific antibodies for EGFR and the mixture was rotated slowly at 4 °C for 16 h. A total of 20 μl packed protein A/G agarose beads was added and the mixture was incubated at 4 °C for 1 h with rotation. The beads were collected by gentle centrifugation and washed twice with 1.5 ml ice-cold buffer (50 mM Tris–Cl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% Na-deoxycholate, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). After the final wash, the precipitated protein–beads complexes were resuspended in SDS-sample loading buffer, fractionated by SDS-PAGE, and transferred to nitrocellulose membrane. Immunodetection was performed by blocking the membranes for 1 h in TBS buffer (20 mM Tris–Cl (pH 7.5), 137 mM NaCl, and 0.05% Tween-20) containing 5% powdered nonfat milk followed by addition of the primary antibody (as indicated) in TBS for 2 h at room temperature. Specifically bound primary antibodies were detected with peroxidase-coupled secondary antibodies and developed by ECL system (Amersham Pharmacia Biotech, Inc.) according to the manufacturer’s instructions.

**Transfections**

Breast cancer cells were transiently transfected with survivin-wild type or psilencer-survivin plasmid or EGFR siRNA (Santa Cruz). Breast cancer cells were transiently transfected with 1.0 μg of survivin-wild type or psilencer-survivin plasmid using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen). After 48 h of transfection, the cells were treated with 100 ng/ml human recombinant full-length leptin for the indicated durations. Cells were harvested and total protein lysates were subjected to western blot analysis using specific antibodies as described. The experiments were performed in triplicate, and similar results were obtained from at least three independent experiments. Cells transfected with survivin-wild type or psilencer-survivin were used to perform migration assay as described above. Cells were transfected with EGFR siRNA (Santa Cruz) using a Lipofectamine 2000 according to the manufacturer’s instructions. Cells were subjected to western blot analysis as described above.

**ECIS wound-healing assays**

Wound-healing assays were performed using the ECIS (Applied BioPhysics; Saxena et al. 2008). For wound-healing assays, cells were grown to confluence on ECIS plates and serum starved for 16 h. The ECIS plates were submitted to an elevated voltage pulse of 40 kHz frequency, 3.5 V amplitude, and 30 s duration, which led to the death and detachment of cells present on the small active electrode resulting in a wound normally healed by cells surrounding the small active electrode that have not been submitted to the elevated voltage pulse. Cells were immediately treated with 100 ng/ml leptin. Wound healing was then assessed by continuous resistance measurements for ~24 h. All experiments were performed at least three times in triplicates.

**Breast tumorigenesis assay**

MDA-MB-231 (5 × 10⁶) cells in 0.1 ml HBSS were s.c. injected into the right gluteal region of 4–6-week-old female athymic nude mice. After 2 weeks of initial implantation, animals were placed into two experimental groups. Mice were treated with i.p. injections of saline or recombinant leptin (dosage of 5 mg/kg), 5 days a week for the duration of the experiment. Tumors were measured using vernier calipers, with tumor volume calculated using the formula \( V = \frac{a \times b^2}{2} \), where \( V \) is the tumor volume in \( \text{mm}^3 \), \( a \) and \( b \) are the largest and smallest diameters in mm respectively. All animals were killed after 6 weeks of treatment. Tumors were collected; weighed, fixed in 10% neutral-buffered formalin; and subjected to further analysis by immunohistochemistry. All animal studies were conducted in accordance with the guidelines of University IACUC.

**Statistical analysis**

All experiments were independently performed three times in triplicate. Statistical analysis was performed using Microsoft Excel software. Significant differences were analyzed using Student’s t-test and two-tailed distribution. Data were considered to be statistically significant if \( P < 0.05 \). Data are expressed as mean ± S.E.M. between triplicate experiments.
Results
Leptin treatment increases growth and migration potential of breast cancer cells along with increased survivin expression

Epidemiological studies have shown that high leptin levels are significantly associated with an increased breast tumor growth and metastasis (Berclaz et al. 2004, Chen et al. 2006). The metastatic process is a complex cascade of events involving the ability of cancer cells to migrate and form colonies in an anchorage-independent manner (Gupta & Massague 2006). We examined the effect of leptin on breast cancer cell growth and migration potential. Breast cancer cells were treated with various concentrations of leptin and subjected to growth and clonogenicity assays. Substantial stimulation was observed after treatment of cells at 100 ng/ml leptin, whereas higher concentrations were not particularly more stimulatory (Supplementary Figures 1 and 2, see section on supplementary data given at the end of this article). Leptin treatment resulted in increased clonogenicity (Fig. 1A) in comparison to untreated cells. Then, we performed a soft agar assay to assess changes in the anchorage-independent growth of breast cancer cells in response to leptin treatment. Untreated cells formed very few colonies in soft agar, whereas leptin treatment resulted in significantly higher number of colonies (Fig. 1B). Leptin treatment increased the migration potential of breast cancer cells in a quantitative ECIS-based migration assay (Fig. 1C). Leptin increased invasion of breast cancer cells in a Matrigel invasion assay (Fig. 1D). Uncontrolled leptin signaling can induce various genes involved in proliferation, adhesion, and angiogenesis, thereby contributing to various aspects of tumorigenesis and metastasis (Cirillo et al. 2008). While analyzing the effect of leptin on some key target genes involved in cancer growth regulation, we found that leptin treatment increased survivin mRNA expression in a real-time RT-PCR analysis within 15 min (Fig. 2A). Leptin increased survivin protein expression within 15 min, which remained elevated for the duration of the experiment (Fig. 2B). Leptin increased survivin expression in a dose-dependent manner. Statistically significant increase in survivin expression was observed at 100 ng/ml leptin, whereas the higher concentrations were not particularly more stimulatory.

Figure 1 Leptin promotes clonogenicity, anchorage-independent growth, and migration of breast cancer cells. (A) Breast cancer cells were subjected to colony-formation assay in the presence or absence of leptin. Colonies containing > 50 normal-appearing cells were counted. Leptin increases clonogenicity. (B) Breast cancer cells were subjected to soft agar colony-formation assay in the presence (L) or absence (U) of leptin for 3 weeks. Results are expressed as average number of colonies counted (in six micro-fields). *P < 0.005 compared with untreated controls. Leptin augments anchorage-independent growth. (C) Breast cancer cells were subjected to ECIS migration assay in the absence (U) or the presence of leptin (L) as indicated. Cells were allowed to grow to confluence and initial resistance was measured for few hours. Cells were subjected to high voltage to initiate wound at 3 h resulting in the drop of resistance. Remaining cells were allowed to migrate in the presence or absence of leptin and resistance changes were measured for ~24 h after the creation of wound. Cells migrate faster in the presence of leptin. (D) Breast cancer cells were subjected to Matrigel invasion assay in the presence (L) or absence (U) of leptin for 24 h. The number of cells that invaded through the Matrigel was counted in six different regions. The slides were blinded to remove counting bias. The results show mean of three independent experiments performed in triplicates. *P < 0.001 compared with untreated controls.
Overexpression of survivin and leptin is associated with tumor progression in breast tumors

The above results prompted us to investigate the pattern of survivin and leptin expression in a panel of normal breast tissue and ER, PR-positive tumors. Immunohistochemical studies on normal breast tissue, DCIS (n = 26), and IDC (n = 26) tumor samples showed increased survivin expression in 60% of DCIS and 85% of IDC samples compared with normal breast tissue. Survivin expression was enhanced in the cytoplasmic region in breast cancer cells, filling the ducts. In agreement with our studies, leptin overexpression was observed in DCIS and IDC samples exhibiting overexpression of survivin, whereas normal breast epithelial cells showed minimal staining (Fig. 2C and D). Adipocytes and stromal cells surrounding the tumoral region also showed increased leptin expression (data not shown). The overexpression of survivin and leptin in DCIS and IDC as well as overexpression of leptin in the surrounding adipocytes indicates their involvement in the progression of breast cancer.

Notch1 activation plays an important role in leptin-induced survivin overexpression in breast cancer cells

We next sought to determine the biological mechanism by which survivin is upregulated in the context of the oncogenic effects of leptin. Recent studies have
suggested that activation of the PI3K/Akt pathway results in the upregulation of survivin expression (Wang & Greene 2005). We found that inhibition of Akt or ERK activation using specific inhibitors did not affect leptin-induced activation of survivin (data not shown). Interactions among various growth factor signaling pathways, such as cross talk between IGF1R and EGFR (Higashiyama et al. 2008), and EGFR and Notch1 (Wu et al. 2007), have been observed in response to specific stimuli. In particular, Notch1 signaling has emerged as a pivotal target in breast cancer with Notch1 receptors acting as survival factors promoting breast cancer. Mammalian Notch1 receptors are type-1 membrane proteins (Wu et al. 2007). Intriguingly, we found that

*Figure 3* Leptin-induced Notch1 expression and activity play an important role in regulating survivin expression in the presence of leptin. (A) Breast cancer cells were treated with leptin for indicated time intervals. Untreated cells are denoted with U. Change in Notch1 mRNA expression was analyzed by RT-PCR analysis using specific primer sets. (B) Notch1 protein and NICD levels were detected by western blot analysis using specific antibodies. Leptin treatment increases Notch1 expression and NICD levels. (C) Breast cancer cells were treated with leptin for indicated time intervals. Total RNA was isolated and subjected to RT-PCR analysis using specific primer sets for Notch1 targets, Hey1 and Notch4. Leptin induces transcriptional activation of Hey1 and Notch4. (D) Breast cancer cells were treated with γ-secretase inhibitor (GSI) and leptin alone or in combination. Untreated cells are denoted with U. Lysates were probed with survivin and actin antibodies. GSI treatment inhibits survivin expression alone and even in the presence of leptin. (E) Breast cancer cells were untreated or treated with leptin and subjected to ChIP analysis using NICD antibodies. Immunoprecipitated chromatin was analyzed using specific primer sets encompassing CSL (CBF1/RBP-Jk, Su(H), Lag-1) binding site in survivin promoter. IgG was used as a control. NICD gets recruited to survivin promoter in the presence of leptin.
leptin treatment increased *Notch1* mRNA expression within 15 min of treatment (Fig. 3A). Leptin also increased *Notch1* protein expression within 15 min post-treatment. Notch1 receptors are heterodimeric proteins consisting of extracellular Notch (NotchEC) and transmembrane Notch (NotchT). Activation of Notch1 receptors involves cleavage of the Notch1 receptor by γ-secretase to release transcriptionally active intracellular Notch (NotchIC or NICD; Callahan & Raafat 2001, Miele 2008). Leptin treatment resulted in the release of NICD confirming Notch1 activation (Fig. 3B). To unequivocally determine the activity of Notch1 in response to leptin, we also examined the expression of endogenous Notch1 targets, Hey1 and Notch4 (Weijzen et al. 2002). Leptin increased expression of Hey1 and Notch4 (Fig. 3C) showing elevated Notch1 activity.

To examine the direct role of Notch1 activation in survivin expression, we treated breast cancer cells with a specific γ-secretase inhibitor (GSI) (LY411,575) and found that inhibition of Notch1 activation inhibits survivin expression (Fig. 3D). Importantly, GSI treatment was able to inhibit the leptin-induced increase in survivin expression in a co-treatment experiment (Fig. 3D). The intracellular domain of Notch1, NICD, translocates to the nucleus and interacts with CSL (CBF1/RBP-Jk, Su(H), Lag-1) resulting in the transcriptional activation of Notch1 targets. In order to define the mechanism of regulation of survivin mediated by Notch1, we searched for possible regulatory elements related to the Notch1 signaling pathway in the survivin promoter. We identified a putative CSL binding site located at position -1218 in the human survivin promoter. The sequence of the putative CSL-binding site is GCTGAGAT; this sequence is different from the consensus sequence for the high-affinity CSL-binding site (GTGGGAA) previously described (Ronchini & Capobianco 2001). However, similar sequences have been identified in the human cyclin D1 promoter and human β-globin locus control region, which have been shown to be authentic CSL-binding sites (Ronchini & Capobianco 2001). We examined the ability of leptin-induced NICD to bind the identified site by ChIP assay. Importantly, we found the recruitment of NICD to the survivin promoter in the presence of leptin (Fig. 3E), whereas no binding was observed in the absence of leptin. Collectively, these results showed that leptin induces expression and activation of Notch1 and leptin-activated Notch1 binds to survivin promoter; hence, Notch1 plays an important role in the regulation of survivin expression in breast cancer cells.

**Cross talk between leptin and Notch1 signaling involves EGFR**

EGFR transactivation, as observed in response to IGF1, E-cadherin, integrins, and activation of G-protein-coupled receptors impacts various physiological processes (Higashiyama et al. 2008). We recently showed that co-treatment with leptin and IGF1 results in increased phosphorylation of EGFR (Saxena et al. 2008). Treatment of MCF7 and MDA-MB-231 cells with leptin resulted in increased phosphorylation of EGFR, as evident in the immunoprecipitation of EGFR followed by immunoblotting with an anti-phosphotyrosine antibody (Fig. 4A). Phosphorylated tyrosine bands corresponded to the expected size band for EGFR. Then, we investigated the role of EGFR in leptin-induced Notch1 activation in breast cancer cells by silencing EGFR using *EGFR* siRNA. *EGFR* siRNA efficiently inhibited EGFR expression (Fig. 4B). Interestingly, leptin-induced Notch1 expression was inhibited in the presence of *EGFR* siRNA, demonstrating the requirement of EGFR in leptin-induced Notch1 expression (Fig. 4B). Activation of the EGFR signaling

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**Figure 4 Involvement of EGFR transactivation in Notch1 and survivin induction in the presence of leptin.** (A) Breast cancer cells were untreated (U) or treated with leptin (L). Lysates were immunoprecipitated with EGFR antibody and probed with anti-phospho-tyrosine antibody. (B) Breast cancer cells were transfected with *EGFR* siRNA, demonstrating the effect of *EGFR* siRNA on EGFR expression (Fig. 4B). Interestingly, leptin-induced Notch1 expression was inhibited in the presence of *EGFR* siRNA, demonstrating the requirement of EGFR in leptin-induced Notch1 expression (Fig. 4B). Activation of the EGFR signaling...
pathway leads to the up-regulation of survivin gene expression (Asanuma et al. 2005). Then, we examined the importance of EGFR activation in leptin-induced increased expression of survivin. We found that pre-incubation of breast cancer cells with the EGFR inhibitor erlotinib significantly inhibited the stimulatory effect of leptin on survivin expression (Fig. 4C) showing that EGFR activation is required for leptin-induced survivin upregulation. Together, these data showed that transactivation of EGFR is upstream of the activation of Notch1 in the context of leptin, revealing the hierarchy of these events and the involvement of the leptin–EGFR–Notch1 axis in survivin upregulation.

Inhibition of survivin disrupts leptin-induced migration of breast cancer cells

Although cancer cell metastasis is a complex process involving various intermediary steps from alteration of microenvironment to colonization in distant organ sites, migration is one of the most important early events (Gupta & Massague 2006). After examining the molecular mechanisms involved in leptin-induced survivin upregulation, we then examined the role of survivin in the biological functions of leptin. Migratory cells typically undergo morphological changes as individual cells show podia formation, irregular membrane ruffling, and the formation of protrusions at the leading edge of these podias (Insall & Machesky 2009). Confocal immunofluorescence microscopy of breast cancer cells forced to be motile in the presence of leptin revealed that the survivin protein was highly concentrated at the tips of podia of migrating cells (Fig. 5A). Based on the above morphologic observations, we speculated that the leptin-mediated induction of survivin might increase cell motility. To test this hypothesis, we modulated survivin expression in breast cancer cells by transfecting full-length wild-type survivin and p-silencer-survivin plasmids (Fig. 5B). These breast cancer cells were subjected to a scratch migration assay in the presence or absence of leptin. Leptin induced migration of breast cancer cells within 6 h of treatment. Survivin overexpression further increased leptin-induced migration, whereas silencing survivin abrogated leptin-induced migration (data not shown). Then, we performed a quantitative real-time impedance assay using an ECIS-based technique to follow the migration of breast cancer cells. Leptin-treated vector-transfected cells displayed an increase in resistance, showing increased migration in comparison with untreated vector-transfected cells. Cells overexpressing survivin migrated at a higher rate than cells transfected with either vector or p-silencer-survivin. Cells overexpressing survivin rapidly migrated to reach the resistance values of the non-wounded cells at the start of the experiment in response to leptin. Whereas cells transfected with p-silencer-survivin showed no significant change in migration even in the presence of leptin (Fig. 5C).

Then, we examined the requirement of survivin upregulation in leptin-mediated migration of breast cancer cells. HRIs, 3-hydroxy-3-methylglutaryl-coenzyme-A-reductase inhibitors, which are widely used...
to reduce the serum cholesterol levels in hypercholes-
terolemia patients, decrease survivin expression in
cancer cells (Kaneko et al. 2007). As shown in Fig. 6A,
leptin increased survivin expression in breast cancer
cells, whereas treatment with the HRI lovastatin inhibited
survivin expression. Pre-treatment with the HRI lovasta-
tatin significantly inhibited leptin-induced survivin
expression (Fig. 6A). Then, we performed a scratch
migration assay in the presence of leptin and lovastatin
treatments. As expected, leptin increased migration,
whereas lovastatin treatment inhibited leptin-induced
migration (Fig. 6B). These results collectively showed
that leptin-induced survivin upregulation is required
for leptin-induced migration of breast cancer cells and
that disruption of survivin expression using lovastatin
might be a valid therapeutic approach to counter the
effects of leptin on breast cancer cells.

Leptin treatment induces breast tumor progression in athymic nude mice

We investigated the physiological relevance of our in vitro findings by evaluating the tumor-promoting effects of leptin on the development of breast cancer in vivo. Leptin treatment significantly increased tumor growth compared with the saline-treated group (Fig. 7A and B). We further confirmed our in vitro findings regarding important signaling molecules. We used tumor sections from this study to determine the effect of leptin administration on the expression of survivin, NICD, and pEGFR proteins by immunohistochemistry. Leptin-treated tumors revealed elevated survivin, NICD, and pEGFR levels in comparison to saline-treated controls (Fig. 7C). Collectively, these in vitro and in vivo studies demonstrated a cogent tumor-promoting effect of leptin and the involvement of Notch–EGFR–survivin.

Discussion

In this study, we have revealed a novel mechanism of regulation of leptin-induced migration of breast cancer cells. The following novel findings are described in this study: a) leptin-mediated upregulation of survivin requires Notch1 activation; b) leptin induces recruitment of NICD to the survivin promoter; c) leptin regulates Notch1 via EGFR transactivation; d) survivin overexpression further increases leptin-induced migration of breast cancer cells, whereas survivin-inhibition abrogates leptin-induced migration; e) pharmacological inhibition of survivin effectively inhibits leptin-induced migration of breast cancer cells; f) both survivin and leptin are overexpressed in DCIS and IDC tumors compared with normal breast tissue. These results show that leptin treatment significantly increases survivin expression via EGFR–Notch1 axis.
We also show a novel integral role of the anti-apoptotic protein survivin in leptin-mediated migration of breast cancer cells. Thus, pharmacological inhibition of survivin may be a suitable strategy for treating metastatic breast cancer progressing in the presence of high leptin levels.

Survivin is a structurally unique anti-apoptotic protein with known dual function in apoptosis and control of mitosis (Altieri 2004). Survivin acts as caspase inhibitor thus blocking caspase activation and inhibiting the biological activities of activated caspases (Altieri 2004). Our current studies demonstrate that leptin induces survivin expression at both mRNA and protein levels in breast cancer cells. Interaction between various growth factor signaling pathways have previously been described. Our previous studies showed that bidirectional cross talk between leptin and IGF1 leads to transactivation of EGFR (Saxena et al. 2008). Recent studies have shown that activation of EGFR with EGF treatment upregulates the expression of survivin (Peng et al. 2006). Thus, leptin-mediated EGFR activation may be responsible for leptin-mediated survivin upregulation. Our studies show for the first time that leptin-mediated survivin upregulation involves EGFR activation. Importantly, the inhibition of EGFR activity inhibits leptin-induced survivin upregulation. Our studies support the concept that the high levels of leptin and EGF associated with obesity can act in concert to increase survivin expression and increase the negative influence of obesity on breast carcinogenesis.

At present, it is unclear how EGFR signaling leads to the overexpression of survivin. EGFR-mediated activation of the PI3K pathway may lead to survivin upregulation (Wang & Greene 2005). Also, involvement of EGFR–HIF1-α cross talk in the regulation of survivin expression (Peng et al. 2006) has been shown. Our studies demonstrate a novel regulatory mechanism involving the EGFR–Notch1 axis for leptin-induced survivin upregulation. Both leptin and Notch1 have previously been linked with poor prognosis in cancer in various epidemiological, clinical, and preclinical studies; however, no studies have shown a direct link between the two pro-oncogenic signals. We found that leptin treatment leads to upregulation of Notch1 in breast cancer cells. Notch1 receptors are cell-fate regulatory proteins that also act as survival proteins in cancer. Activation of Notch1 not only causes mammary tumors in mice, but is also associated with poor prognosis for breast cancer development (Dievart et al. 1999, Stylianou et al. 2006). Our studies show that the oncogenic potential of leptin might be due in part to the activation of Notch1 receptors. In a microarray analysis, Notch1 inhibition has been shown to inhibit EGFR expression in glioma and colon cancer cells (Purow et al. 2008), thus presenting EGFR as a downstream target of Notch1 signaling. Interestingly, we found cross talk between Notch1 and EGFR in breast cancer cells where EGFR silencing inhibits leptin-induced Notch1 expression, thus showing the direct upstream involvement of EGFR in leptin-mediated Notch1 upregulation. We further found that inhibiting Notch1 using GSI inhibits survivin expression in breast cancer cells. Our study reveals the direct involvement of the leptin–EGFR–Notch1 axis in the regulation of survivin expression in breast cancer cells.
The most remarkable feature of survivin is its differential expression in cancer versus normal tissues. Survivin plays an important role in development; it is strongly and diffusely expressed in embryonic and fetal organs, whereas most terminally differentiated normal tissues do not express survivin (Altieri 2003). In particular, overexpression of survivin has been observed in various tumors including those of the lung, colon, stomach, esophagus, pancreas, liver, uterus, ovaries, and melanoma in contrast to no survivin expression in corresponding normal tissues (Altieri 2003). Survivin has been shown to be involved with mitotic progression and apoptosis (Altieri 2004). Our study showed yet another important function of survivin in the leptin-induced migration of breast cancer cells. Survivin overexpression increases leptin-induced migration, whereas silencing of survivin abrogates leptin-induced migration of breast cancer cells. Migration being one of the important early events in metastatic process, our study showed a direct role of survivin in the metastatic process. This becomes an important finding in light of the previous epidemiological and clinical studies linking survivin with increased metastasis and poor prognosis. At present, it is not clear how survivin exerts its promigratory role in the presence of leptin. A recent study linked survivin expression in human melanocytes and melanoma cells with enhanced capacity for migration, and showed that survivin knockdown impaired melanoma cell migration (McKenzie et al. 2010). It has been suggested that survivin-mediated migration promotion may involve Akt-dependent upregulation of α5 integrin (McKenzie et al. 2010). Integrins promote cell migration through interactions with extracellular matrix proteins that enhance adhesion and transmit intracellular signals (Hood & Cheresh 2002). Signaling molecules known to be associated with deregulated cancer cell migration include activation of focal adhesion kinase (Sieg et al. 1999), Src (Kleekotka et al. 2001), MAPK (Zheng et al. 2000), and Akt (Zhang et al. 2009). Further analysis of the molecular mechanisms underlying leptin-induced migration and contribution of survivin will provide novel insights into breast cancer metastasis.

Because of the important roles of survivin in mitosis, apoptosis, and now in migration, a survivin-based therapy becomes an attractive option. Also, it would be expected to have limited toxicity toward normal tissues owing to the differential expression of survivin. Amongst various translational approaches targeting survivin, the generation of antigen-specific cytolytic T cells against survivin peptides has been considered for vaccination with the advantage of there being minimal risk of autoimmune effects (Kanwar et al. 2001, Andersen & thor 2002). Using molecular antagonists to interfere with the survivin pathway is another approach that uses systemic administration of antisense oligonucleotides, ribozymes, or local administration of mutant survivin adenoviruses to inhibit endogenous survivin expression (Chen et al. 2000, Kanwar et al. 2001). Taking a pharmacological approach, we used the HRI lovastatin to inhibit leptin-induced survivin expression. Lovastatin treatment also inhibited the leptin-mediated survivin-dependent migration of breast cancer cells. Our studies showed that leptin augments survivin expression and migration of breast cancer cells via the involvement of the leptin–EGFR–Notch1 axis, thus providing mechanistic insights for the development of novel translational applicability of the survivin pathway in cancer therapy.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-11-0075.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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