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14. ABSTRACT. Our central hypothesis is that in overweight and obese triple negative breast cancer (TNBC) patients the elevated levels of leptin increase drug resistance through induction of breast cancer stem cells (BCSC). Leptin secreted by adipose or TNBC activates VEGFR-2/Notch axis increasing tumor growth, angiogenesis and BCSC, which induce metastasis and drug resistance. Leptin induces tumor angiogenesis by mechanisms not completely understood. We found for the first time that leptin induces Notch expression in endothelial cells via trans-activation of VEGFR, which was independent from VEGF and, linked to increased cell proliferation, and the development of angiogenic features. A novel leptin signaling inhibitor conjugated to nanoparticles (IONP-LPrA2) has been characterized. IONP-LPrA2 increases the effectiveness of several chemotherapeutic (doxorubicin, cisplatin, paclitaxel) and angiogenic drugs (sunitinib) on TNBC cell lines. Data obtained suggest that IONP-LPrA2 has potential beneficial effects for TNBC patients, specifically those that are obese and show the higher leptin levels and, poorer outcome. IONP-LPrA2 may increase the efficacy of chemotherapeutics in vivo and, allow reduction of dose and side effects. IONP-LPrA2 adjuvant used alone or with chemotherapeutics will be applied to syngeneic obese mice hosting TNBC. Investigations on IONP-LPrA2 therapeutic potential and the elucidation of the molecular mechanisms by which leptin-induces BCSC survival and increases drug resistant in obese contexts may identify new targets for therapeutic intervention, for the hard-to-treat TNBC, which has not targeted therapies.

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1. INTRODUCTION

The Centers for Disease Control reports that 1/3 of the US population, or 78.6 million, adults are obese[1]. As of 2014, there were at least 600 million obese adults across the globe[2]. Therefore, obesity is a global pandemic, which is characterized by high levels of body fat (adiposity) and derived-cytokines (*i.e*., leptin). Leptin's main function is to regulate energy balance. Obese individuals habitually develop leptin resistance, which is the consequence of the breakdown in the signaling mechanism controlling satiety resulting in the accumulation of leptin. Research shows that adiposity and leptin provide insight on the link between obesity and cancer progression. Leptin levels are often chronically elevated in human obesity and related to several human cancers, including breast cancer. In addition to adipose tissue, breast cancer cells also overexpressed leptin receptors can secrete leptin, which acts as a mitogen, inflammatory and pro-angiogenic factor inducing cancer cell proliferation and tumor angiogenesis. We have found that breast cancer cells respond to leptin stimulus by increasing production of angiogenic factors (VEGF, VEGFR2, Notch), increasing proliferation, and survival [3-5]. Moreover, we have suggested that a novel and complex crosstalk between leptin, Notch and IL-1 (NILCO: Notch, IL-1 and leptin crosstalk outcome) seems to be an important driver of leptin-induced oncogenic actions, especially in triple negative breast cancer cells (TNBC). Additionally, leptin could induce breast cancer stem cells (BCSC) in TNBC. BCSC are linked to cancer recurrence and drug resistance. TNBC is an aggressive form of the disease has no targeted therapy. Therefore, TNBC patients are treated with chemotherapeutics, which virtually always inducing drug resistance and show several undesirable side effects.

Our central hypothesis is that in overweight and obese TNBC patients the elevated levels of leptin increase drug resistance through induction of BCSC, which are more resistant to chemotherapeutics and anti-angiogenic therapies. Then, leptin secreted by adipose or TNBC activates the Notch pathway in cancer increasing tumor growth, angiogenesis and BCSC, which induce metastasis and drug resistance. We further hypothesize that leptin's effects could involve the activation of a VEGFR-2/Notch axis. The elucidation of the molecular mechanisms by which BCSC survive in obese contexts may identify new targets for therapeutic intervention, for the "hard-to-treat" TNBC.

To this end, we have designed and tested potent inhibitors of leptin signaling: LPrA. To explore the potential impact of obesity signals (leptin) on TNBC drug resistance and BCSC, novel nanoparticles from conjugated IONP-LPrA2 will be used alone or as adjuvant therapies of chemotherapeutics. The main purposes of the project are to 1) e*xamine the role of leptin-induced VEGFR-2/Notch in BCSC and drug resistance in TNBC cells and, 2) determine the impact of adjuvant therapies for leptin signaling inhibition via IONP-LPrA2 nanoparticles combined with cisplatin and sunitinib on syngeneic and xenografts TNBC hosted by obese mice.*

During the first year of research we investigated the effects of leptin on mammosphere formation by TNBC and expression of BCSC markers. Inhibition of leptin signaling using a novel inhibitor designed and produced by us, IONP-LPrA2, showed how leptin can contribute to TNBC resistance to chemotherapeutics (*i.e*., cisplatin and sunitinib).

During the second year of funded research we have further investigated the effects of leptin on mouse E0771 parent and E0771-TAM cells (TNBC-like cells, obtained after long-term treatment with Tamoxifen, TAM). These cells are being used in a mouse syngeneic model to investigate the effects of obesity signals (leptin) on the growth of TNBC. We have also further characterized

specificity and relative effectiveness of IONP-LPrA2 antagonist to be applied in mouse models. Additionally, the impact of leptin on tumor angiogenesis via activation of the VEGFR2/Notch pathway and the pattern of expression of NILCO molecules in TNBC versus estrogen responsive breast cancer was investigated breast cancer tissue arrays. In addition, we found for the first time that leptin induces Notch expression in endothelial cells via trans-activation of VEGFR, which was independent from VEGF and linked to increased cell proliferation, and the development of angiogenic features.

2. KEY WORDS:

Leptin, LPrA2, IONP-LPrA2, Notch, VEGFR2, NILCO, triple negative breast cancer, breast cancer stem cells, drug resistance, obesity-related breast cancer, E0771 cells, chemotherapeutics.

3. ACCOMPLISHMENTS

Task 1

1) Characterization of Leptin Peptide Receptor Antagonist Linked to Iron Oxide Nanoparticles (IONP-LPrA2), a Novel Auxiliary Treatment for Breast Cancer: specificity and effectiveness for inhibiting leptin receptor signaling in TNBC cells.

Background: Obesity and high leptin levels are strongly associated with TNBC relapse, drug resistance, and poor patient outcomes. Over expression of leptin and its receptor, OB-R, induce cell proliferation, angiogenesis, and metastasis in TNBC. We have created a Leptin Peptide Receptor Antagonist, LPrA2, which has been shown to effectively prevent leptin signaling. LPrA2 was coupled to iron oxide nanoparticles (IONP) and used to determine its potential use as an adjuvant to chemotherapeutics**.**

Methods: IONP bound to LPrA2, were confirmed by Western Blot. Breast cancer cells were then treated with leptin and IONP-LPrA2 to determine the effect on cell cycle progression and leptin-induced signaling pathway marker expression. Subsequently, breast cancer cells were treated with IONP-LPrA2 in addition to chemotherapeutics. IC_{50} concentrations were determined. The cells were analyzed for apoptosis with Annexin V FITC and Propidium Iodide.

Results: IONP-LPrA2 specifically inhibited OB-R signaling and leptin-induced DNA synthesis during the S phase of the cell cycle in breast cancer cells. Additionally, when combined with chemotherapeutics IONP-LPrA2 showed an additive effect on the reduction of cell survival.

Conclusions: IONP-LPrA2 may increase the efficacy of chemotherapeutics in vivo. These findings indicate that IONP-LPrA2 may be useful in combination with chemotherapeutics for treatment of TNBC. Current data suggest potential beneficial effects of IONP-LPrA2 for TNBC patients, specifically those that are obese and show the higher leptin levels and, poorer outcome.

Materials and Methods
Nanoparticle conjugation

LPrA2 was synthesized as earlier described[6]. LPrA2 was solubilized in DMSO solution and was further de-salted via dialysis (membrane cut-off : <1 Kd; Thermo Fisher Scientific, Waltham,

MA.). IONPs were obtained from Ocean Nanotech LLC (San Diego, CA). LPrA2 was conjugated to IONPs by a method previous outlined[7]. Nanoparticle tracking analysis was performed by NanoSight analysis (NanoSigth Ltd., Malvern, United Kingdom)

Anti-LPrA2 antibodies:
Immune naïve rabbits were inoculated with pure LPrA2 solutions to elicit the production of antibodies. High affinity anti-sera were isolated from rabbits and, anti-LPrA2 antibodies were titrated by ELISA and purified using affinity chromatography. Eluted fractions containing purified Abs anti-LPrA2 were pooled and lyophilized using conventional methodologies.

Immunoblot analysis
IONP-LPrA2 was analyzed using SDS-PAGE. LPrA2 and LPrA2-Scramble (Sc) were used as positive and negative controls, respectively. After electrophoresis the blots were transferred onto nitrocellulose membranes. Then, the membranes were probed with purified anti-LPrA2 polyclonal antibodies. Anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad) was used for further antigen detection. Chemiluminescent detection of antigen bound to IONP was displayed by Western blotting substrate (Thermo Fisher Scientific). Relative levels of IONP-LPrA2 were determined using the Image J program (NIH).

Cell culture
Human ER+ MCF-7 cells in addition to TNBC cells MDA-MB-231 and HCC1806 were purchased from the American Type Culture Collection (ATCC, USA). The cells were cultured in MEM (Life Technol, Thermo Fisher Scientific) containing 10% fetal bovine serum (Med Supply Partners, Atlanta, GA) and, were maintained in an incubator at 37^0C with 5% CO_2.

Cell cycle analysis
Cells were seeded in 6 well cell culture plates and grown to 70-80% confluence. Then, cells were treated with leptin (1.2nM) and various concentrations of IONP-LPrA2 solutions (0.05-1 ppm) for 24-48 hours. After treatment, the cells were trypsinized, washed with 1X PBS, and resuspended in cold 100% methanol. Cells were stored at -20^0C prior to analysis. Afterward, the cells were centrifuged to remove the methanol. Cells were resuspended and incubated at 37^0C for 40 minutes in 50µL propidium iodide solution (PI). Cells were centrifuged to remove the PI, resuspended in 1X PBS, and analyzed by Cellometer Vision ® image based cytometer (Nexcelom Biosc.).

Apoptosis assay
Cells were cultured in 6 well cell culture plates in medium containing 5% FBS and grown to 70-80% confluence. Cells were treated with the chemotherapeutics cisplatin (EMD Millipore, Billerica, MA), sunitinib, paclitaxel, and doxorubicin (all from SelleckChem, Houston, TX) for time periods ranging from 1-6 days. Before trypsinizing, the supernatants were transferred into microfuge tubes for subsequent analysis. The trypsinized cells were washed by centrifugation. The pellets were further washed with 1X PBS and resuspended in Annexin V binding buffer (Nexcelom). Five µL of Annexin V FITC and PI were added and mixed with the trypsinized cells. Then, samples were incubated in the dark at room temperature for 15 minutes. The cells were washed with 1X PBS, centrifuged, and resuspended in Annexin V binding buffer to a concentration of 30,000 cells per 20 µL. The samples were analyzed for apoptosis by the Cellometer Vision (Nexcelom). The IC_{50} values for each chemotherapeutic were determined by plotting the concentration of the chemotherapeutics versus the percentage of live cells.

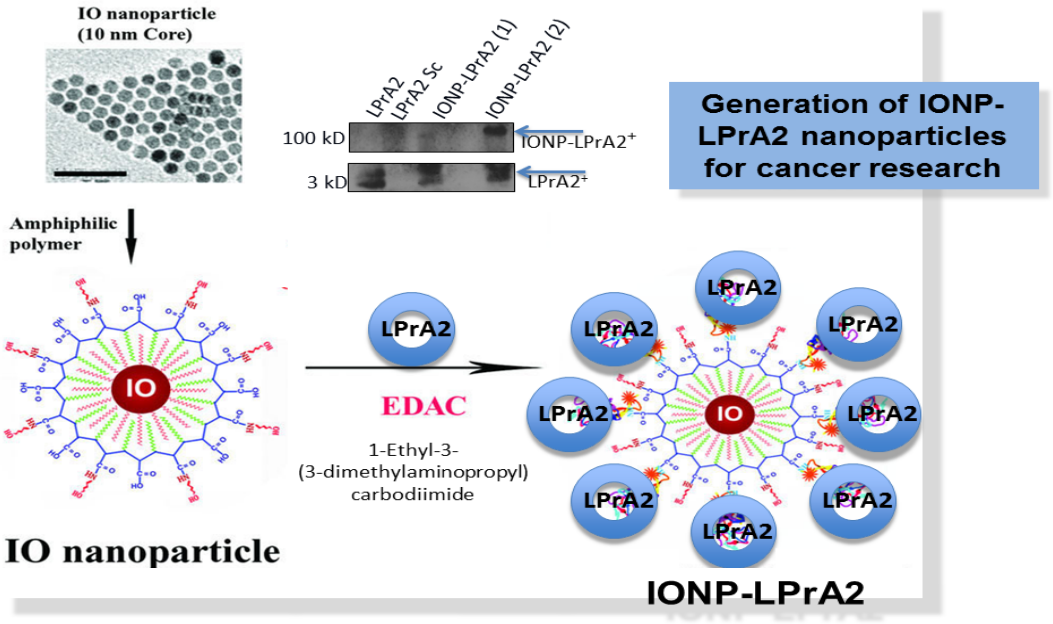
Surface Marker Analysis

Breast cancer cells were seeded in 6 well cell culture plates, cultured in medium containing 10% FBS and grown to 70-80% confluence. Cells were washed with PBS and starved for 24 h previous to treatment with leptin (1.2nM) alone or plus IONP-LPrA2 (0.5 ppm). The cells were trypsinized, fixed with 3.7% paraformaldehyde (PFA), and permeabilized by incubation at 4^{0}C for 10 minutes with 0.05% Triton X100. Non-specific binding was reduced by incubation of cells with 1% bovine serum albumin (BSA). The cells were then incubated with primary antibody, anti-Notch1 (Santa Cruz) for 1 hour. Following primary antibody incubation, the cells were washed with 1X PBS and incubated with a goat anti-mouse PE fluorescent antibody for 45 minutes. Subsequently the cells were centrifuged and the supernatant was removed. The cells were resuspended in 1X PBS and analyzed for surface marker expression using a Cellometer (Nexcelom).

Results and Discussion

Generation of IONP-LPrA2 conjugates

Leptin antagonist LPrA2 has been shown to inhibit breast cancer growth in vitro as well as in vivo[8-10]. To increase effectiveness for leptin signaling inhibition, LPrA2 was conjugated to IONPs. IONPs are amphiphilic and have a 10 nm core[7]. The binding of LPrA2 to IONPs was facilitated by ethyl-3-dimethyl amino propyl carbodiimide (EDAC), which activates carboxyl groups and allows the formation of an amide bond (Figure 1).



Adapted from Yang et al. *Clin Cancer Res* 2009;15: 4722-32

Figure 1. Conjugation and purification of IONP-LPrA2.

IONP-LPrA2 conjugation and nanoparticle characterization

To confirm binding and to determine the molecular weight of the nanoparticle-bound LPrA2 peptides, the IONP-conjugates were analyzed by SDS-PAGE and Western Blot (see Fig 1). Anti-LPrA2 incubation displayed bands at ~100 Kd indicating nanoparticle-bound LPrA2 for dialyzed sample (2) and ~3kD unbound LPrA2 precipitate for both non-dialyzed samples (1 and

2). LPrA2 and LPrA2 Sc were used as positive and negative controls, respectively. It was determined that ~40% of LPrA2 bonded with the IONPs while ~60% remained unbound and the bound portion showed similar expression to the positive control.

To further characterize the bound IONP-LPrA2, nanoparticle-tracking analysis was performed with the NanoSight. It was found that particle size range is 116-147 nm and the particle concentration is $1.35x10^8$/ml (Figure 2).

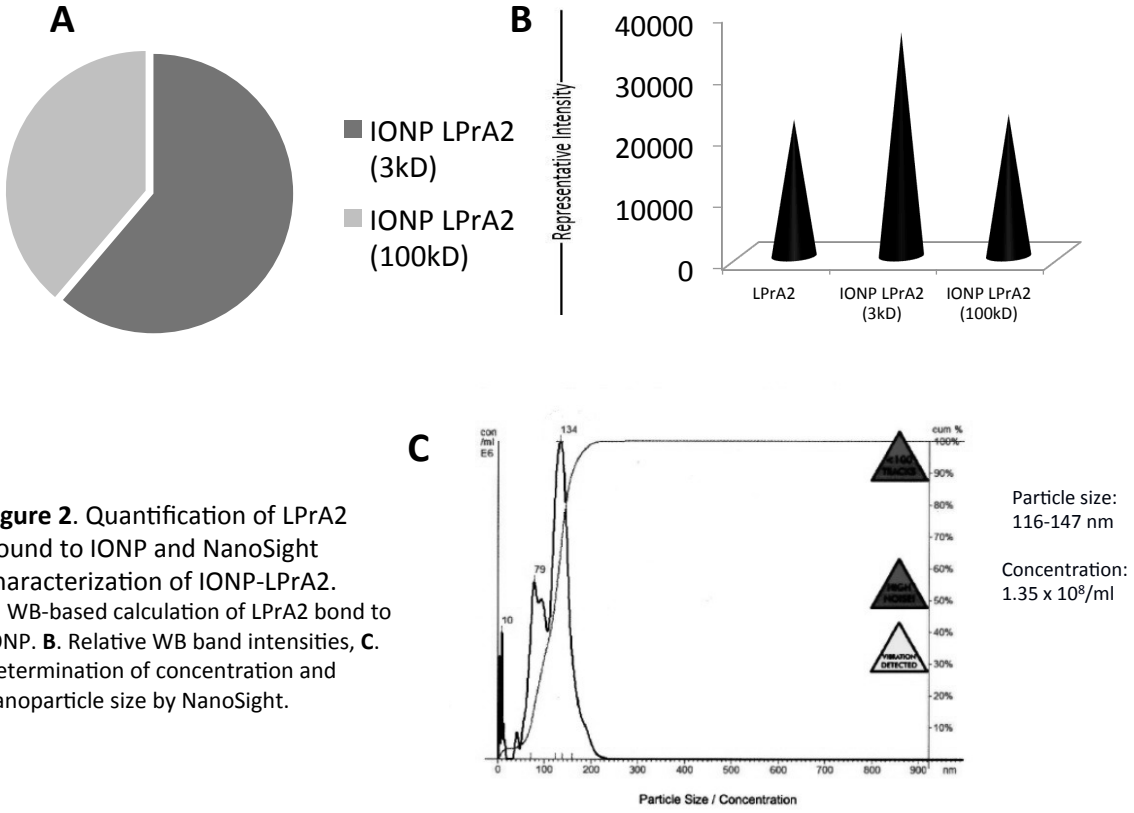


Figure 2. Quantification of LPrA2 bound to IONP and NanoSight characterization of IONP-LPrA2. **A**. WB-based calculation of LPrA2 bond to IONP. **B**. Relative WB band intensities, **C**. Determination of concentration and nanoparticle size by NanoSight.

IONP-LPrA2 inhibits leptin-induced cell cycle progression

Leptin has been shown to increase Cdk2 and cyclin D1 levels in MCF-7 cells[8]. To display the effect of leptin on cell cycle progression MDA-MB-231, HCC1806, and MCF-7 human breast

cancer cell lines were treated with leptin and leptin plus IONP-LPrA2 in order to determine the antagonistic effect. The cells were treated with IONP-LPrA2 concentrations ranging from 0.25-1.0 ppm. MDA-MB-231 and HCC1806 TNBC cell lines were treated for 24 hours while the ER^+ MCF-7 cells were treated for 48 hours. Treatment with leptin caused an increase in cell cycle progression in all cell lines, while treatment with leptin plus IONP-LPrA2 abrogated the effect of leptin (Figure 3A-C). HCC1806 cells displayed the greatest increase in the percentage of cells in the S phase in response to leptin as well as the greatest decrease with IONP-LPrA2 treatment (Figure 3B)

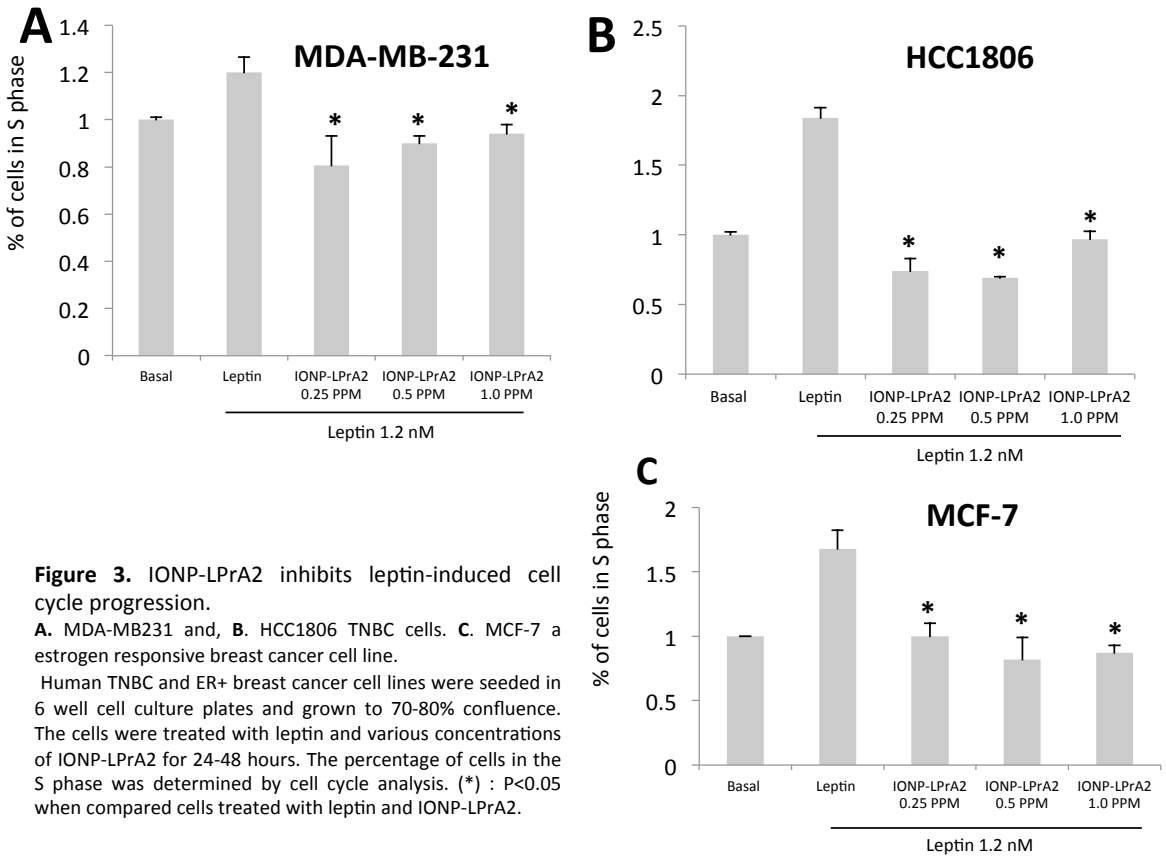


Figure 3. IONP-LPrA2 inhibits leptin-induced cell cycle progression.

A. MDA-MB231 and, **B**. HCC1806 TNBC cells. **C**. MCF-7 a estrogen responsive breast cancer cell line.

Human TNBC and ER+ breast cancer cell lines were seeded in 6 well cell culture plates and grown to 70-80% confluence. The cells were treated with leptin and various concentrations of IONP-LPrA2 for 24-48 hours. The percentage of cells in the S phase was determined by cell cycle analysis. (*) : $P<0.05$ when compared cells treated with leptin and IONP-LPrA2.

The effect of chemotherapeutics on survival of breast cancer cell lines

Chemotherapy is among the most common treatments for breast cancer in addition to radiation and surgery. To determine the effective concentration of the chemotherapeutics in the three breast cancer cell lines, the cells were treated with various concentrations of the drugs for time

periods ranging from 1-6 days. The cells were subsequently analyzed by the Annexin V FITC and PI assay. Dot blots were generated to determine live, apoptotic, and necrotic cells. All cell lines displayed more sensitivity to the anti-angiogenic and chemotherapeutic sunitinib than by doxorubicin, cisplatin and paclitaxel (Figure 4). The TNBC cell line MDA-MB-231 showed more sensitivity to cisplatin and doxorubicin (Figure 4A, D) while MCF-7 cells had a more robust response to the anti-microtubule agent Paclitaxel (Figure 4B).

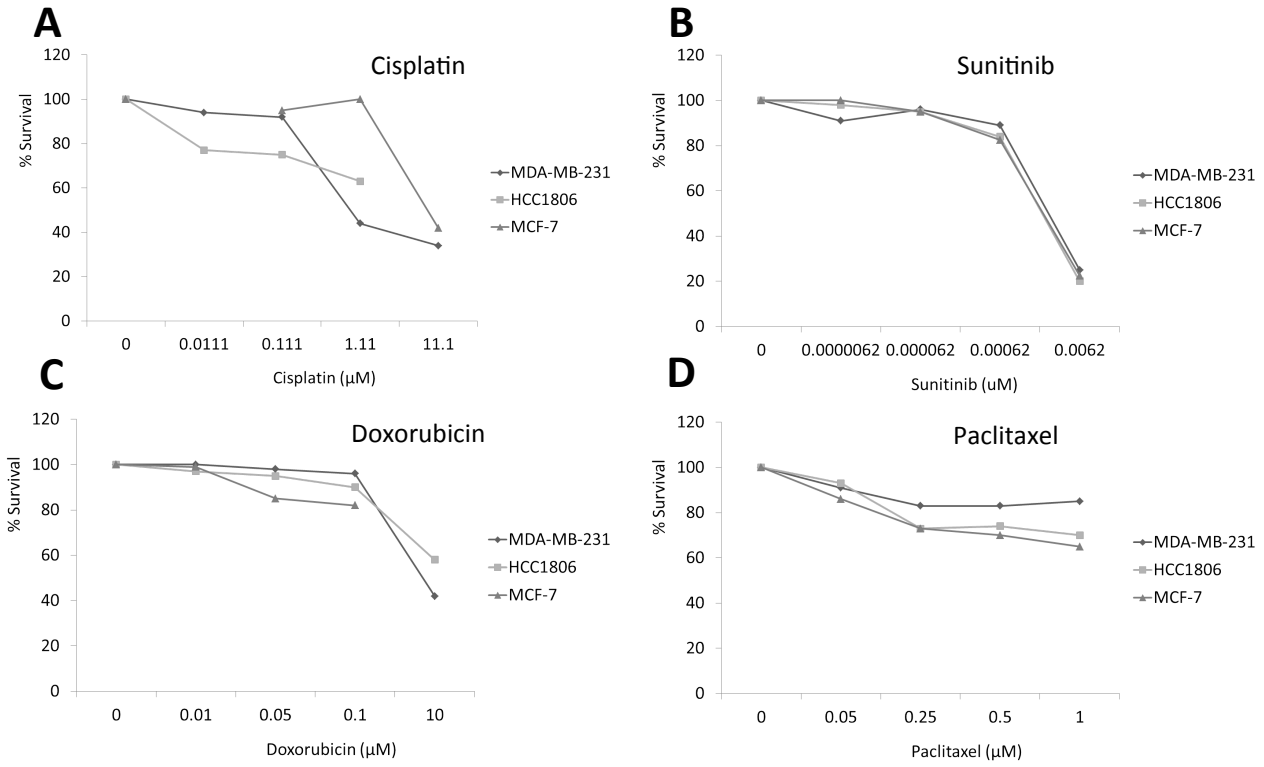


Figure 4. The effect of chemotherapeutics on survival of breast cancer cell lines**.**
A. Cisplatin, B. Sunitinib, C. Doxorubicin and, D. Paclitaxel**.**
Human TNBC and ER+ breast cancer cell lines were seeded in 6 well cell culture plates and grown to 70-80% confluence. Cells were treated with the chemotherapeutics for time periods ranging from 1-6 days. Survival was determined using Annexin V FITC and PI assays. IC50 were determined using GraphPad software.

IONP-LPrA2 decreases viability in human ER+ and TNBC cell lines

Chemotherapy has many detrimental side effects and elicits drug resistance, which is presumably due to the actions of cancer resistant cells (cancer stem cells). Therefore, it is advantageous to utilize combination therapy in order to reduce the effective dose and resistance of chemotherapeutics. Combinations of chemotherapeutics with IONP-LPrA2 (specifically targeting oncogenic actions of leptin) may be advantageous. Breast cancer cell lines were treated with IC_{50} concentrations of chemotherapeutics in media containing 5% FBS, to mimic physiological leptin levels plus 0.5 ppm IONP-LPrA2 for time periods ranging from 1-6 days. The cells were then analyzed by the Annexin V FITC and PI assays. HCC1806 TNBC cells treated with IONP-LPrA2 showed the greatest reduction in viable cells when dosed with cisplatin

and doxorubicin (Figure 5A, C.). MDA-MB-231 cells treated with IONP-LPrA2 had a similar significant effect when sunitinib was administered (Figure 5B.) while the ER+ MCF-7 cells treated with IONP-LPrA2 showed no significant decrease. Paclitaxel, which acts on the M phase of the cell cycle, showed no significant decrease in viability when treated with IONP-LPrA2 (Figure 5D.).

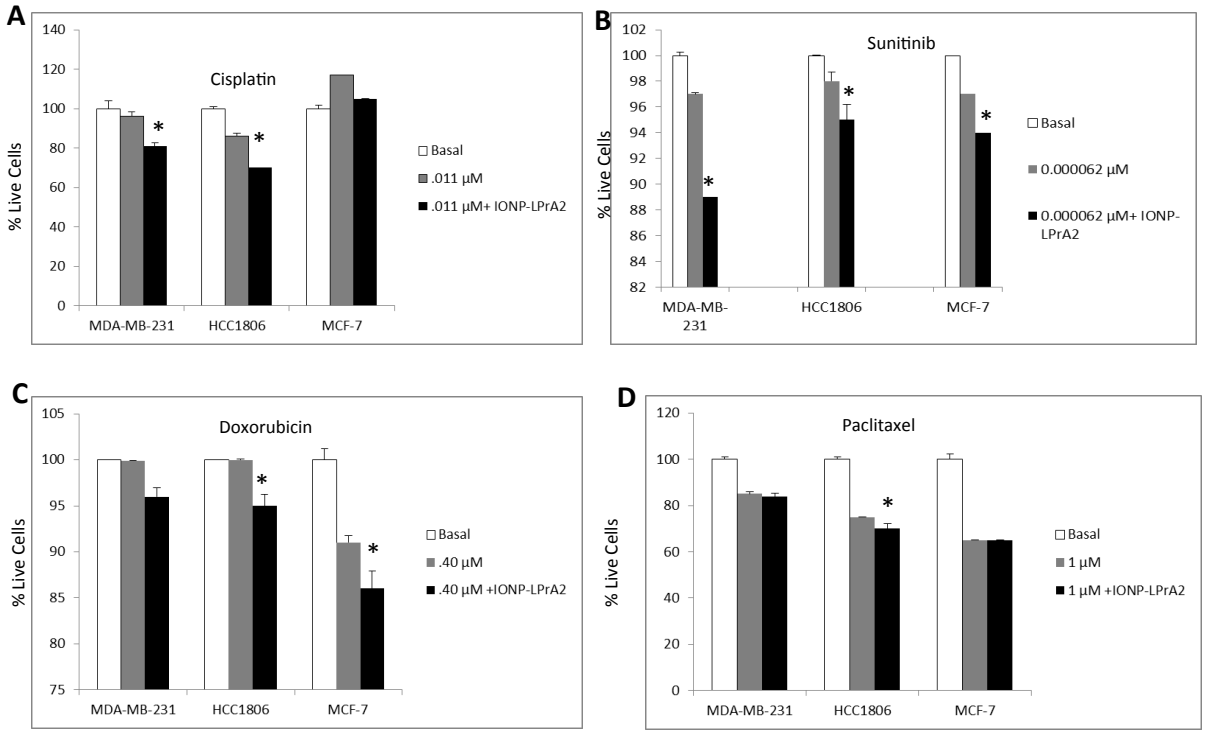


Figure 5. IONP-LPrA2 decreases viability in human ER+ and TNBC cell lines**. A.** Cisplatin, B. Sunitinib, C. Doxorubicin and D Paclitaxel. Human ER+ and TNBC cell lines were seeded in 6 well cell culture plates and grown to 70-80% confluence Cells were treated with chemotherapeutics at IC50 concentrations plus IONP-LPrA2 for time periods ranging from 1-6 days. The effect of IONP-LPrA2 on cell survival was determined using the Cellometer Vision image cytometer.

IONP-LPrA2 inhibition of leptin-induced signaling pathways

Leptin signaling upregulates Notch, IL-1, and VEGF/VEGFR2 in TNBC, which promote survival and angiogenesis. Therefore, the ability of IONP-LPrA2 to inhibit these leptin effects was determined[11]. Cells were seeded in 6 well cell culture plates and grown to 70-80% confluence and challenged with leptin and leptin plus IONP-LPrA2. The pellets were centrifuged and blocked with BSA. The cells were then incubated with anti-Notch1. Subsequently the cells are washed with 1X PBS and analyzed for surface marker expression. Notch1 expression increased with leptin treatment. Treatment with IONP-LPrA2 abrogated leptin-induced Notch1 expression (Fig. 6).

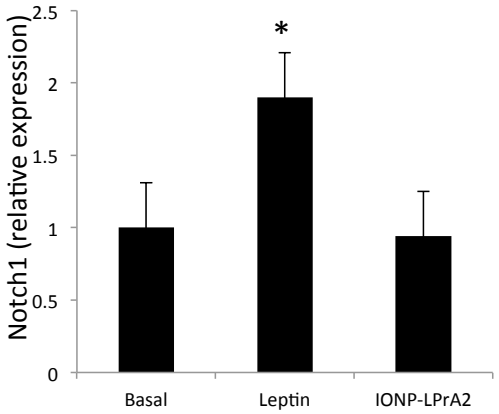


Figure 6. IONP-LPrA2 inhibition of leptin-induced signaling pathways.

Cells were seeded in 6 well cell culture plates and grown to 70-80% confluence. The pellets were centrifuged and blocked with BSA. The cells were then incubated with anti-Notch 1. Subsequently the cells are washed with 1X PBS and analyzed for surface marker expression with a Cellometer.

2. **Mechanisms of leptin-induced VEGFR-2/Notch axis.**

Abstract

Leptin induces tumor angiogenesis by mechanisms not completely understood. Here we investigated whether VEGFR and Notch signaling are involved in leptin-induced angiogenic features of endothelial cells (EC). Human umbilical vein (HUVEC) and porcine aortic EC (PAEC: wild type not expressing VEGFRs, and PAEC-VEGFR-1, PAEC-VEGFR-2 transfected cells) were challenged with leptin and inhibitors of Notch (γ-secretase inhibitor, DAPT), VEGFR-2 kinase (Semaxanib/SU5416 and siRNA VEGFR) and leptin (pegylated leptin peptide receptor antagonist 2, PEG-LPrA2). Involvement of VEGFR on leptin-mediated induction of Notch and HUVEC proliferation and tube formation was assessed via MTT, cell cycle progression and matrigel assays, respectively. Leptin-induced trans-phosphorylation/activation of VEGFR-2 was determined by ELISA and Western blot. Present data show that leptin induces VEGFR-2 trans-phosphorylation at Y1175, Y951 and Y996. We found for the first time that leptin induces Notch expression in EC via trans-activation of VEGFR, which was linked to increased cell proliferation, and the development of angiogenic features. Leptin-upregulated Notch1 and Notch4 were linked to both VEGFR-1 and -2 activities, but leptin induction of Notch2 and JAG1 was related to VEGFR-2 transactivation. However, the specific biological relevance of these findings needs to be further investigated. Remarkably, leptin's effects were independent of VEGF-induced signaling. Present data suggest that high levels of leptin found in overweight and obese cancer patients could lead to increased tumor angiogenesis via VEGFR/Notch axis in EC. Combinatory inhibition of leptin signaling and Notch or VEGFR-2 could be used to develop new strategies targeting tumor angiogenesis.

Background

Endothelial cells (EC) play a major role in normal biological processes such as blood-tissue exchange, blood-cell activation, vasculogenesis, and angiogenesis. These cells line the blood vasculature, are key regulators in vascular homeostasis, and form a barrier to create the endothelium; which acts as a vessel for the circulating blood. EC under the actions of several circulating factors can help to modify the phenotype of the vessel wall[11]. In these processes, vascular endothelial growth factor (VEGF) and its receptor type 2 (VEGFR-2) play major roles in driving angiogenesis[12]. VEGFR-2 is a kinase insert domain receptor encoded by the human KDR gene, which corresponds to the FLK-1 mouse gene. It plays an essential role in the angiogenic process in physiologic and pathological scenarios[13]. VEGFR-2 is a major mitogenic and chemotactic receptor found in EC, which can induce several cellular processes common to many growth factor receptors, such as, cell proliferation, migration, and survival vascular development during embryogenesis as well as new blood vessel formation[14,15]. In addition, VEGF/VEGFR-2 signaling is essential for autocrine/paracrine survival processes of breast cancer cells[16].

Another factor that plays an important role in angiogenesis is Notch. Activated Notch leads to EC proliferation, differentiation, survival, and angiogenesis. Abnormal expression of Notch correlates to cancer development and poor outcomes. The Notch receptor family is composed of four members, Notch1, Notch2, Notch3, and Notch4. Notch1 is considered a hallmark of breast cancer, and is generally overexpressed in tumor tissues. In contrast, Notch receptors are lowly expressed in normal breast tissue[17]. Several ligands of Notch: Jagged (JAG1 and JAG2) and Delta-like (DLL1, DLL3, and DLL4) are also abnormally expressed in cancer tissues. Ligand binding to Notch receptors on adjacent cells leads to the activation of

signaling pathways. A series of proteolysis events involving γ-secretase, cleave and activate Notch receptors leading to the formation of Notch intracellular domain (NICD); which binds CSL/RBPJk, a transcription factor in the nucleus, and thus leads to activation of several genes (*i.e*. survivin and Hey2). The protease γ-secretase is an essential enzyme involved in NICD formation[18].

In EC, Notch and VEGF/VEGFR-2 signaling has been shown to promote a switch from stalk to a tip cell; thus promoting differentiation and migration of tip EC. These EC become highly polarized and extend their filopodia; which allows for the formation of capillaries[19]. The molecular structure of tip cells is characterized by the expression of VEGFR-2, DLL4, and other receptors[19]. Notch signaling in EC stalk cells impairs filopodia extension, promotes tube length by EC proliferation, and inhibits vessel branching by lowering expression of VEGFR-2 and other receptors[20,21]. Notch induces lateral inhibition of EC during tip-stalk cell fate switching that involves DLL4/Notch signaling[22-24].

During embryogenesis and cancer progression Notch target genes, survivin and Hey2 are crucial for the angiogenic process. DLL4 is upregulated in human umbilical vein endothelial cells (HUVEC) under stressed conditions[25]. Hey2 has been shown to interact with histone deacetylase complexes and repress transcription of zinc finger factors and their target genes. Survivin is a cell survival factor and an inhibitor of proper caspase function, which is highly expressed in most cancer[26].

Leptin is a 16kD, small, nonglycosylated cytokine secreted by adipocytes and cancer cells. It is a non-classical angiogenic factor that signals through its receptor (Ob-R), which is found in peripheral cells and tissues, including EC[28]. Obese individuals exhibit the highest leptin levels and develop leptin-resistance, where leptin cannot control appetite or energy balance[28]. Increased leptin signaling correlated to enhanced expression of proteins involved in cancer progression and tumor angiogenesis[29]. Leptin signaling regulates VEGF/VEGFR-2 and Notch and its targets in breast cancer[27]. Moreover, a crosstalk between Notch, leptin, and IL-1 signaling (NILCO) affects the expression of pro-angiogenic molecules, leading to cell proliferation and migration of breast cancer cells[27]. Therefore, leptin secreted by adipose tissue or cancer cells could be an important factor contributing to tumor angiogenesis by acting directly on cancer cells to induce VEGF secretion[8,30] or on EC to induce angiogenesis[15]. However, the mechanisms involved in leptin modulation of EC function are not completely understood. Here we show that leptin transactivation of VEGFR regulates the expression and activity of Notch, and the development of EC angiogenic features.

Materials and Methods

Reagents and antibodies

Human recombinant leptin and human VEGFR-2 Quantikine ELISA Kits and human VEGF cytokine (293-VE) was purchased from R&D Systems, Minneapolis, MN. Notch1 (sc-373891), Notch4 (sc-56594) and Jagged1 (JAG1, sc-8303) polyclonal antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. DLL4 (ab7280), Notch2 (ab8926) and Notch3 (ab23426) polyclonal antibodies were from Abcam, Cambridge, MA. VEGFR-2 monoclonal antibody (55B11) was purchased from Cell Signaling, Danvers, MA. Anti-mouse and anti-rabbit polyclonal antibodies conjugated to horseradish peroxidase were from Bio-Rad Laboratories, Hercules, CA. Semaxinib/SU5416 was purchased from Selleckhem, Houston TX. Enhanced

chemiluminescence (ECL)-western blot stripping buffer was from Thermo Scientific, Rockford, IL. Leptin receptor inhibitor (PEG-LPrA2) was homemade as previously described[8]. b-actin (A5316) and GAPDH (G8795) monoclonal antibodies, protease and phosphatase inhibitor cocktails 1 and 2, fetal bovine serum (FBS), DAPT [N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester], 4',6-diamidino-2-phenylindole and other chemicals were purchased from Sigma-Aldrich, St. Louis, MO.

Endothelial Cell (EC) Cultures

HUVEC (Invitrogen, Grand Island, NY) were cultured in Medium 200 (Invitrogen) containing 10% fetal bovine serum (FBS, Med Supply Partners, Inc) plus P/S [1% penicillin/streptomycin (Gibco, Inc.), 20 µg/ml of EC growth supplement (Millipore Inc.) and 0.4 µg/ml Geneticin (Life Technologies)]. Additionally, wild type porcine aortic EC (PAEC), which do not normally express detectable levels of VEGFR-1 or VEGFR-2; and PAEC transfected with VEGFR-1 or VEGFR-2 were cultured in Hams F-12 Medium (Invitrogen) containing 10% FBS and P/S. PAEC and HUVEC (maximum passage equal to 5) were cultured at 37º C in 5% CO2/95% air in 25 mm^2 tissue culture flasks.

Dose and time-response effects of leptin on EC.

Confluent EC were detached and re-seeded into 75 mm^2 tissue culture flasks and treated with human recombinant leptin (0, 0.6, 1.2 and 6.2 nM), γ-secretase inhibitor, DAPT (5 µM DAPT/0.1% DMSO) and a potent inhibitor of VEGFR-2 kinase activity (Semaxinib/SU5416, 5 µmol/ml). The leptin concentration yielding a maximum effect on EC proliferation (1.2 nM) was used for time response experiments (0, 12, 24 and 48 h).

Cell Proliferation

HUVECs were plated at a density of 10,000 cells per well 96 well plates in serum-supplemented medium for 24 hours, then cultured in serum deprived medium for additional 16 hours. Cells were treated with leptin (1.2nM), and inhibitors of VEGFR-2 (5 µmol/L), SU5416 (5 µmol/L) and Ob-R (pegylated leptin antagonist PEG-LPrA2; 1.2nM). Cell proliferation was determined using MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. Optical density (OD) values corresponding to formazan formation were recorded at 570 nm on a Microplate Reader (Spectramax). The results were expressed relative to the OD value of untreated HUVEC.

Cell Cycle Progression

HUVECs were plated in 12 well plates at a density of 100,000 cells per well in serum-supplemented medium for 24 hours, and then in serum deprived-medium additional for 24 hours. Cells were treated as described above. S-phase progression was measured by quantitative staining of DNA using propidium iodide using a Cellometer device (Nexcelom; Lawrence, MA). Gated cells in S-phase were counted and expressed as percentage of untreated HUVEC.

WB analysis

Total proteins from EC were extracted on ice using radioimmunoprecipitation assay (RIPA) buffer containing an enzymatic inhibitor cocktail (Sigma). Fifty microgram of protein lysates were used for WB analysis as previously described[8]. After electrophoresis, protein bands were transferred onto nitrocellulose membranes (BioRad), which were incubated with specific primary antibodies, GAPDH, β-Actin, Notch1, Notch2, Notch3, Notch4, Jagged1, DLL4, and VEGFR-2/phosphorylated (p) VEGFR-2 at 4ºC overnight. β-Actin or GAPDH were used as the

experimental loading controls. The NIH Image program was used for quantitative analysis. Protein concentrations of tissue lysates were determined using the Bradford method (Bio-Rad).

ELISA determinations

An enzyme linked immunoassay (ELISA; sensitivity <1 pg/mL pVEGFR-2) using human recombinant pVEGFR-2 as standard (R&D systems) was used to detemine levels of pVEGFR-2 in lysates from EC treated with leptin for 24 hours. VEGF (25 ng/ml) was used as positive control. Additionally, to detect whether secreted VEGF, cell supernatants were analyzed by ELISA (R&D systems). All determinations and calibrations were carried out in triplicate.

Real-time PCR

Total RNA form EC was extracted and purified using RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. cDNA was synthesized from total RNA using SuperScript First-Strand Synthesis System with SuperScript II reverse transcriptase according to the manufacturer's protocols (Bio-Rad). cDNA was used as a template in for RT-PCR to determine mRNA Notch expression with SYBR-Green PCR master-mix, and iQ5 RT-PCR detection System. RT-PCR reactions consisted of 1x SybrGreen Supermix, 0.20 nmol/L forward and reverse primers, and 1µg of cDNA. To generate a standard curve, amplified cDNA from the reference sample was obtained using a 5-fold dilution series of cDNA per reaction. Relative gene expression was calculated by dividing the specific expression value by the corresponding expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers include Notch-1 forward primer: 5'-GTCAACGCCGTAGATGACC-3', reverse primer: 5'-TTGTTAGCCCCGTTCTTCAG-3', Notch-2 forward primer: 5'-TCCACTTCATACTCACAGTTGA-3', reverse primer: 5'-TGGTTCAGAGAA AACATACA-3', Notch-3 forward primer: 5'-GGGAA AAAGGCAATAGGC-3', reverse primer: 5'-GGAGGGAGAAGCCAAGTC-3', Notch-4 forward primer: 5'-AACTCCTCCCCAGGAATCTG-3', reverse primer: 5'-CCTCCATCCAGCAGAGGTT-3', Dll4 forward primer: 5′-tccaactgcccttcaatttcac-3′, reverse primer: 5′-ctggatggcgatcttgctga-3′, Jagged-1 forward primer: 5′-GGAGGCGTGGGATTCCA-3′; reverse primer: 5′-CCGAGTGAGAAGCCTTTCAATAAT-3′ and GAPDH, forward primer: 5′-atggggaaggtgaaggtcg-3′ and reverse primer: 5′-ggggtcattgatggcaacaata-3′. PCR conditions were: 1 cycle, 95°C for 3 min (45 cycles), 95°C for 30 sec; 52°C for 30 sec and 72°C for 30 sec. Annealing temperatures varied around 61°C. RT-PCR determinations were performed in triplicates.

Small interfering RNA transfection

Knock down of VEGFR-1/FLT-1 and VEGFR-2/FLK-1 gene expressions in EC was achieved using specific small interfering RNA (siRNAs; Santa Cruz Biotechn.). Cells were seeded at a density of 2×10^5/ml and cultured until 60% confluence in growth medium without antibiotics in 12 well plates. Cells were then transfected with 1 µg of FLT-1 and FLK-1 oligonucleotides that were composed of three to five specific siRNA targets of 19–25 nucleotide length (Santa Cruz Biotechn.). Negative controls included VEGFR-1 and VEGFR-2-scramble siRNAs. EC were incubated at 37ºC for 6 hours in serum-free transfection medium (Santa Cruz Biotechn.). Normal growth medium with antibiotics, 10% FBS, and transfection medium were added to cells. Then, EC were incubated at 37ºC for an additional 24 hours. WB analyses of VEGFR-1 and VEGFR-2 expression were performed in cell lysates from non-transfected and transfected cells.

Matrigel assay

The effects of leptin, VEGF, and inhibitors of VEGFR-2 and Notch on angiogenic features of EC were determined via capillary-tube formation assays in Matrigel (BD Sciences). EC were cultured in M200 medium containing 5% FBS and 5 mg/ml of EC growth factor (ECGF, Millipore). Growth factor-reduced Matrigel was plated onto 96-well plates (200 µl per well) and

incubated at 37ºC for 30 minutes. Then, HUVEC were seeded in the Matrigel-coated plates at 10,000/well, and treated with leptin (1.2 nM), SU5416 (5 μmol/L), and DAPT (5 μmol/L) in 1% reduced FBS medium. The medium was removed after 8 hours, and the cells were washed twice in PBS. Cell fixation was carried out in 4% paraformaldehyde. Images of tube formation were captured at 10X using an inverted microscope.

Statistical Analysis

Statistical analysis was performed from triplicate data (three trials and triplicate wells) using one-way ANOVA to compare results between multiple treatment groups. $P<0.05$ was considered to be statistically significant. Quantitative data was expressed as standard error means. All experiments were repeated 3 or more times.

Results

Leptin induces Notch protein and mRNA expression in EC in dose and time-dependent manner

To determine the effects of leptin on Notch expression and its targeted molecules in EC, western blot (WB) and real time polymerase chain reaction (RT-PCR) were performed. Leptin treatment of HUVEC resulted in a dose-response increase in expression of Notch protein (Fig 7A,B) and mRNA (Fig 7C) in HUVEC. Moreover, leptin elicited a time-dependent induction of Notch2 and DLL4 protein and, Notch2, Notch4 and Jagged1 mRNA levels in HUVEC (Fig 8A-C).

Wild type-PAEC was not found to express detectable levels of VEGFR-1 or VEGFR-2 (Fig 9A). In contrast, VEGFR-1 and VEGFR-2 transfected PAEC were positive for VEGFR-1 and VEGFR-2, respectively (Fig 9A). However, Ob-R was detected in all PAEC types (Fig 9B). Leptin was unable to regulate Notch in wild-type PAEC (data not shown). However, leptin induced in a dose dependent manner Notch1, Notch3 and DLL4 in PAEC-VEGFR-1 (Fig 9C, D). Additionally, leptin at the higher concentration (6.2 nM) upregulated Jagged1 in PAEC-VEGFR-1 (Fig 9C, D). However, in PAEC-VEGFR-2, leptin induced Notch1 and Notch4 expression (Fig 9E, F). In comparison, leptin induction of Notch was more evident in HUVEC (see Fig 7). These data suggest that leptin induction of Notch in EC requires functional VEGFRs.

Leptin-induction of Notch in EC involves VEGFR-2 kinase activity.

To further explore the mechanisms underlying leptin induction on Notch expression, a selective tyrosine kinase inhibitor of VEGFR-2 (SU5416) was added to HUVEC cultures treated with leptin. Inhibition of VEGFR-2 activity significantly reduced leptin-induced Notch (Fig 10A, B). These findings suggest that leptin induction of Notch in EC requires functional VEGFR-2 kinase activity. Moreover, leptin induced the expression of VEGFR-2 in HUVEC that was abrogated by inhibition of γ-secretase (an essential enzyme involved in Notch activation) (Fig 10C, D).

It was further assessed that leptin trans-phosphorylates VEGFR-2 in HUVEC in the absence of VEGF (Fig 11A). pVEGFR-2 levels were increased by leptin in HUVEC as determined by ELISA, which was abrogated by SU5416 and PEG-LPrA2 (Fig 11A). Moreover, WB analysis showed that leptin significantly increased trans-phosphorylation of several tyrosine sites in the intracytoplasmatic VEGFR-2 tail of PAEC-VEGFR-2 cells. Leptin induced the trans-phosphorylation of additional amino acid residues Y996 and Y951 in PAEC-VEGFR-2 (Fig 11B, C). Taken together, these data further support the notion that leptin induction of Notch requires VEGFR-2 activity, which is in turn regulated by a Notch feedback in EC.

Abrogation of VEGFR-2 and Notch activities reduce leptin-induced EC proliferation and tube-like structures.

Leptin-induced EC proliferation was measured using the MTT cell proliferation assay. DAPT and SU5416 inhibitors were used to determine whether Notch and VEGFR-2 activities, respectively, are required for leptin-induction of HUVEC proliferation. Leptin failed to promote cell proliferation when Notch or VEGFR-2 were inhibited in HUVEC (Fig 12A). Moreover, inhibition of VEGFR-2 or Notch abrogated leptin-induced S-phase progression in HUVEC as determined by Cellometer (Nexcelom) (Fig 12B). Similarly, exposure of leptin-treated HUVEC to the Ob-R inhibitor, PEG-LPrA2, abrogated leptin-induced cell proliferation and S-phase progression. The effects of leptin on HUVEC proliferation were similar to those obtained with VEGF (positive control). The ability of leptin to induce tube-like structures in absence of VEGF was assessed. It was further determined whether leptin-induction of tube-like structures in HUVEC was dependent on VEGFR-2 and Notch signaling (Fig 12C). The addition of Notch inhibitor, DAPT, to HUVEC cultures treated with leptin significantly decreased tube-like structure formation (Fig 12C). Moreover, the inhibition of VEGFR-2 signaling via SU5416 produced similar reduction of leptin-induced tube-like formation (Fig 12C). These data suggest that leptin-induced VEGFR-2 activation and Notch expression/signaling are involved in leptin pro-angiogenic effects in HUVEC. Therefore, leptin could re-enforce VEGF actions by stimulating Notch expression and activity, which were linked to the progression of cell cycle, proliferation and angiogenic differentiation of EC.

Silencing of VEGFR-1 and VEGFR-2 abrogates leptin-induced Notch protein expression

To further investigate the role of VEGFR in leptin-induced Notch expression in HUVEC, the cells were transfected with siRNA VEGFR-2 and VEGFR-1. Leptin-induced Notch2 and Jagged1 expression was significantly decreased when VEGFR-2 was silenced in HUVEC (Fig 13A, B). Additionally, double silencing of VEGFR-2 and VEGFR-1 genes significantly decreased Notch1 expression (Fig 13C, D). Effective abrogation of VEGFR-2 expression via siRNA VEGFR2 or double siRNA for VEGFR-1 and VEGFR-2 was assessed (Fig 13E, F).

Discussion

Leptin is a known regulator of normal and pathological angiogenesis[28]. However, the specific mechanisms involved in these leptin actions are not completely understood. We earlier described that leptin upregulates the expression of VEGF in breast cancer cells through mechanisms involving several canonical signaling pathways and specific transcription factors (*i.e.*, HIF and SP1)[29]. Furthermore, we found that functional Ob-R signaling was linked to increased levels of VEGF/VEGFR2 in breast cancer[13,30]. Moreover, we also found that leptin upregulates Notch in breast cancer, and that a complex crosstalk between Notch, IL-1 and leptin (NILCO) regulates the expression of angiogenic molecules (VEGF/VEGFR) in breast cancer cells[6,27,31].

We early reported that leptin induced the phosphorylation of VEGFR-2 (Y1175) in HUVEC independently of VEGF signaling. These leptin's actions in EC were related to activated Ob-R canonical signals (AKT and p38MAPK) and COX-2 (cyclooxygenase 1) in absence of VEGF[15]. In these cells, the inhibition of VEGFR-2 tyrosine kinase activity reduced leptin-stimulated p38MAPK and Akt activation, COX-2 induction, and pro-angiogenic responses. In addition, the blockade of VEGFR-2 or COX-2 activities abolished leptin-driven neo-angiogenesis in a chick chorioallantoic membrane vascularization assay in vivo[15].

Here we expanded these investigations by showing that leptin-induced proliferation and pro-angiogenic features of EC are related to VEGFR phosphorylation and activation that mediate the upregulation of Notch expression and signaling, in absence of VEGF. These leptin's effects

involve VEGF-independent transactivation of VEGFR-1 or -2.

Present data suggest that leptin pro-angiogenic actions in EC are dependent on a functional Ob-R/VEGFR/Notch signaling axis (Fig 14). Indeed, abrogation or inactivation of Ob-R via PEG-LPrA2, VEGFR kinase activity or gene expression by siRNA or SU5416, and abrogation of Notch activity via DAPT mediated-inhibition of γ-secretase (an essential step for Notch activation), completely impaired leptin-induced HUVEC proliferation, S-phase progression, and tube-like structure formation.

It is known that Notch signaling plays critical roles in cell fate determination an angiogenesis[32]. Notch1 and Notch4 receptors, and DLL1, DLL4, and JAG1 ligands are predominant found in the endothelium. Moreover, Notch pathway is involved in a feedback loop with VEGF. Here, we further assessed that leptin can induce VEGFR-2 expression through Notch signaling[8].

The Notch pathway is tightly regulated in EC by VEGF signals. Positive or negative modulation of Notch results in vascular pathology. It was earlier shown that VEGF, but not basic fibroblast growth factor (bFGF), could induce gene expression of Notch1 and DLL4 in EC. Moreover, regulation of Notch/DLL gene expression by VEGF was found critical for arteriogenesis and angiogenesis[33]. VEGF regulates fate and phenotypic changes of EC during the formation of capillaries and the formation of tip cells during angiogenesis via Notch1/Dll4 activity[23]. Additionally, Notch1 induced signals have been implicated in other angiogenic functions (*i.e.,* formation of hematopoietic stem cells from EC[34]. However, the signaling mechanisms controlling Notch and ligand gene expression in EC are not completely known.

Present results suggest that leptin regulates specific Notch receptors and ligands in EC either through trans-activation of VEGFR-1, VEGFR-2 or heterodimers. It was assessed that leptin induces the trans-phosphorylation of Y1175 VEGFR-2. Moreover, leptin also induces additional trans-phosphorylation of VEGFR-2 at Y951 and Y996, which was more evident in PAEC-VEGFR-2 transfected cells. Interestingly, leptin upregulation of Notch1 and 4 was linked to both VEGFR-1 and -2 activities, as the simultaneous knock down of these receptors abolished leptin effects. Then, these leptin's actions could involve VEGFR-1/VEGFR-2 heterodimer signaling. In contrast, leptin upregulation of Notch2 and JAG1 in EC was only related to leptin-induced VEGFR-2 transactivation. However, the specific biological relevance of these findings needs to be further investigated.

We previously reported that the crosstalk between Notch, leptin, and IL-1 signaling, NILCO[29] regulate the expression of pro-angiogenic molecules, leading to increased cell proliferation and migration in breast cancer[27]. Moreover, we found that leptin-induced Notch signaling, and protein and mRNA expression, and upregulation of Notch target genes was linked to obesity context in mice hosting xenograft breast tumors[8,31]. Here we further show that leptin trans-activates VEGFRs to induce Notch, which seems to be instrumental for leptin-induced proliferation and angiogenic transformation of EC. These leptin's actions were independently of VEGF signaling. Therefore, present data suggest that leptin has redundant actions to VEGF on EC, which could profoundly affect angiogenesis, especially in obesity contexts that are characterized by abnormal high levels of leptin. Obesity and overweight conditions have been strongly related to the incidence and poor prognosis of several cancer types[35].

In conclusion, present data suggest that leptin induces proliferation and transformation of EC through mechanism involving VEGFR-2 and Notch (see Fig 14). These findings suggest that Ob-R could be a potential target for anti-angiogenesis therapies. Moreover, the finding that a functional leptin/VEGFR/Notch crosstalk axis is required for leptin-induced angiogenic features in EC opens the potential development of novel therapies for control of tumor angiogenesis. Combination therapies targeting the Ob-R/VEGFR-2/Notch axis could be beneficial for cancer patients, particularly those showing high levels of leptin due to obesity or overweight conditions. However, these results need to be further validated using in vivo models.

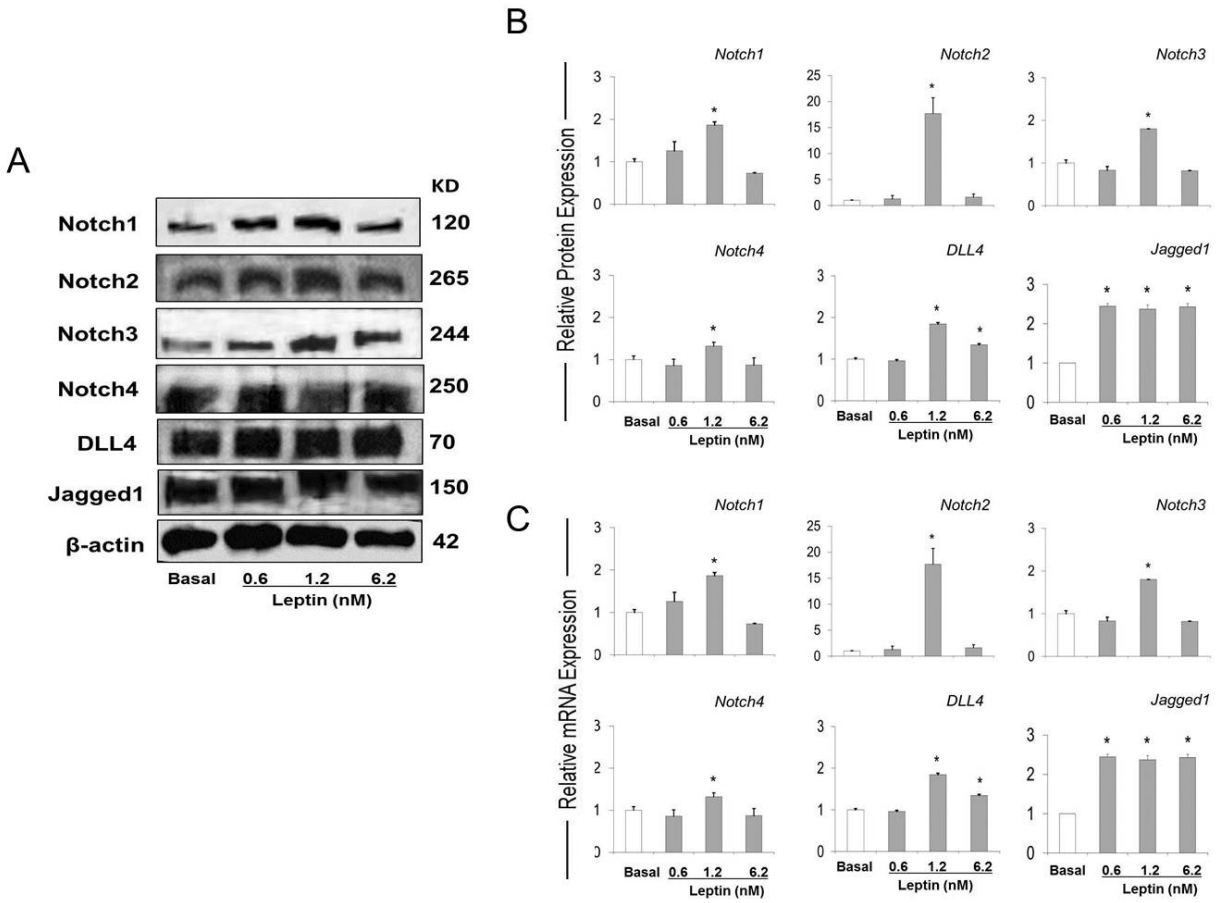


Figure 7. Dose-response effects of leptin on Notch expression in endothelial cells.
(A) Representative Western blot (WB) results, and **(B)** quantitative analysis of leptin-induced effects on the expression of Notch proteins, and **(C)** mRNA as determined by RT-PCR in HUVEC exposed to leptin (0.6, 1.2, and 6.2 nM) for 24 hours. WB and RT-PCR results were normalized to b-actin and GAPDH, respectively. Densitometric analysis of protein expression was carried out using NIH image J software. Data is presented as an average ± SD from three independent experiments. (*) $P<0.05$ when comparing levels of protein to control (basal).

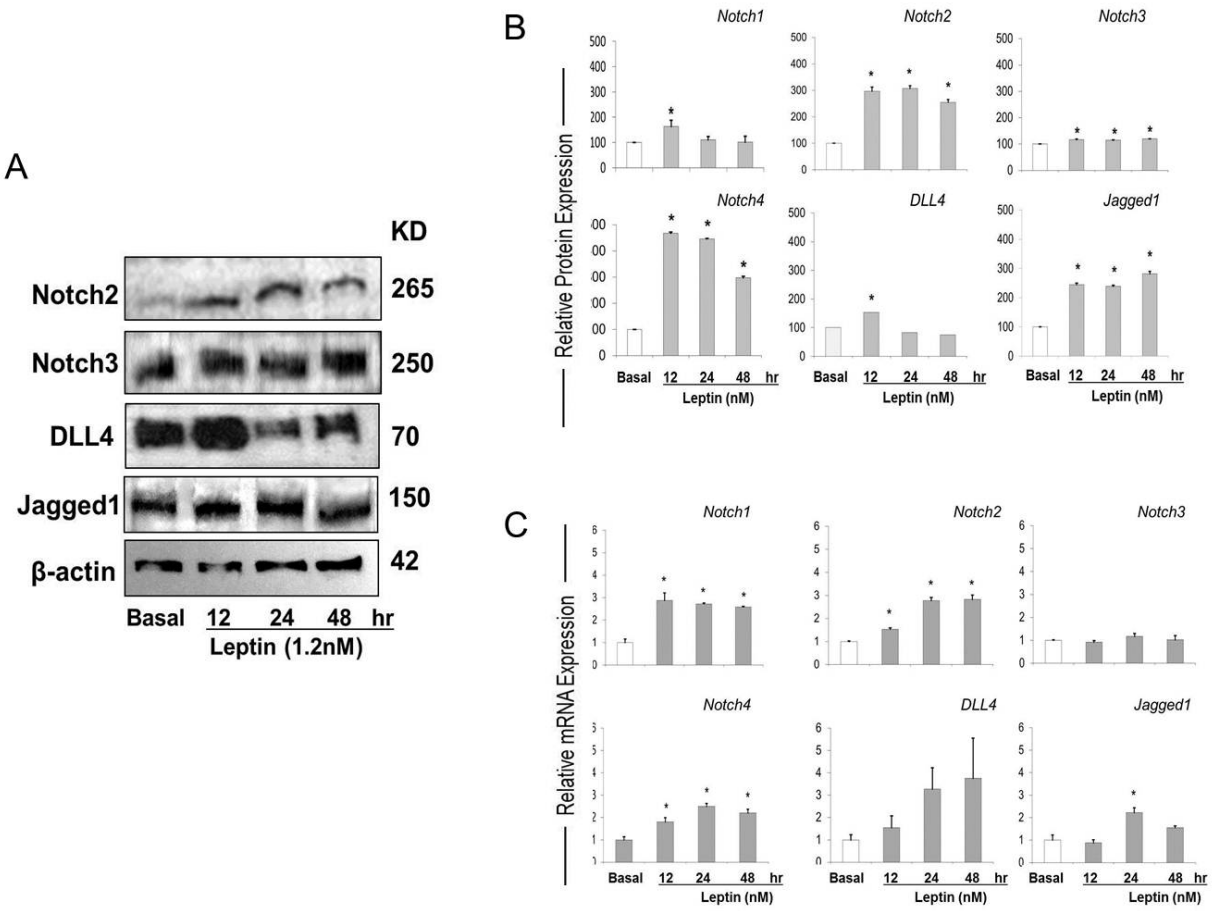


Figure 8. Time-response effects of leptin on Notch expression in endothelial cells.
(A) Representative Western blot (WB) results, and **(B)** quantitative analysis of leptin-induced effects on the expression of Notch proteins, and **(C)** mRNA as determined by RT-PCR in HUVEC exposed to leptin (1.2 nM) for 12, 24, and 48 hours. WB and RT-PCR results were normalized to b-actin and GAPDH, respectively. Densitometric analysis of protein expression was carried out using NIH image J software. Data is presented as an average ± SD from three independent experiments. (*) $P<0.05$ when comparing levels of protein to control (basal).

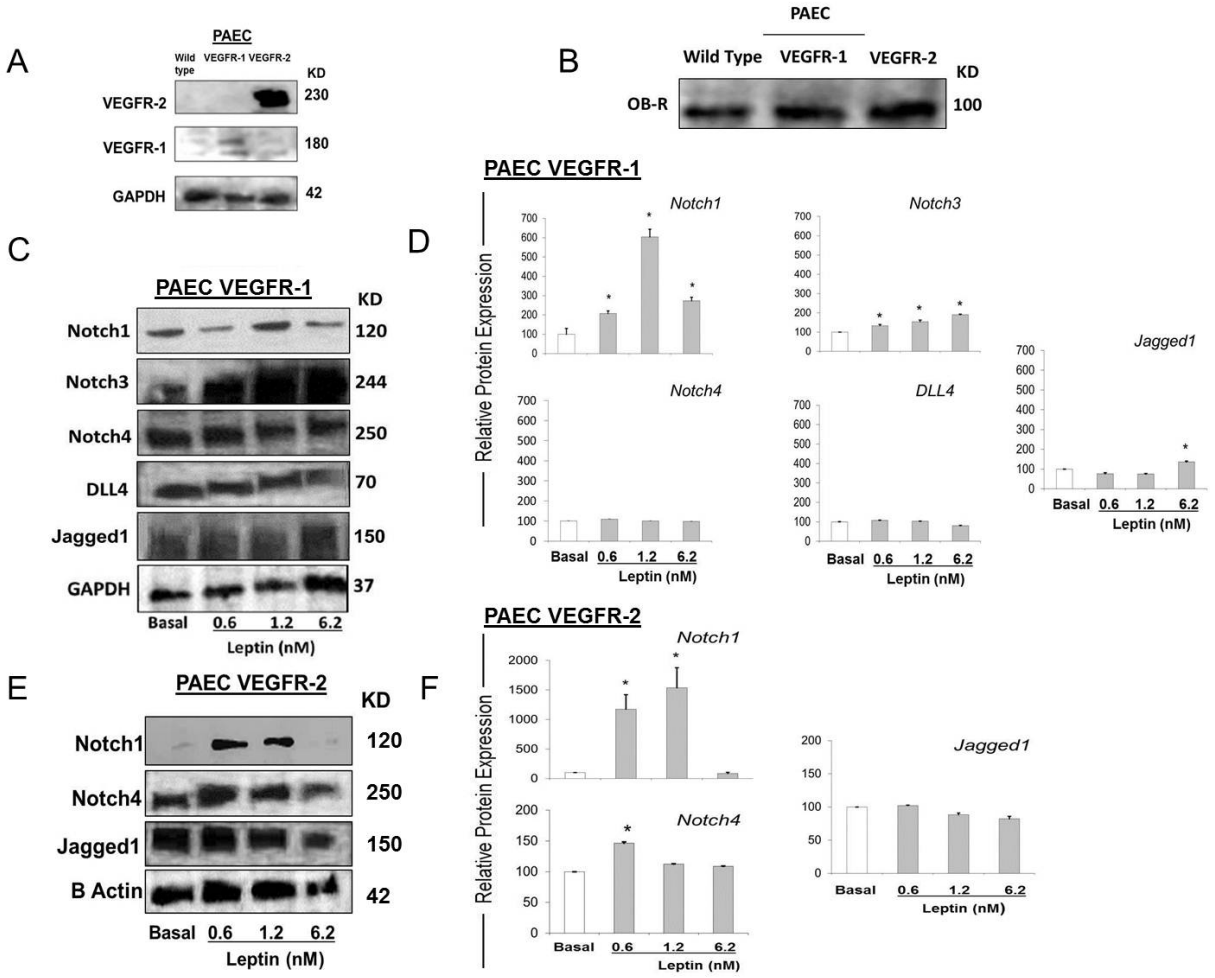


Figure 9. Functional VEGF receptors are needed for leptin induction of Notch
(A) Representative Western blot (WB) results from VEGFR-2 and VEGFR-1 and (B) Ob-R expression in wild type PAEC, and transfected PAEC VEGFR-1 and PAEC VEGFR-2. **(C** and **E)** Representative WB results, and **(D** and **F)** quantitative analysis of leptin induction of Notch proteins in transfected PAEC VEGFR-1 and PAEC VEGFR-2 exposed to leptin (0.6, 1.2, and 6.2 nM) for 24 hours. WB results were normalized to b-actin or GAPDH. Densitometric analysis of protein expression was carried out using NIH image J software. Data is presented as an average ± SD from three independent experiments. (*) $P<0.05$ when comparing levels of protein to control (basal).

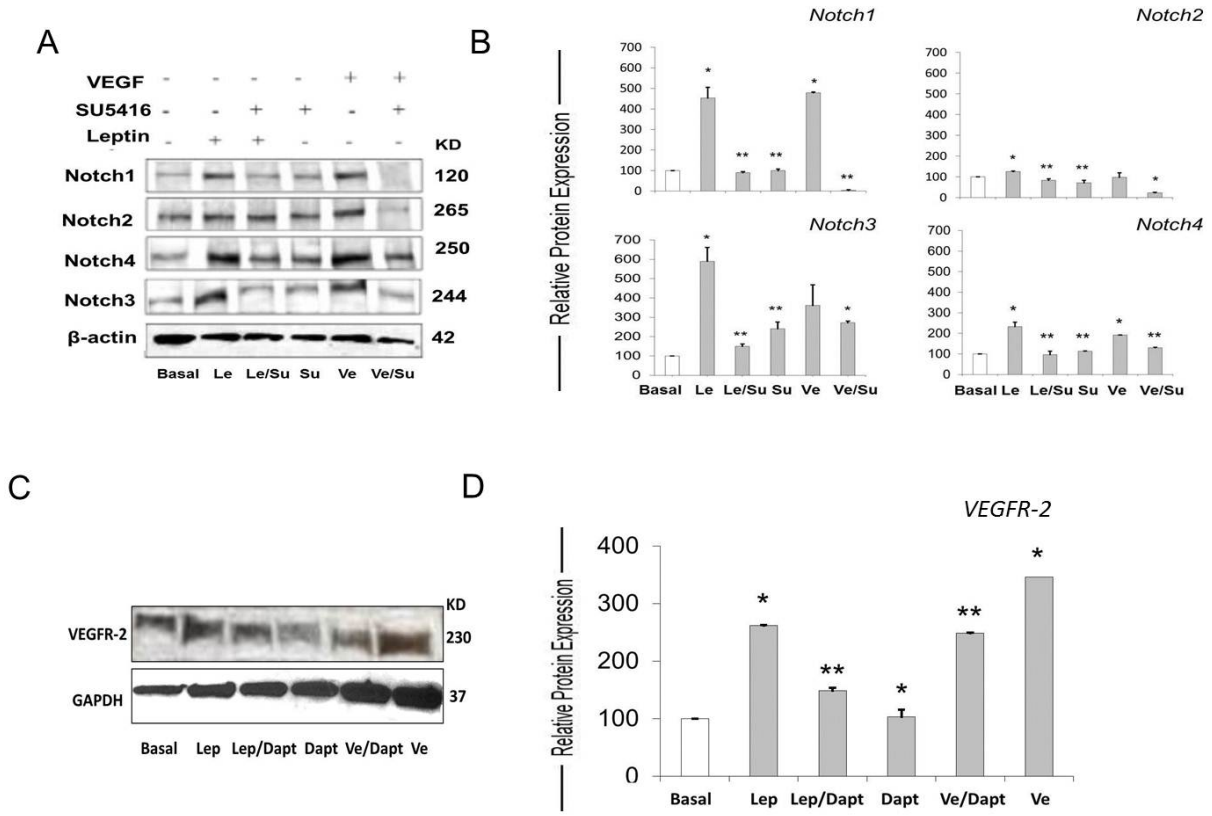


Figure 10. Inhibition of VEGFR-2 kinase and g-secretase abrogated leptin induction of Notch in HUVEC

(A and **C)** Representative Western blot (WB) results, and **(B** and **D)** quantitative analysis of the effects of VEGFR-2 and Notch inhibition on leptin-induced Notch and VEGFR-2 in HUVEC, respectively. HUVEC were treated with leptin (1.2 nM), VEGFR-2 inhibitor, Semaxinib/SU5416 (5 µmol/L) or γ-secretase inhibitor, DAPT (5 µmol/L), and positive control, VEGF (25 ng/mL). WB results were normalized to b-actin or GAPDH. Densitometric analysis of protein expression was carried out using NIH image J software. Data is presented as an average ± SD from three independent experiments. (*) P<0.05 when comparing levels of protein to control (basal). (**) P<0.05 when comparing to HUVEC treated with leptin. Abbreviations: Leptin (Lep), SU5416 (Su) and VEGF (Ve).

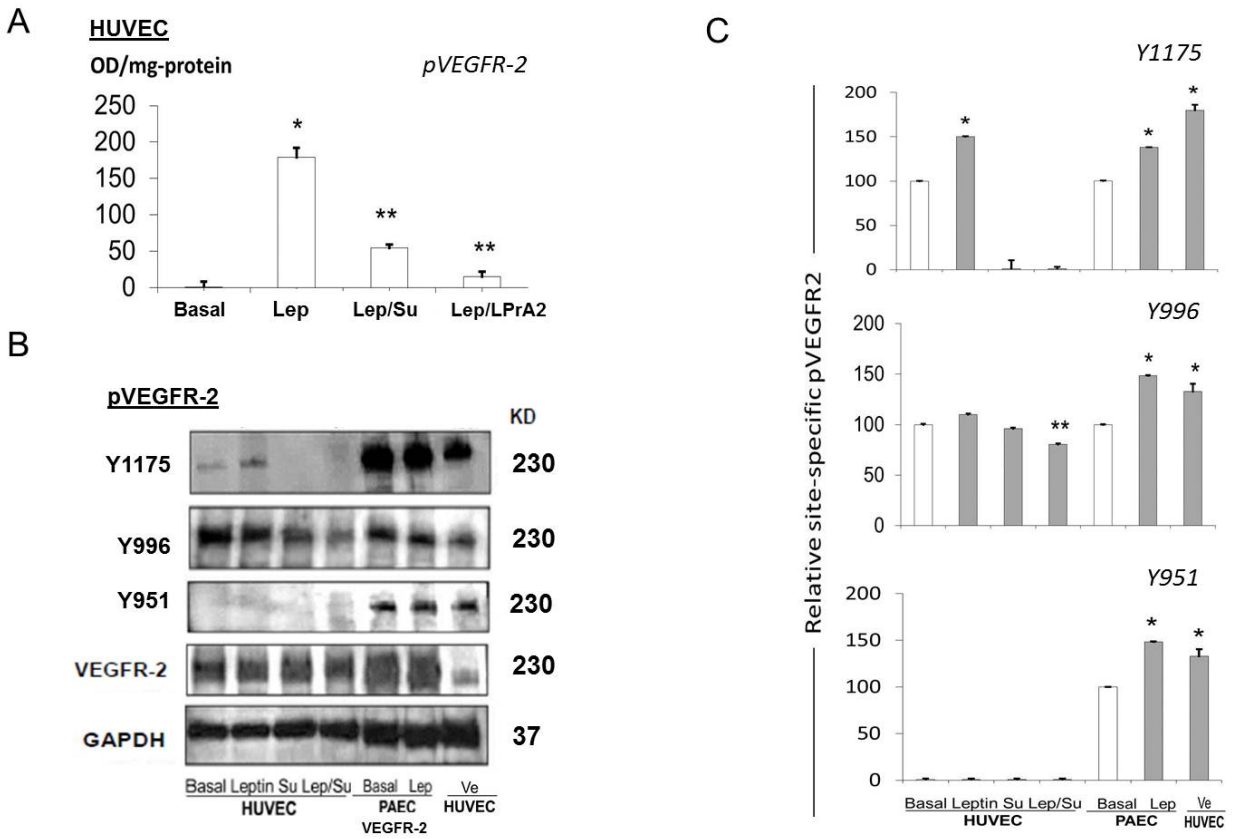


Figure 11. Leptin induces trans-phosphorylation of VEGFR-2 in endothelial cells
(A) Quantitative results from leptin-induced trans-phosphorylation of VEGFR-2 in HUVEC as determined by ELISA in cell lysates. (B) Representative Western blot (WB) results, and **(C)** quantitative analysis of leptin-induced trans-phosphorylation of pVEGFR-2 at Y1175, Y951, and Y996 determined in transfected PAEC-VEGFR-2. WB results were normalized to GAPDH. Densitometric analysis of protein expression was carried out using NIH image J software. pVEGFR-2 ELISA sensitivity <1 pg/ml (R&D System). Data is presented as an average ± SD from three independent experiments. (*) P<0.05 when comparing levels of protein to control (basal). (******) P<0.05 when comparing to HUVEC treated with leptin. Abbreviations: Leptin (Lep), LPrA2 (pegylated LPrA2), SU5416 (Su) and VEGF (Ve).

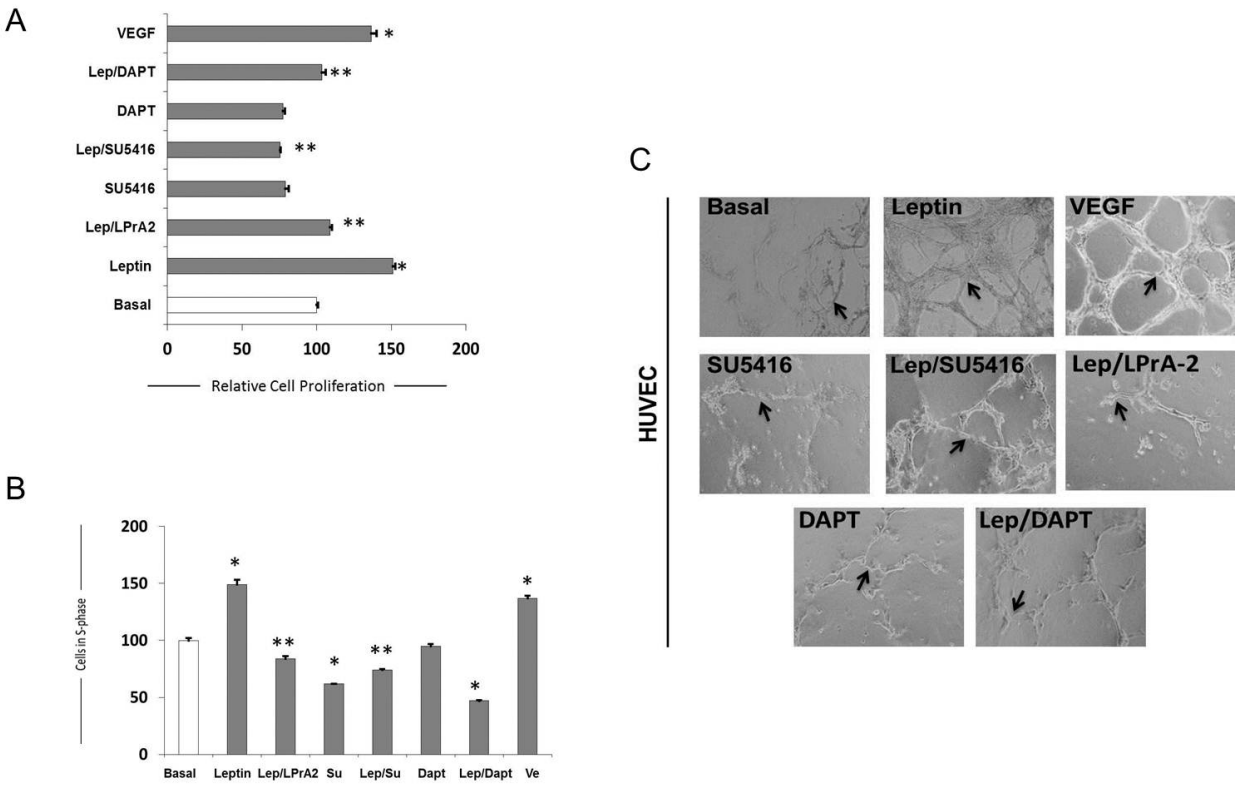


Figure 12. Abrogation of VEGFR-2 and Notch signaling are essential for leptin induced cell proliferation, S-phase progression, and tube formation in endothelial cells.
(A) Quantitative results from the effects of VEGFR-2 and Notch inhibition on leptin-induced proliferation and **(B)** S-phase progression of HUVEC as determined by MTT assay and Cellometer-cell cycle determinations, respectively. **(C)** Representative results of the effects of VEGFR-2 and Notch inhibition on leptin-induced tube-like structure formation by HUVEC cultures in Matrigel coated plates. For cell proliferation analysis HUVEC were exposed to leptin (1.2 nM), inhibitors of VEGFR-2, SU5416 (5 µmol/L) and γ-secretase, DAPT (5 µmol/L) for 24 hours in 96 well plates. VEGF (25 ng/ml) and PEG-LPrA2 (1.2 nM) were used as positive and negative controls, respectively. MTT assay was performed using Spectramax software to determine the amount of cells proliferating. Absorbance was determined at 540 nm. Quantitative staining of DNA was carried out using propidium iodide and gated cells in S-phase were analysed using Cellometer (Nexcellom) and represented as percentages respect to basal conditions (no leptin or inhibitors). For analysis of leptin-induced tube-like structure formation, HUVEC were cultured on growth-factor reduced matrigel and treated with leptin (1.2 nM), inhibitors, and positive and negative controls as described above for 24 hours. Endothelial cells and tube-like structures were photographed under microscopy at 10X magnification. Data is presented as an average ± SD from three independent experiments. (*) $P<0.05$ when comparing levels of protein to control (basal). (******) $P<0.05$ when comparing to HUVEC treated with leptin. Abbreviations: Leptin (Lep), LPrA2 (pegylated LPrA2), SU5416 (Su) and VEGF (Ve).

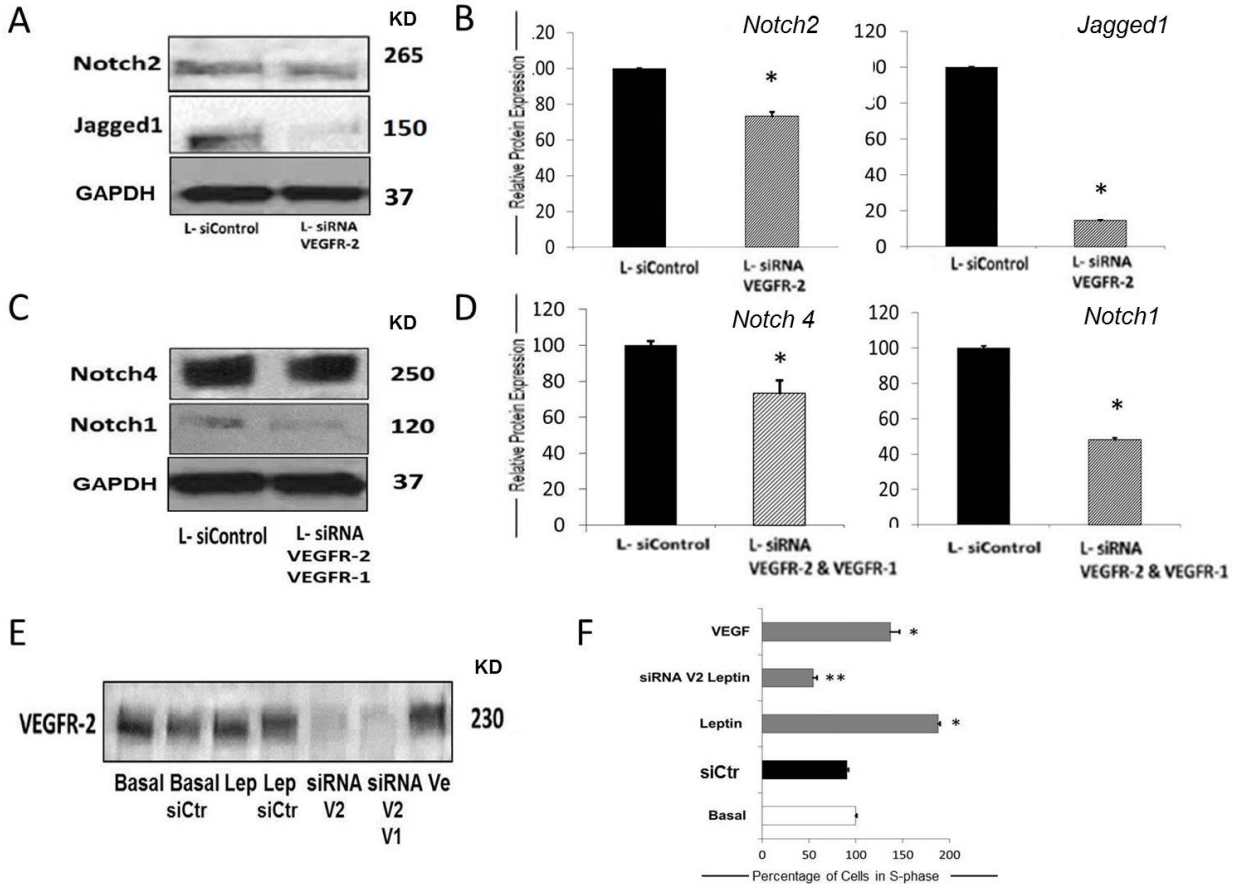


Figure 13. Knockdown of VEGFR-1 and VEGFR-2 abrogates leptin induction of Notch expression and S-phase progression in HUVEC

(A and **C)** Representative Western blot (WB) results and **(B** and **D)** quantitative analysis of changes in Notch expression in HUVEC transfected with siRNA VEGFR-2 and double silencing with siRNA VEGFR-1 and VEGFR-2. **(E)** Representative WB results from VEGFR-2 expression and, **(F)** S-phase progression of HUVEC treated with leptin and transfected with siRNA VEGFR-1, and double silencing with siRNA VEGFR-1 and VEGFR-1. HUVEC transfected with siRNA scramble were used as transfection control. WB results were obtained from cell lysates after 6 hours silencing and normalized to GAPDH. Densitometric analysis of protein expression was carried out using NIH image J software. Data is presented as an average ± SD from three independent experiments. (*) $P<0.05$ when comparing levels of protein to control (basal). (**) $P<0.05$ when comparing to HUVEC treated with leptin. Abbreviations: Basal (Ba), Leptin (Lep), siRNA control, (siCtr), siRNA VEGFR-1 (V1), siRNA VEGFR-2 (V2), and VEGF (Ve).

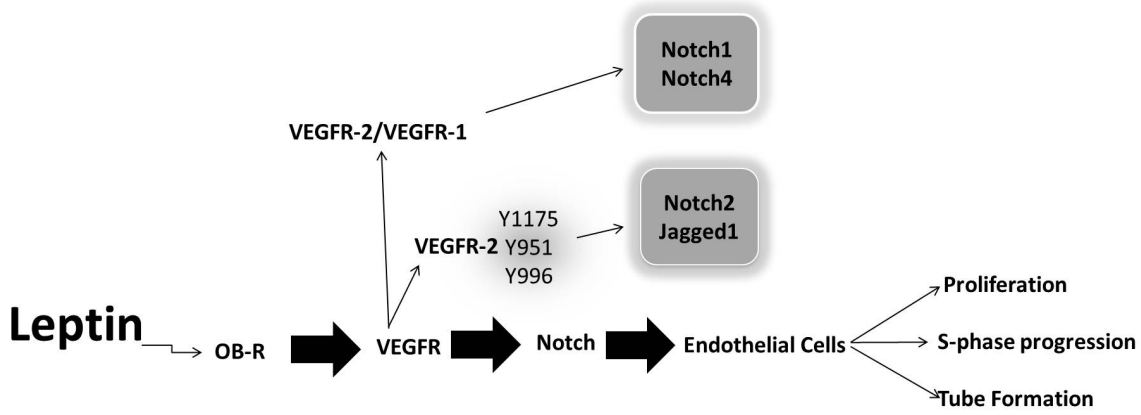


Figure 14. A Functional Ob-R/VEGFR/Notch signalling axis is required for the VEGF-independent leptin induction of proliferation, S-phase progression and angiogenic features in endothelial cells

Representative diagram showing that leptin induction of endothelial cell (EC) proliferation, S-phase progression and tube-like structure formation in vitro, require leptin/Ob-R trans-phosphorylation and activation of VEGFR-2, which upregulates Notch expression leading to the acquisition of angiogenic features (tube-like structure formation) in absence of VEGF. Y1175, Y951, and Y996 amino acid residues in the VEGFR-2 intracytoplasmatic tails are trans-phosphorylated by leptin/Ob-R that upregulate Notch 2 receptor and Jagged1 ligand. Functional VEGFR-2/VEGFR-1 heterodimer is needed for leptin-induced upregulation of Notch1 and Notch4 expression. Leptin actions in EC seem to be redundant to VEGF, which may be relevant for angiogenesis, particularly tumor angiogenesis in obese contexts characterized by high levels of leptin.

Task 2

Targeting BCSC in vivo in TNBC hosted by obese mice

Syngeneic E0771-TAM/DIO-mouse model: Diet-induced-obesity (DIO): Feeding C57BL/6J mice high-fat diet to induce DIO.

To investigate whether obesity induces a leptin–VEGFR2/Notch signaling axis in TNBC that affects breast cancer stem cells (BCSC) and drug resistance, E0771-TAM (PR^-, $Her2^-$ and ER^+, but estrogen unresponsive; TNBC-like cells) hosted by syngeneic diet-induced-obesity (DIO) C57BL/6J female mice are being used. DIO-mice hosting orthotopic E0771-TAM tumors will be treated with leptin inhibitor (IONP-LPrA2).

Notch loss-of-function [DAPT: a γ-secretase inhibitor, and expression of dominant negative RBP-Jk or CSL (R218H), an essential Notch transcription factor] suggested that a functional leptin-Notch signaling axis was involved in the proliferation and migration of E0771 cells.

Materials and Methods

DMEM was obtained from Life Technologies, Grand Island, NY, USA. MTT assay kit was purchased from Promega Corporation, Fitchburg, WI, USA. Dual-luciferase assay system and pGL-3 plasmid were obtained from Promega (Madison, WI). RNeasy Mini kits, DNase kits and Superfect transfect reagents were obtained from Qiagen (Valencia, CA). Vectastin ABC-APK and Vectamount were obtained from Vector Laboratories, Burlingame, CA, USA. Hematoxilyn was purchased from Dako Corporation, Carpinteria, CA, USA. Monoclonal Notch1 (N6786) and β-actin (A5316) antibodies, protease inhibitor, phosphatase inhibitor cocktails 1 and 2, fetal bovine serum (FBS), DAPT [N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester], DAPI (4',6-diamidino-2-phenylindole) and other chemicals were purchased from Sigma-Aldrich, St. Louis, MO, USA.

Cell cultures

The E0771 cell line (originally provided by Dr. Mikhail Kolonin; Centre for Stem Cell Research, Institute of Molecular Medicine, University of Texas) was generated from an estrogen receptor positive (ER^+) mammary adenocarcinoma isolated from a C57BL/6J mouse[36]. To pharmacologically inhibit Notch signaling, a γ-secretase inhibitor, DAPT, was used. Additionally, RBP-Jk dominant negative construct, pCMX-N/R218H (RIKEN, Tsukuba-city, Ibaraki, JAPAN) deposited by T. Honjo (University of Kyoto, Japan) was used[27]. R218H carries an R-to-H substitution at position 218, which is critical for the DNA binding activity of RBP-Jk. R218H was re-cloned into the pCMX vector and transfected into E0771 cells. To assess the inactivation of RBP-Jk gene expression by pCMX-N/R218H the cells were co-transfected with RBP-Jk-Luciferase reporter and *Renilla* control-plasmid (PGL3-CBF; Signosis, Inc.). Additionally, the levels of RBP-Jk protein in E0771-R218H cells were determined by WB after leptin challenge[38]. E0771-TAM: The cells (1.0×10^5 cells/ml) were cultured on increasing TAM concentrations (0.01 to 1%) in DMEM medium over 6-months.

Animals and experimental procedures

Female C57BL/6J mice four-week old (Jackson Laboratories, Bar Harbor, ME, USA) were randomly allocated into two groups: lean (n=5) and DIO (n=42). Mice were housed four per cage in the animal facilities of Morehouse School of Medicine (MSM, Atlanta, GA, USA) in

rooms maintained at 25^{o}C with 10-15 air exchanges per hour. Artificial light was provided under a 12h/12h light/dark cycle. All housing materials, as well as food and water, were autoclaved prior to use. All experiments were performed according to the protocol approved by MSM-IACUC and NIH guide for the Care and Use of Laboratory Animals (IACUC, Protocol 15-02).

Diets, overweight and obesity evaluations

The TD.06416 (chow diet: 10% Kcal from fat; 3.6 Kcal/g; Harlan Lab.) and TD.06414 (HFD; 60% Kcal from fat; 5.1 Kcal/g; Harlan Lab.) adjusted calories diets were used[8]. The mice were fed either the lean (control) or DIO diets at libitum. Each cage was provided with a supply of pre-weighted diet. Food intake was determined weekly by weighting the differences of remain diets in each cage divide by number of mice (n=4 mice/cage). Currently, the mice are being fed with the described diets. Overweight and obesity are being identified in DIO-mice showing body weights (BW) ≥ 20- 24% and ≥25% BW of lean control group, respectively (see Fig. 15). E0771-TAM cells will be orthotopically inoculated in the mammary glands of DIO mice when obesity is detected. After detection of tumor growth (100mm3) the mice will be treated for five weeks with IONP-LPrA2 (0.5 ppm), IONP-scramble peptide, cisplatin and combinations of these compounds. Effects of obesity and treatments on tumors growth, general health, food intake, will be determined overtime. After euthanasia, histopathological analysis of tumors will be done. Tumors will be evaluated for the expression of leptin-induced molecules (VEGFR2/Notch axis, proliferation and BCSC markers).

Data Analysis

Summary statistics were performed using Arithmetic means ± Standard deviation for normally distributed continuous data, and the Geometric mean with 95% Confidence Interval. Obesity and treatment status were defined as dichotomous variables with DIO/lean groups, respectively. Differences between of final BW of lean and obese/DIO mice groups were tested using the student's t-test. Similarly, the student's t-test was used to test the difference in antigen expression in cell cultures. The likelihood ratio test will be used to test the assumption that the differences in final BW between mice differed by obesity status in a multiple regression model with an interaction term between obesity status and treatment status. All statistical tests were two-sided and p-values ≤ 0.05 were considered statistically significant. Chi-squared tests will be used to test the association between tumor detection rates and obesity status. Similar analyses will be used to test the association between tumor detection rates and treatment status. Fisher's exact test will be utilized if expected values in any of the cells of the 2×2 tables were less than 5. The student's t-test will also be used to test the differences in plasma leptin levels between mice with detectable tumors and mice without detectable tumors. Due to the expected skewed distribution of plasma leptin levels, the student's t-tests will be performed on the natural logarithm values of plasma leptin. All statistical tests are performed using STATA SE version 11

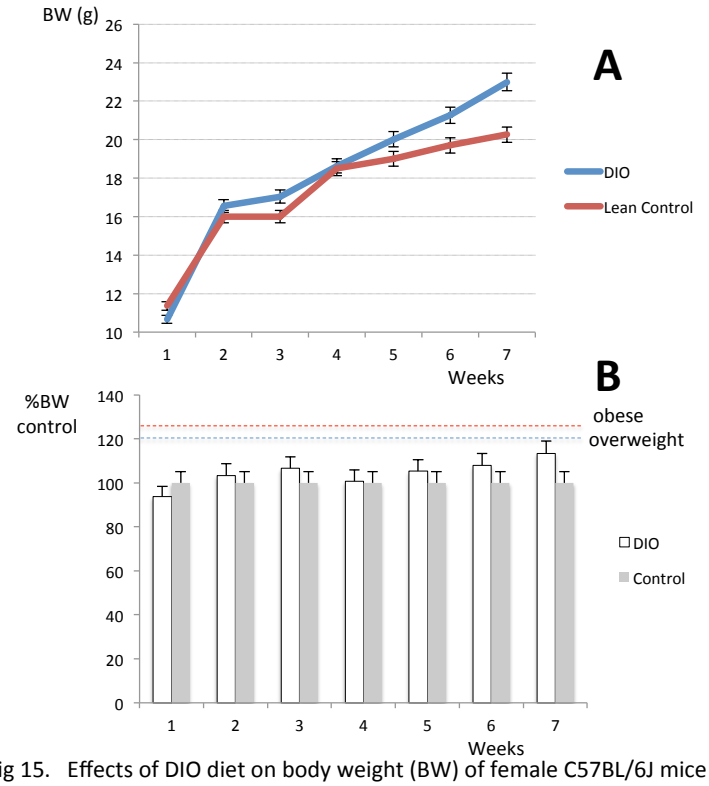


Fig 15. Effects of DIO diet on body weight (BW) of female C57BL/6J mice overtime. A. BW DIO vs control mice and, B. % BW DIO vs control mice. Four weeks mice are being fed with phytoestrogen-free DIO diet (60% Kcal from fat) to induce obesity. Controls mice are receiving similar diet, but containing 10% Kcal from fat. Overweight is defined as BW 20-24 % and obesity as ≥ 25 over BW of control mice

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4. **IMPACT**

Leptin signaling upregulates Notch, IL-1, and VEGF/VEGFR2 in TNBC, which promote survival and angiogenesis. These leptin effects could explain why obesity (characterized by abnormal high levels of leptin) correlates to higher incidence of TNBC and poor prognosis. Remarkably, the novel delivery platform we have developed for our LPrA2 inhibitor of leptin signalin, IONP-LPrA2 (at very low concentrations: 0.5 ppm), was successfully used to inhibit these leptin effects on several TNBC cell lines. Moreover, combinatory treatments with IONP-LPrA2 plus chemotherapeutic (doxorubicin, cisplatin and paclitaxel) or anti-angiogenic drugs (sunitinib) suggested that IONP-LPrA2 could be used as an effective adjuvant for TNBC therapy.

Targeted inhibition of leptin signaling via IONP-LPrA2 in TNBC could open new strategies for prevention or treatment of TNBC, especially in obese patients. IONP-LPrA2 could also allow reduction of chemotherapeutic dose and their undesirable side effects. Data is being obtained on the effectiveness of IONP-LPrA2 and combined chemotherapeutic (cisplatin) treatments applied to obese mice (DIO) hosting TNBC-like (E0771-TAM) tumors.

We found for the first time that leptin-VEGFR2-Notch axis is essential for leptin-induced angiogenic features in endothelial cells (EC). Leptin induces Notch expression in EC via trans-activation of VEGFR, which was linked to increased cell proliferation, and the development of angiogenic features. Leptin induction of EC proliferation and angiogenic features requires VEGFR-2 upregulation of Notch, which occurs independently of VEGF. Leptin actions in EC seem to be redundant to VEGF, which may be relevant for angiogenesis, particularly tumor angiogenesis in obese contexts characterized by high levels of leptin.

Moreover, analysis of breast cancer tissue arrays from Chinese women suggests that NILCO components are differentially expressed in breast cancer. TNBC showed distinctive patterns for NILCO expression and localization. The complex crosstalk between leptin, IL-1 and Notch could differentially drive breast cancer growth and angiogenesis. Furthermore, the analysis of NILCO and targets using Pathway Studio9 software (Ariadine Genomics) showed multiple molecular relationships that suggest NILCO has potential prognostic biomarker value in breast cancer.

5. CHANGES/PROBLEMS

There have been some delays encountered during the reporting period due to the hiring of a postdoctoral fellow (Dr. Adrian A. Sandoval-Montiel). Dr. Sandoval- Montiel was interviewed and selected among several qualified candidates to collaborate with in the development of the project. Unfortunately, after less than two months of being hired, for personal reasons Dr. Sandoval-Montiel resigned his position at the Department of Microbiology, Biochemistry and Immunology, Morehouse School of Medicine. This issue negatively impacted on the development of the planned investigations using obese (diet–induced-obesity, DIO) C57BL/6J syngeneic mice hosting E0771-TAM derived tumors. Currently, we have already hired Dr. Antonio Rampoldi to replace Dr. Sandoval-Montiel. Dr. Rampoldi is a postdoctoral fellow that is currently involved in the accomplishment of the TNBC-DIO-mice tasks. This part of the project is ongoing and should be finalized before the end of 2015.

6. PRODUCTS

Publications, conference papers, and presentations

Journal publications

1) Colbert L, Wilson K, Wan Z, Liu Y, Oprea-Ilies G, Gillespie G, Dickson Toi, Newman G, **Gonzalez-Perez RR**. NILCO biomarkers in breast cancer from Chinese patients. BMC Cancer. 2014, 14:249. [*Acknowledgement of federal support: yes]*

2) Daley-Brown, D, Oprea-Ilies GM, Lee R, Pattillo R, **Gonzalez-Perez RR**. Molecular cues on obesity signals, tumor markers and endometrial cancer. Hormone Molecular Biology and Clinical Investigation (HMBCI) 2015; 21 (1): 89–106, ISSN (Online) 1868-1891. [*Acknowledgement of federal support: yes]*

Books or other non-periodical, one-time publications

1) Harmon T, **Gonzalez-Perez, RR**. Evaluation of nanoparticle-based therapy for triple negative breast cancer using cellometer vision CBA. 2014. Nexcelom. http://www.nexcelom.com. [*Acknowledgement of federal support: yes]*

Manuscripts submitted

1) Crystal C. Lipsey; Adriana Harbuzariu; Danielle Daley-Brown, **Ruben R. Gonzalez-Perez.** Oncogenic role of leptin and NILCO signaling in Cancer (Invited Review). World J. Methodology, 2015. [*Acknowledgement of federal support: yes]*

2) Tia Harmon, Adriana Harbuzariu, **Ruben R. Gonzalez-Perez**. Development of leptin peptide receptor antagonists linked to iron oxide nanoparticles, a novel adjuvant therapy for triple negative breast cancer. 2015. [*Acknowledgement of federal support: yes]*

3) Viola Lanier, Corey Gilespie, Merle Jeffers, Danielle Daley-Brown, Joy Milner, Crystal Lipsey, Nia Webb, Leonard Anderson, Gale Newman, Johannes Waltenberger, **Ruben Rene Gonzalez-Perez**. Leptin induces a VEGFR-Notch axis in endothelial cells. 2015. [*Acknowledgement of federal support: yes]*

4) Danielle Daley-Brown, Gabriela Oprea-Ilies, Regina Lee, Kiara T Vann, Viola Lanier, Alexander Quarshie, Roland Matthews, James W. Lillard, Roland Pattillo and **Ruben Rene Gonzalez-Perez.** Type II endometrial cancer overexpresses NILCO: a potential link to obesity. 2015. [*Acknowledgement of federal support: yes]*

Presentations in Congresses and Meetings
Proceedings

1) Daley-Brown D, Lee, R, Oprea-Ilies G, Screws E, Matthews R, Patillo R, **Gonzalez- Perez RR**. Role of obesity in Leptin-Notch crosstalk in endometrial cancer from African American and Chinese women. 105th AACR Annual Meeting", San Diego, CA 2014. Cancer Research 2014; 74, 19:5044. Abstract 5044. [*Acknowledgement of federal support: yes]*

2) Harmon T, Harbuzariu A, Yang L, **Gonzalez-Perez RR**. Novel adjuvant therapy with leptin peptide receptor antagonist-2 conjugated to nanoparticles (IONP-LPrA2) to minimize chemoresistance in triple negative breast cancer". 26th EORTC-NCI-ACR Symposium on "Molecular targets and Cancer therapeutics", Barcelona, Spain, 2014. European Journal of Cancer 2014, 50, 89. [*Acknowledgement of federal support: yes]*

Presentations

1) RR Gonzalez-Perez, Invited speaker "Role of NILCO in cancer", Cancer Program MSM Seminar Series MSM/TU/UAB U54 partnership, Atlanta, GA. 2014. [*Acknowledgement of federal support: yes]*

2) Harmon T, Harbuzariu A, Yang L, **Gonzalez-Perez RR**. Iron Oxide Nanoparticle-Leptin Receptor Antagonist: A Novel Targeted Adjuvant Therapy for Triple Negative Breast Cancer. 106th AACR Annual Meeting, Philadelphia, PA, 2015. [*Acknowledgement of federal support: yes]*

2) Lanier V, Gillespie C, Dale-Brown D, Leffres M, Waltenberger, J, **Gonzalez-Perez RR**. Leptin induces a VEGFR2/Notch crosstalk in endothelial cells. New England Science Symposium, 2014, Joseph B. Martin Conference Center at Harvard Medical School, Boston, MA. [*Acknowledgement of federal support: yes]*

3) Lanier V, Leffers M, Waltenberger J, Daley-Brown D, Anderson L, **Gonzalez-Perez R.R**. Abrogation of leptin signaling can reduce transactivation of VEGFR-2/Notch crosstalk and angiogenic features in endothelial cells. World Molecular Imaging Congress. Honolulu, Hawaii September 2-5, 201. [*Acknowledgement of federal support: yes]*

4) Viola Lanier, Corey Gillespie, Danielle Daley, Merle Leffers, Johannes Waltenberger, and **Ruben R. Gonzalez-Perez**. Leptin Induces a VEGFR-2/Notch Crosstalk in Endothelial Cells. The Minority Health and Health Disparities Grantees' Conference 2014, Baltimore, MD. [*Acknowledgement of federal support: yes]*

5) Lanier V, Webb N, Anderson L, **Gonzalez-Perez RR**. Leptin-Notch axis affects cancer stroma. NIH/NCI 2014 International Symposium on Minority Health and Health Disparities, ISMHHD, NIH/NIMHD/DHHS, MD, 2014. [*Acknowledgement of federal support: yes]*

6) Dill C, Harbuzariu A, **Gonzalez-Perez RR**. PEG-LPrA2: A New Adjuvant Therapy for Triple Negative Breast Cancer. "Student Symposium MSM; Curtis L Parker", 2015. [*Acknowledgement of federal support: yes]*

7) Lanier V, Leffers M, Waltenberger J, Anderson L, **Gonzalez-Perez RR**. Abrogation of leptin signaling can reduce transactivation of VEGFR-2/Notch crosstalk and angiogenic features in endothelial cells. "Student Symposium MSM; Curtis L Parker", 2015. [*Acknowledgement of federal support: yes]*

8) Courtney Dill, Adriana Harbuzarui M.D., Tia Harmon and **Ruben Rene Gonzalez-Perez** Ph.D. PEG-LPrA2: A New Adjuvant Therapy for Triple Negative Breast Cancer. Curtis L. Parker Research Symposium. 02/2015. Morehouse School of Medicine. (Poster). [*Acknowledgement of federal support: yes]*

9) Courtney Dill, Adriana Harbuzarui M.D., Tia Harmon, and **Ruben Rene Gonzalez-Perez** Ph.D. PEG-LPrA2: A New Adjuvant Therapy for Triple Negative Breast Cancer. (Poster). Lasker Lectureship Poster Session. 03/2015. Morehouse School of Medicine. [*Acknowledgement of federal support: yes]*

10) Courtney Dill, Adriana Harbuzarui M.D., Tia Harmon, Crystal Lipsey, Ayobami Loye, and **Ruben Rene Gonzalez-Perez** Ph.D. The Efficacy and Toxicity of PEG-LPrA2: A New Adjuvant for Triple Negative Breast Cancer Treatment. (Poster and Oral Presentation). Summer Cancer Research Training Program. 07/2015. Tuskegee University. [*Acknowledgement of federal support: yes]*

11) Nnanna Onyekaba, Danielle Daley-Brown, Viola Lanier, Regina Lee, Gabriela Oprea-Ilies, Roland Pattillo, **Ruben Rene Gonzalez-Perez**. Obesity and leptin-targeted molecules correlated to more aggressive endometrial cancer in African-American women. NIH STEP-UP Program 2014. Bethesda, MD. [*Acknowledgement of federal support: yes]*

12) Harbuzariu A, Garrison RC, Daley-Brown DS, Beech DJ, Cason FD, Harmon T, Yang L,

Gonzalez-Perez RR. Novel adjuvant therapy for obesity related pancreatic cancer. NIH/NCI 2014 International Symposium on Minority Health and Health Disparities, ISMHHD, NIH/NIMHD/DHHS, MD, 2014. [*Acknowledgement of federal support: yes]*

13) Harbuzariu A, Garrison RC, Daley-Brown DS, Beech DJ, Cason FD, Harmon T, Yang L, **Gonzalez-Perez RR**. Identification of a novel adjuvant therapeutic agent for obesity related pancreatic cancer. 106th AACR Annual Meeting, Philadelphia, PA, 2015. [*Acknowledgement of federal support: yes]*

14) Daley-Brown D, Oprea-Ilies G, Pattillo R, Lillard J, **Gonzalez-Perez RR**. NILCO as a novel biomarker for type 2 endometrial cancer. Translational Science 2015 (ACTS), Washington, DC, 2015. [*Acknowledgement of federal support: yes]*

15) Danielle Daley-Brown, Viola Lanier, Gabriela Oprea-Ilies M.D., Regina Lee M.D.,Roland Pattillo M.D., James Lillard Ph.D., **Ruben Rene Gonzalez-Perez** Ph.D. NILCO: A Novel Chemotherapeutic Target for Endometrial Cancer. Eastern-Atlantic Student Research Forum 2015, University of Miami Miller School of Medicine. Miami, FL. [*Acknowledgement of federal support: yes]*

16) A. Harbuzariu, RC Garrison; DJ Beech; FD Cason; TL Harmon; L Yang; **RR Gonzalez-Perez**. Novel adjuvant therapy for obesity related pancreatic cancer. Summer Institute, Tuskegee, AL, 2015-BEST POSTER THIRD PLACE PRIZE. [*Acknowledgement of federal support: yes].*

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	*Ruben Rene Gonzalez-Perez*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	*Dr. Gonzalez-Perez has performed a leading role in all work areas for the development of the project*
Funding Support:	

Name:	*Dr. Adrian A. Sandoval-Montiel*
Project Role:	*Postdoctoral fellow*
Researcher Identifier (e.g. ORCID ID):	

Nearest person month worked:	*2*
Contribution to Project:	*Dr. Sandoval-Montiel performed some investigations and planning using DIO-TNBC mice.*
Funding Support:	

Name:	*Dr. Antonio Rampoldi*
Project Role:	*Postdoctoral fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2*
Contribution to Project:	*Dr. Rampoldi has developed tasks related to the transfection and characterization of E0771-TAM and E0771-TAM (R218H) and generation of DIO-mice.*
Funding Support:	

Name:	*Dr. Adriana Harbuzariu*
Project Role:	*Postdoctoral fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3*
Contribution to Project:	*Dr. Harbuzariu has collaborated with the characterization of TNBC-BCSC and generation of DIO-mice.*
Funding Support:	NIH/NCI 5U54 CA118638 UAB-Tuskegee–MSM Cancer Partnership.

Name:	*Tia Harmon, MSc*
Project Role:	*Ph.D. Graduate student*
Researcher Identifier (e.g. ORCID ID):	

Nearest person month worked:	*12*
Contribution to Project:	Tia Harmon has collaborated with the characterization of TNBC-BCSC, chemotherapeutic effects, and generation of IONP-LPrA2, E0771-TAM cell and DIO-mice.
Funding Support:	National Institute on Minority Health and Health Disparities (RISE 5R25 GM058268)

Name:	*Viola Lanier, MSc*
Project Role:	*Ph.D. Graduate student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	Viola Lanier has collaborated with the characterization of leptin-VEGFR/NOTCH axis and generation of DIO-mice.
Funding Support:	National Institute on Minority Health and Health Disparities (RISE 5R25 GM058268)

Name:	*Danielle Daley-Brown, MSc*
Project Role:	*Ph.D. Graduate student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	Danielle Daley-Brown has collaborated with the characterization of IONP-LPrA2 and generation of DIO-mice.
Funding Support:	National Institute of General Medical Sciences, National Institutes of Health (5T32HL103104-04)

Name:	*Courtney Dill, MSc*
Project Role:	*Ph.D. Graduate student*
Researcher Identifier (e.g. ORCID ID):	

Nearest person month worked:	*12*
Contribution to Project:	Ms. Dill has collaborated with the characterization of IONP-LPrA2 and generation of DIO-mice.
Funding Support:	Gates Millennium Scholarship Award

Name:	*Crystal Lipsey*
Project Role:	*Ph.D. Graduate student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	Ms. Lipsey has collaborated with the characterization of IONP-LPrA2, TBNC cell lines and generation of DIO-mice.
Funding Support:	NIH/NCI 1R41 CA183399-01A1 and 5U54 CA118638 to RRGP; and S21 MD000101, 5G12 MD0076021, G12 RR026250-03, NIH RR03034 and 1C06 RR18386 to Morehouse School of Medicine

Name:	*Cynthia Tchio*
Project Role:	*Ph.D. Graduate student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
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8. SPECIAL REPORTING REQUIREMENTS

Nothing to report

9. APPENDICES

Two published papers are attached.

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RESEARCH ARTICLE Open Access

NILCO biomarkers in breast cancer from Chinese patients

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Abstract

Background: Notch, IL-1 and leptin are known pro-angiogenic factors linked to breast cancer development, tumor aggressiveness and poor prognosis. A complex crosstalk between these molecules (NILCO) has been reported in breast cancer cell lines. However, whether NILCO biomarkers are differentially expressed in estrogen responsive (ER+), unresponsive (ER-) and triple negative (TNBC) breast cancer tissues is unknown.

Methods: Expression levels of nine NILCO and targets [Notch1, Notch4, JAG1, DLL4, VEGF, VEGFR2 (FLK-1), leptin, leptin receptor (OB-R) and interleukin-1 receptor type I (IL-1R tI)] were examined via immunohistochemistry in breast cancer tissue microarrays from Chinese patients (ER+, n=33; ER-, n=21; TNBC, n=13) and non-malignant breast tissue (n=5; Pantomics, Inc.) using a semi-quantitative analysis of intensity staining, HSCORE.

Results: Categorical expression of NILCO and targets (+ or -) was similar among all cancer tissues. However, TNBC showed differential localization pattern of NILCO. TNBC showed fewer nuclei and cytoplasms positive for Notch4 and JAG1, but more cytoplasms positive for leptin. In addition, fewer TNBC stromas were positive for Notch1 and Notch4, but 100% of TNBC stromas were positive for VEGFR2. Moreover, TNBC had lower DLL4 and IL-1R tI expression. TNBC and ER- showed higher expression of EGFR, but lower expression of AR. Leptin and OB-R were detected in more than 61% of samples. Leptin positively correlated to OB-R, JAG1, VEGF, and marginally to IL-1R tI. Notch1 positively correlated to IL-1R tI. EGFR and Ki67 were positively associated to Notch1, but no associations of NILCO and targets with p53 were found.

Conclusions: Present data suggest that NILCO components are differentially expressed in breast cancer. TNBC showed distinctive patterns for NILCO expression and localization. The complex crosstalk between leptin, IL-1 and Notch could differentially drive breast cancer growth and angiogenesis. Furthermore, the analysis of NILCO and targets using Pathway Studio9 software (Ariadine Genomics) showed multiple molecular relationships that suggest NILCO has potential prognostic biomarker value in breast cancer.

Background

Breast cancer is a heterogeneous disease with four major genetic-signature subtypes [1]. However, breast cancer can be broadly divided into two main groups: 1) Estrogen receptor positive (ER+) and triple negative breast cancer (TNBC: ER-, PR- and HER2-). The majority of breast cancers are ER+, respond to estrogens, and are commonly treated with anti-hormonal and HER2 (ErbB-2) targeted therapies. ER+ positive and HER2/neu+ breast cancer cells show suppressed Notch signaling, which is probably limited by the overwhelming proliferative and survival effects of ER and HER2-dependent pathways [2].

In contrast, TNBC is mostly dependent on growth factors [*i.e.*, insulin, insulin-like growth factor-I (IGF-I) and adipokines] [3,4]. This aggressive form of the disease accounts for 15% of all invasive breast cancers showing an acutely early onset. TNBC is associated with poor survival and resistance to common therapeutic treatments. This difficult-to-treat form of breast cancer shows a tendency to overcome drug effectiveness [5].

Notch signaling is a hallmark of breast cancer [6,7]. The role of Notch in breast cancer development has long been studied, particularly relative to its effects on angiogenesis [8]. Notch expression correlates to poor prognosis and drug resistance of breast cancer patients [9,10]. A particular

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feature of Notch signaling is its variable outcomes, which are dependent on cell and microenvironment types [7]. Ductal and lobular breast carcinomas show variable levels of Notch expression [2].

Notch signaling is a key mediator of proliferation, survival, and possibly malignant invasion of TNBC. These data suggest that TNBC is heavily dependent on Notch signaling [11]. In line with this notion, TNBC seems to differentially-activate Notch. Indeed, Notch1 and Notch4 are overexpressed in TNBC [12]. Moreover, in contrast to normal and ER+ breast cancer tissues, the activation of Notch in ER– breast cancer is linked to survivin upregulation (an apoptosis inhibitor and cell cycle regulator), which suggests ER- breast cancer cells are dependent on Notch-survivin signaling [13].

Recent data indicate that breast cancer development is likely related to lifestyle and the result of being overweight. Obesity is associated with more than 100,000 incidents of cancer in the United States every year, particularly cancers of the breast, colon, and endometrium. The specific molecular mechanisms involved in the development of obesity-related breast cancers are unknown. However, the general picture suggests that obesity-related breast cancer is the consequence of multi-factorial causes [14,15]. Several molecules with altered patterns of expression in obesity are involved in breast cancer (*i.e.*, insulin and IGF-1, and adipokines) [16]. Leptin, the major adipokine secreted by adipose tissue, is also produced by malignant cells, and linked to increased levels of Notch and survivin in breast cancer [17-19], and can affect tumor angiogenesis [20]. Leptin signaling can influence pro-angiogenic, inflammatory and mitogenic events in breast cancer [21-24].

We have previously unveiled a complex crosstalk between Notch, IL-1 and leptin (NILCO) in breast cancer cell lines, which could be essential for leptin-induced proliferation, inflammation and angiogenesis [17]. Moreover, a functional Notch-leptin axis is found in mouse carcinogenic-induced [18] and syngeneic breast cancer [19]. In these mouse models, leptin signaling induces both the expression and activation of Notch. However, it is unknown whether NILCO and its targets could correlate and/or are differentially-expressed in human TNBC, ER-, and ER+ breast cancer tissues.

We propose that specific associations between NILCO biomarkers occur in breast cancer, which may differ in TNBC. To this end, we examined the expression and cellular localization of NILCO, and targets, via immunohistochemistry in a commercial array of breast cancer biopsies from Chinese patients. Data were also analyzed *in silico* via Pathway Studio9 software (Ariadine Genomics, MD) [4]. Data analyses suggest that significant associations exist between NILCO and targets in breast cancer tissues. Higher levels of leptin and Notch1 were found in malignant compared to non-malignant tissues. TNBC showed lower levels of DLL4 and IL-1R tI compared to ER- and ER+ breast cancer. TNBC and their stromas showed differential cellular localization of Notch1, Notch4, JAG1, leptin and VEGFR2. Taken together, these results suggest that differential patterns of NILCO and targets are found in TNBC versus ER- and ER+ breast cancer. Present data support the idea for the potential use of NILCO and related molecules as biomarkers in breast cancer.

Methods

Reagents and antibodies

Polyclonal antibodies for Notch4, OB-R amino terminus, DLL4, IL-1 R tI, VEGF, VEGFR2, Jagged1 (JAG1) and leptin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal anti-Notch2 and -Notch3 were from Abcam Inc. (Cambridge, MA). Monoclonal anti-Notch1 antibody and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Vectastin ABC-APK and Vectamount were obtained from Vector Laboratories (Burlingame, CA). Hematoxilyn was purchased from Dako Corporation, Carpinteria, CA.

Tissue microarray

Breast cancer tissue arrays from female Chinese were obtained from Pantomics, Inc. (Richmond, CA). Biopsies features included age, grading, TNM staging, and receptor status of estrogen (ER), androgen (AR), progesterone (PR), epidermal growth factor receptors (ErbB1/EGFR/HER1 and ErbB2/HER2), and p53 and Ki67 expression data. However, no information on body weight of patients was available. Each slide contained 150 cores, including 75 cases in duplicate of normal/hyperplastic specimens (n=3), fibroadenomas (n=2), ductal carcinoma in situ (DCIS, n=2), Paget's disease (n=1) and invasive carcinomas (ER^+, n=33; ER^-, n=21 and TNBC, n=13) showing diverse levels of PR and HER2 expression. The studies were focused on non-malignant (n=5) and invasive carcinoma samples (ER+, ER- and TNBC; n=67).

Immunohistochemisty (IHC)

IHC staining was performed on 12 separate microarray slides. The following specific antibodies were used to analyze nine antigens: anti-Notch1, Notch4, DLL4, JAG1, leptin, leptin receptor (OB-R), VEGF, VEGFR2 (FLK-1) and IL-1R tI. Staining patterns of 1206 tissue samples were evaluated by two independent observers in a blind manner. Three slides were used for negative controls (no primary antibody) incubated with secondary antibodies (anti-rabbit; anti-mouse and anti-goat-HRP, respectively; Vector Lab.).

HSCORE determination

Staining intensity of cells in tissue arrays was evaluated as negative or positive in three different bright fields (≥100 cells/

field). Semi-quantitative HSCORE was calculated for each antigen using the following equation: HSCORE = ∑ pi(i +1), where "i" was the intensity with a value of 0, 1, 2, or 3 (negative, weak, moderate or strong, respectively) and "pi" was the percentage of stained cells for each intensity [25,26].

In silico analysis of NILCO and targets interaction networks in breast cancer

Pathway Studio9 software (Elsevier, Ariadine Genomics, MD) was used to evaluate NILCO and its targets' interactions in breast cancer tissue arrays. HSCORE of antigens showing significantly relationships in breast cancer were imported into the pathway software and analyzed.

Statistics

HSCOREs for each antigen were determined twice, averaged, named A_HSCORE and used in the analyses. Pearson or Spearman correlation coefficients were used to compare the concordance between results from duplicate breast

Table 1 Clinicopathological and histology characteristics of breast cancer tissue microarray samples

	Breast cancer				Non-malignant
Characteristic	ER- (n=21)	ER+(n=33)	TNBC (n=13)	P-value*	Hyperplasias (n=3) or Fibroadenomas (n=2)
Age	50.86 (± 12.47)	48.67 (± 11.15)	48.69 (± 11.6)	0.778	34.2 (± 11.67)
Grade					
I	0 (0)	1 (3.03)	0 (0)	0.969	NA
II	6 (28.57)	10 (30.3)	3 (23.08)		
III	15 (71.43)	22 (66.67)	10 (76.92)		
Stage					
1	1 (4.76)	1 (3.03)	0 (0)	0.142	NA
2	17 (80.95)	20 (60.61)	5 (38.46)		
3	2 (9.52)	7 (21.21)	4 (30.77)		
4	1 (4.76)	5 (15.15)	4 (30.77)		
ER					
Negative	21 (100)	0 (0)	13 (100)	**<.001**	2 (40)
Positive	0 (0)	33 (100)	0 (0)		3 (60)
PR					
Negative	19 (90.48)	11 (33.33)	13 (100)	**<.001**	2 (40)
Positive	2 (9.52)	22 (66.67)	0 (0)		3 (60)
HER2					
Negative	2 (9.52)	12 (36.36)	13 (100)	**<.001**	5 (100)
Positive	19 (90.48)	21 (63.64)	0 (0)		NA
EGFR					
Negative	13 (61.9)	31 (93.94)	8 (61.54)	**0.004**	5 (100)
Positive	8 (38.1)	2 (6.06)	5 (38.46)		NA
AR					
Negative	14 (66.67)	13 (39.39)	13 (100)	**<.001**	3 (60)
Positive	7 (33.33)	20 (60.61)	0 (0)		2 (40)
Ki67					
Negative	8 (38.1)	10 (30.3)	5 (38.46)	0.791	5 (100)
Positive	13 (61.9)	23 (69.7)	8 (61.54)		NA
P53					
Negative	16 (76.19)	24 (72.73)	9 (69.23)	0.903	5 (100)
Positive	5 (23.81)	9 (27.27)	4 (30.77)		NA

Data are presented as number of patients (%) or mean (± SD). ER: estrogen receptor; TNBC: triple negative breast cancer; PR: progesterone receptor; HER2: human epidermal growth factor receptor type 2; EGFR: epidermal growth factor receptor 1; AR: androgen receptor; Ki67: a proliferation marker; p53: a tumor suppressor protein. *The p-value is calculated by ANOVA for age and chi-square test or Fisher's exact test for other covariates, where appropriate. Numbers in "bold" show significant differences.

tissue samples and pairwise correlation between A_HSCOREs from the nine antigens analyzed in the microarray. The outcome was defined for three types of breast cancers according to the expression of ER, PR and HER2 (ER^+, $ER^-$and TNBC). Predictors were defined for A_HSCORE of nine antigens (continuous; 1–4) and dichotomized A_HSCORE (categorical: negative if HSCORE=1; positive otherwise). Covariate analyses were performed for p53, EGFR, Ki67, AR, grade, stage and age. The patients' clinicopathological and histology characteristics, and the categorical and continuous A_HSCOREs were summarized for breast cancer and non-malignant disease patients. Univariate association between categorical HSCOREs and continuous A_HSCOREs for ER, PR, HER2, EGFR, AR, Ki67 and p53 expressions and grade and stage were compared by Chi-square or Fisher's exact test. ANOVA (analysis of variance) was used to analyze age and Notch1 expression. Kruskal-Wallis test

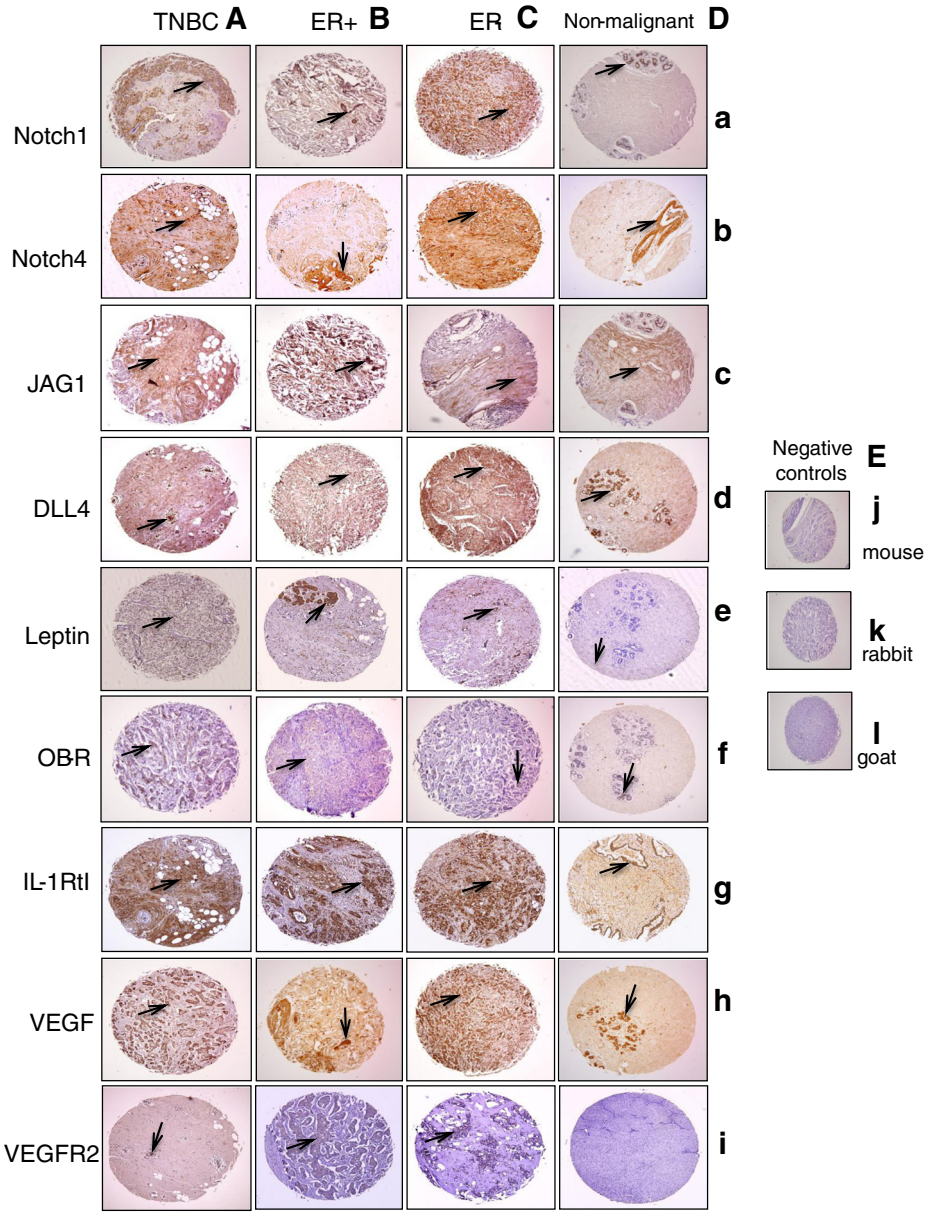

Figure 1 Immunohistochemical (IHC) detection of NILCO and targets in tissue arrays from breast cancer and non-malignant patients. Representative pictures of immunohistochemical staining from breast cancer TNBC **(A)**, ER+ **(B)**, ER- **(C)** and non-malignant tissues **(D)** and, IHC negative controls **(E)** for the expression of NILCO and targets. Arrows show positive staining of NILCO components: Notch1 **(a)**, Notch4 **(b)**, DLL4 **(c)**, JAG1 **(d)**, leptin **(e)**, OB-R **(f)**, IL-1R tl **(g)** and targets VEGF **(h)** and VEGFR2 **(i)**, respectively. No staining was found in IHC-negative controls using anti-mouse **(j)**, anti-rabbit **(k)** and anti-goat **(l)** secondary antibodies. Magnification ×10.

was also used to analyze continuous HSCOREs. All analyses were done using SAS 9.3 (SAS Institute, Inc.) with a significance level of 0.05.

Results

Breast cancer tissue arrays

The clinicopathological and histological characteristics of non-malignant and breast cancer patients are summarized in Table 1. Invasive breast adenocarcinomas were assigned to three main groups according their expression of ER, PR and HER2 (*i.e.*, ER+, ER- and TNBC). Age, grade, stage, Ki67, and p53 expression statuses were well-balanced among breast cancer tissues in the three groups (ER+, ER- and TNBC). However, ER, PR, HER2, EGFR and AR statuses were significantly different among them.

The majority of TNBC were grade III and stage III invasive ductal carcinomas. ER+ and ER- breast cancers were mainly invasive ductal carcinomas grade III and stage II (see Table 1). Approximately one third of TNBC and ER- breast cancers were positive for EGFR1. Notably, ER+ breast cancers were negative for EGFR1. Additionally, p53 expression was low in all breast cancers. In contrast, more than 50% of breast cancer samples were positive for Ki67 proliferation marker, which differed from non-malignant tissues (Table 1).

Detection of NILCO and targets in breast cancer tissue arrays

Figure 1 shows representative images of staining of the nine antigens detected in invasive breast carcinomas [n=67; TNBC (n=13; 19%) and ER+(n=33; 49%) and ER- breast cancers (n=21; 31%)] and non-malignant breast tissues (n=5). Other breast cancer types were not included. NILCO and targets were detected in invasive breast adenocarcinomas and non-malignant breast tissues. Majority of breast cancers were positive for Notch1, DLL4 and VEGF.

Continuous A_HSCORE (positive or negative) of Notch1, Notch4, JAG1, OB-R, VEGF and, VEGFR2 were not significantly different among breast cancers irrespective of their expression of ER, HER2 and PR. Notch1, DLL-4 and, VEGF were detected in most malignant tissues. Leptin, OB-R, Notch4, JAG1 and, IL-1R tI were detected in more than 60% of breast cancer (ER+, ER- and, TNBC). In contrast, approximately 30% of these breast cancers showed immunoreactivity for VEGFR2. Leptin, OB-R, Notch1 and VEGFR2 were significantly lower in non-malignant tissues.

Remarkably, cellular localization of NILCO and targets was significantly different in TNBC compared to ER- and ER+ breast cancer (Table 2). A lower number of TNBC showed nuclear and cytoplasmatic staining for Notch4 and JAG1, but more TNBC cells showed cytoplasmatic staining for leptin. Additionally, VEGFR2 was mainly found in TNBC stromas, but fewer showed Notch1 and Notch4 staining (Table 2).

Table 2 Cellular localization of NILCO and targets within TNBC, ER+ and ER- breast cancer tissues

Antigen	ER- (n=21)	ER+ (n=33)	TNBC (n=13)	P-value*
Notch1	% positive	% positive	% positive	
Nucleus	90	97	83	0.0661
Cytoplasm	90	97	92	0.2480
Stroma	100	100	67	**<.001**
Notch4				
Nucleus	74	74	42	**0.0151**
Cytoplasm	90	77	50	0.0762
Stroma	100	100	67	**<.001**
JAG1				
Nucleus	84	77	50	**0.0281**
Cytoplasm	90	88	75	0.0694
Stroma	100	100	100	-
DLL4				
Nucleus	90	88	100	0.098
Cytoplasm	90	88	100	0.098
Stroma	0	0	0	-
Leptin				
Cytoplasm	68	65	100	**<.001**
Stroma	68	59	67	0.4055
OB-R				
Cytoplasm	84	71	67	0.3023
Stroma	84	79	83	0.3311
IL-1R tI				
Cytoplasm	90	97	83	0.3867
Stroma	100	100	83	0.2736
VEGF				
Nucleus	58	94	92	0.4221
Cytoplasm	100	91	92	0.4414
Stroma	91	100	100	-
VEGFR2				
Nucleus	84	85	50	0.0901
Cytoplasm	90	85	75	0.2668
Stroma	32	35	100	**<.001**

Staining for NILCO and targets within cancer (nucleus and cytoplasm) and tumor stroma is expressed as % of positive immunoreactivy in each group of breast cancer. ER: estrogen receptor; TNBC: triple negative breast cancer; Notch1 and 4: Notch receptor type 1 and 4; JAG1: Jagged1, a Notch ligand; DLL4: Delta like ligand 4, a Notch ligand; OB-R: leptin receptor; IL-1RtI: interleukin 1 receptor type I; VEGF: vascular endothelial growth factor; VEGFR2: vascular endothelial growth factor 2. *The p-value is calculated by ANOVA. Numbers in "bold" show significant differences.

Associations of HSCOREs of NILCO and targets with breast cancer type

Evaluation of A_HSCOREs showed diverse grade of expression of NILCO and targets in ER+, ER- and, TNBC (Table 3). A_HSCOREs of antigens in malignant and non-

malignant tissues were used to calculate their univariate associations with breast cancer types.

Expression of DLL4 and IL-1R tI were significantly different among the three breast cancer types (p=0.028 and 0.027, respectively; Table 3). Remarkably, TNBC showed the lowest levels of DLL4 and IL-1R tI.

Correlations of NILCO and targets in breast cancer

Table 4 shows the pairwise analysis of continuous A_HSCOREs for the nine antigens in all invasive breast cancer tissues evaluated. Several correlations between NILCO and its targets were found in breast cancer tissue arrays. Positive detection of leptin within breast cancer tissues significantly correlated to higher levels of IL-1R tI, VEGF, and, OB-R. Additionally, OB-R was positively correlated to VEGF. Similarly, Notch ligands JAG1 and DLL4 positively correlated to leptin, OB-R, and, VEGF and OB-R expression, respectively. Furthermore, Notch receptors (Notch1 and Notch4) correlated to IL-1R tI and DLL4, respectively, whereas leptin was negatively correlated to VEGFR2.

Associations of A_HSCOREs of NILCO and targets with EGFR, AR, Ki67 and p53 expression

Table 5 shows the analysis of univariate associations of categorized and continuous A-HSCOREs of NILCO and targets in breast cancer tissue arrays with EGFR and AR

Table 3 Univariate associations of A_HSCORE of NILCO and targets with TNBC, ER+and ER- breast cancer tissues

	Breast cancer				Non-malignant
Antigen	**ER- (n=21)**	**ER+ (n=33)**	**TNBC (n=13)**	**P-value***	**Hyperplasias (n=3) or Fibroadenomas(n=2)**
Notch1	2.6 (2.01-3.19)	2.55 (1.9-3.19)	2.43 (1.68-3.18)	0.743	1.45 (1.04-1.86)
Negative	0 (0)	0 (0)	1 (7.69)	0.194	NA
Positive	21 (100)	33 (100)	12 (92.31)		5 (100)
Notch4	1.67 (1–3.48)	1.62 (1–2.9)	1.36 (1–2.87)	0.449	2 (1.23 - 2)
Negative	5 (25)	5 (16.13)	5 (41.67)	0.204	NA
Positive	15 (75)	26 (83.87)	7 (58.33)		3 (100)
JAG1	1.23 (1–2.09)	1.37 (1–3)	1.23 (1–2.24)	0.776	1.28 (1.11 - 2.28)
Negative	6 (28.57)	9 (27.27)	4 (30.77)	0.972	NA
Positive	15 (71.43)	24 (72.73)	9 (69.23)		5 (100)
DLL4	3.16 (1.91 - 3.66)	3.21 (2–3.87)	2.85 (1.19 - 3.36)	**0.028**	3.18 (2.59 - 3.68)
Negative	NA	NA	NA	NA	NA
Positive	21 (100)	33 (100)	13 (100)		5 (100)
Leptin	2.69 (1–4)	3.16 (1–4)	1.5 (1–3.84)	0.675	1 (1–1.24)
Negative	6 (28.57)	10 (30.3)	5 (38.46)	0.820	3 (60)
Positive	15 (71.43)	23 (69.7)	8 (61.54)		2 (40)
OB-R	1.06 (1–2.3)	1.11 (1–2.2)	1.05 (1–2.27)	0.924	1 (1–1.5)
Negative	7 (33.33)	10 (30.3)	5 (38.46)	0.867	4 (80)
Positive	14 (66.67)	23 (69.7)	8 (61.54)		1 (20)
IL-1R tI	3.65 (2.55 - 4)	3.72 (2.46 - 4)	3.31 (1–4)	**0.027**	3.99 (2.74 - 4)
Negative	7 (33.33)	10 (30.3)	5 (38.46)	0.194	NA
Positive	14 (66.67)	23 (69.7)	8 (61.54)		4 (100)
VEGF	3.74 (2.22 - 4)	3.67 (2.64 - 4)	3.77 (1.04 - 4)	0.767	3.96 (3.74 - 4)
Negative	NA	NA	NA	NA	NA
Positive	21 (100)	33 (100)	13 (100)		5 (100)
VEGFR2	1 (1–2)	1 (1–2)	1 (1–2)	0.506	1 (1–1.1)
Negative	15 (71.43)	20 (60.61)	9 (69.23)	0.685	3 (60)
Positive	6 (28.57)	13 (39.39)	4 (30.77)		2 (40)

Data are presented as median (range), and number of positive and negative tissues (%). ER: estrogen receptor; TNBC: triple negative breast cancer; Notch1 and 4: Notch receptor type 1 and 4; JAG1: Jagged1, a Notch ligand; DLL4: Delta like ligand 4, a Notch ligand; OB-R: leptin receptor; IL-1Rtl: interleukin 1 receptor type I; VEGF: vascular endothelial growth factor; VEGFR2: vascular endothelial growth factor 2. Numbers in "bold" show significant differences. *The p-value is calculated by ANOVA for Notch1 and Kruskal-Wallis test for the remaining numerical covariates; chi-square test or Fisher's exact test for categorical covariates, where appropriate.

Table 4 Pairwise correlation for NILCO in breast cancer tissue array

	Notch1								
Notch1	1.000	**Notch4**							
Notch4	0.132 (0.303)	1.000							
			JAG1						
JAG1	−0.128 (0.302)	−0.181 (0.155)	1.000						
				DLL4					
DLL4	−0.053 (0.672)	0.247 **(0.051)**	0.096 (0.441)	1.000					
					Leptin				
Leptin	0.055 (0.659)	−0.267 **(0.035)**	0.337 **(0.005)**	0.157 (0.206)	1.000				
						OB-R			
OB-R	0.098 (0.428)	0.026 (0.841)	0.288 **(0.018)**	0.228 (0.064)	0.359 **(0.002)**	1.000			
							IL-1R tI		
IL-1R tI	0.407 **(0.001)**	0.003 (0.985)	−0.024 (0.848)	0.087 (0.484)	0.221 (0.073)	0.153 (0.218)	1.000		
								VEGF	
VEGF	−0.137 (0.269)	−0.083 (0.517)	0.243 **(0.047)**	0.298 **(0.014)**	0.461 **(<.001)**	0.278 **(0.023)**	0.025 (0.844)	1.000	
									VEGFR2
VEGFR2	0.023 (0.852)	0.279 **(0.027)**	−0.132 (0.289)	0.057 (0.646)	−0.370 **(0.002)**	−0.001 (0.992)	0.044 (0.725)	−0.176 (0.154)	1.000

Data are presented as a Spearman correlation coefficient (p-value). Notch1 and 4: Notch receptor type 1 and 4; JAG1: Jagged1, a Notch ligand; DLL4: Delta like ligand 4, a Notch ligand; OB-R: leptin receptor; IL-1RtI: interleukin 1 receptor type I; VEGF: vascular endothelial growth factor; VEGFR2: vascular endothelial growth factor 2. *The p-value is calculated by ANOVA. Numbers in "bold" show significant differences.

expression. Notch1 expression was associated to EGFR1 expression (p=0.018; Table 5). However, the expression of EGFR1 was not significantly associated with the positive or negative detection (categorized A_HSCOREs) of NILCO or targets. DLL4, Notch1, IL-1R tI and VEGF were expressed in almost all breast cancer tissues irrespective of AR status (Table 5). In contrast, IL-1R tI expression was associated with AR expression (p=0.026; Table 5). All TNBC tissues analyzed were negative for AR.

Table 6 shows the analysis of univariate associations of categorized and continuous A-HSCOREs of NILCO and targets in breast cancer tissue arrays with Ki67 and p53 expression. The analysis of univariate association of categorized and continuous A_HSCOREs of NILCO and targets showed that Notch1 and JAG1 expression were significantly higher in breast cancers positive for Ki67 (p=0.01 and p=0.004 respectively; Table 6). In contrast, continuous A_HSCORE of VEGF was marginally higher and negatively associated to Ki67 expression (p=0.056, Table 6). An inverse association of VEGFR2 with Ki67 positive staining was found in breast cancer tissue arrays (p=0.026, Table 6). Univariate association analysis of A_HSCOREs of NILCO and its targets did not show significant differences with p53 expression in breast tissue arrays (Table 6). Nevertheless, no differences in Ki67 or p53 reactivity were found among ER+, ER- and, TNBC (see Table 1).

Pathway studio analyses

In silico analysis of relationships between NILCO and its targets were performed using Pathway Studio9 software. Analysis of data published on expression of Notch, leptin, OB-R, IL-1R tI, and VEGF/VEGFR2 in breast cancer showed several correlations with tumor progression/angiogenesis. The software identified 1626 references reporting 160 connectivity hits that include regulation, biomarker, quantitative, and state changes (Figure 2). Further, analysis of EGFR, AR, Ki67, Notch, leptin, IL-1, VEGF and VEGFR2 genes showed their involvement in the regulation of Notch1, leptin, JAG1, and VEGF in carcinogenesis (1064 references; see Additional file 1).

Discussion

Notch signaling is a hallmark of breast cancer that is frequently identified as an indicator of poor prognosis and advanced disease. Therefore, Notch signaling is being targeted for breast cancer treatment [7,27]. Additionally, increased leptin signaling has also been related to breast cancer growth, angiogenesis and poor outcomes [24]. Leptin increased the expression and activation of several members of the Notch family of proteins in breast cancer cells and derived tumors [17-19]. VEGF and VEGFR2 can be regulated by leptin-Notch crosstalk, which was also affected by IL-1 signaling. Therefore, Notch, IL-1 and leptin crosstalk outcome (NILCO) could be essential for the integration of leptin's proangiogenic, pro-inflammatory and proliferative actions in breast cancer [17]. Leptin could also be involved in the development of drug resistance, metastasis and relapse of breast cancer, which are related to cancer stem cells [24,28]. Furthermore, leptin transactivated

Table 5 Univariate associations of HSCORE for NILCO and targets with EGFR and AR

	EGFR			AR		
Antigen	**Negative (N=52)**	**Positive (N=15)**	**P-value***	**Negative (N=40)**	**Positive (N=27)**	**P-value***
Notch1	2.44 (1.78- 3.06)	2.89 (2.28-3.50)	**0.018**	2.49 (1.84-3.09)	2.62 (1.99-3.25)	0.403
Negative	1 (1.92)	0 (0)	1.000	1 (2.5)	0 (0)	1.000
Positive	51 (98.08)	15 (100)		39 (97.5)	27 (100)	
Notch4	1.55 (1–3.48)	1.81 (1–2.5)	0.782	1.62 (1–3.48)	1.7 (1–2.5)	0.966
Negative	12 (25)	3 (20)	1.000	10 (27.03)	5 (19.23)	0.474
Positive	36 (75)	12 (80)		27 (72.97)	21 (80.77)	
JAG1	1.35 (1–3)	1.17 (1–2.09)	0.215	1.28 (1–2.43)	1.23 (1–3)	0.510
Negative	13 (25)	6 (40)	0.332	13 (32.5)	6 (22.22)	0.360
Positive	39 (75)	9 (60)		27 (67.5)	21 (77.78)	
DLL4	3.15 (1.91 - 3.87)	3.27 (1.19 - 3.66)	0.443	3.19 (1.19 - 3.65)	3.15 (2–3.87)	0.828
Negative	NA	NA	NA	NA	NA	NA
Positive	52 (100)	15 (100)		40 (100)	27 (100)	
Leptin	3.07 (1–4)	2.69 (1–4)	0.825	2.88 (1–4)	3.02 (1–4)	0.938
Negative	16 (30.77)	5 (33.33)	1.000	12 (30)	9 (33.33)	0.773
Positive	36 (69.23)	10 (66.67)		28 (70)	18 (66.67)	
OB-R	1.09 (1–2.3)	1.05 (1–1.26)	0.338	1.1 (1–2.27)	1.06 (1–2.3)	0.359
Negative	17 (32.69)	5 (33.33)	1.000	12 (30)	10 (37.04)	0.547
Positive	35 (67.31)	10 (66.67)		28 (70)	17 (62.96)	
IL-1R tI	3.67 (1–4)	3.52 (1.56 - 4)	0.255	3.53 (1–4)	3.86 (2.46 - 4)	**0.026**
Negative	1 (1.92)	0 (0)	1.000	1 (2.5)	0 (0)	1.000
Positive	51 (98.08)	15 (100)		39 (97.5)	27 (100)	
VEGF	3.64 (1.04 - 4)	3.81 (2.45 - 4)	0.625	3.67 (1.04 - 4)	3.74 (2.83 - 4)	0.635
Negative	NA	NA	NA	NA	NA	NA
Positive	52 (100)	15 (100)		40 (100)	27 (100)	
VEGFR2	1 (1–2)	1 (1–2)	0.957	1 (1–2)	1 (1–2)	0.757
Negative	34 (65.38)	10 (66.67)	0.927	27 (67.5)	17 (62.96)	0.701
Positive	18 (34.62)	5 (33.33)		13 (32.5)	10 (37.04)	

Data are presented as median (range), and number of positive and negative tissues (%). EGFR: epidermal growth factor receptor 1; AR: androgen receptor; Notch1 and 4: Notch receptor type 1 and 4; JAG1: Jagged1, a Notch ligand; DLL4: Delta like ligand 4, a Notch ligand; OB-R: leptin receptor; IL-1Rtl: interleukin 1 receptor type I; VEGF: vascular endothelial growth factor; VEGFR2: vascular endothelial growth factor 2. * The p-value is calculated by ANOVA for Notch1 and Wilcoxon rank-sum test for the remaining numerical covariates; chi-square test or Fisher's exact test for categorical covariates, where appropriate. Numbers in "bold" show significant differences.

and induced the expression of ER [29], EGFR [30], HER2 [31,32] and IGF-1R [33] in breast cancer.

The abrogation of leptin signaling impaired the growth of tumors and expression of angiogenic biomarkers in human breast cancer xenografts [34,35], and in mouse carcinogenic-induced [18] and syngeneic mammary tumors, which was more evident in obese contexts [19,21]. Moreover, accumulated evidence from these pre-clinical studies in mice reinforces the idea that leptin-Notch crosstalk plays an important role in breast cancer. Nevertheless, whether NILCO and targets are differentially-expressed in human breast cancer tissues, in relation to ER, PR and HER2 as well as EGFR and AR statuses, is unknown.

Here we show that NILCO components (Notch1, Notch4, JAG1, DLL4, leptin, OB-R, IL-1R tI) and target molecules (VEGF and VEGFR2) were co-expressed in breast cancer tissues, irrespective of ER, PR and, HER2 statuses. Remarkably, TNBC shows a differential pattern of expression and cellular localization of NILCO. TNBC showed lower protein levels of IL-1R tI and DLL4, and fewer nuclei and cytoplasms were positive for Notch4 and JAG1. In contrast, more TNBC cytoplasms were positive for leptin. Moreover, TNBC stromas showed distinctive patterns of Notch1 and VEGFR2 immnoreactivities. Notch1 and Notch4 expression were lower, but VEGFR2 expression was higher in stromas from TNBC compared with ER- and ER+ breast cancer stromas.

Table 6 Univariate associations of HSCORE for NILCO and targets with Ki67 and p53

	Ki67			p53		
Antigen	**Negative (n=52)**	**Positive (n=15)**	**P-value***	**Negative (n=40)**	**Positive (n=27)**	**P-value***
Notch1	2.27(1.68-2.86)	2.69 (2.06-3.32)	**0.010**	2.51(1.82-3.20)	2.63 (2.13-3.13)	0.528
Negative	1 (4.35)	0 (0)	0.343	1 (2.04)	0 (0)	1.000
Positive	22 (95.65)	44 (100)		48 (97.96)	18 (100)	
Notch4	1.42 (1–2.82)	1.69 (1–3.48)	0.125	1.51 (1–3.48)	1.8 (1–2.87)	0.404
Negative	7 (33.33)	8 (19.05)	0.209	12 (26.67)	3 (16.67)	0.522
Positive	14 (66.67)	34 (80.95)		33 (73.33)	15 (83.33)	
JAG1	1.37 (1–2.24)	1.21 (1–3)	0.350	1.2 (1–3)	1.54 (1–2.43)	0.587
Negative	3 (13.04)	16 (36.36)	**0.044**	14 (28.57)	5 (27.78)	0.949
Positive	20 (86.96)	28 (63.64)		35 (71.43)	13 (72.22)	
DLL4	3.21 (1.91 - 3.66)	3.11 (1.19 - 3.87)	0.584	3.15 (1.19 - 3.66)	3.21 (2.59 - 3.87)	0.651
Negative	NA	NA	NA	NA	NA	NA
Positive	23 (100)	44 (100)		49 (100)	18 (100)	
Leptin	3.34 (1–4)	2.66 (1–4)	0.841	2.64 (1–4)	3.29 (1–4)	0.541
Negative	7 (30.43)	14 (31.82)	0.908	16 (32.65)	5 (27.78)	0.703
Positive	16 (69.57)	30 (68.18)		33 (67.35)	13 (72.22)	
OB-R	1.08 (1–2.27)	1.07 (1–2.3)	0.648	1.08 (1–2.3)	1.11 (1–2.2)	0.666
Negative	7 (30.43)	15 (34.09)	0.762	17 (34.69)	5 (27.78)	0.593
Positive	16 (69.57)	29 (65.91)		32 (65.31)	13 (72.22)	
IL-1R tI	3.63 (1–4)	3.63 (1.56 - 4)	0.714	3.65 (1–4)	3.55 (2.62 - 4)	0.847
Negative	1(4.35)	0 (0)	0.343	1 (2.04)	0 (0)	1.000
Positive	22(95.65)	44(100)		48(97.96)	18(100)	
VEGF	4(1.04 - 4)	3.56(2.22 - 4)	0.056	2.51(± 0.69)	2.63(± 0.5)	0.528
Negative	NA	NA	NA	1(2.04)	0(0)	1.000
Positive	23(100)	44(100)		48(97.96)	18(100)	
VEGFR2	1.09(1–2)	1(1–2)	**0.047**	1.51(1–3.48)	1.8(1–2.87)	0.404
Negative	11(47.83)	33(75)	**0.026**	12(26.67)	3(16.67)	0.522
Positive	12(52.17)	11(25)		33(73.33)	15 (83.33)	

Data are presented as median (range), and number of positive and negative tissues (%). Ki67: a proliferation marker; p53: a tumor suppressor protein; Notch receptor type 1 and 4; JAG1: Jagged1, a Notch ligand; DLL4: Delta like ligand 4, a Notch ligand; OB-R: leptin receptor; IL-1Rtl: interleukin 1 receptor type I; VEGF: vascular endothelial growth factor; VEGFR2: vascular endothelial growth factor 2. *The p-value is calculated by ANOVA for Notch1 and Wilcoxon rank-sum test for the remaining numerical covariates; chi-square test or Fisher's exact test for categorical covariates, where appropriate. Numbers in "bold" show significant differences.

Notch1 was found in the majority of breast cancer tissues evaluated. This data was in agreement with a previous report that showed 100% of Notch1 expression in TNBC [36]. Notch4 expression was previously found in 73% of TNBC cases (n=29) [36]. Present data show that fewer TNBCs were positive for Notch4 (58%) compared to ER- (75%) and ER+ (84%), but the differences were not significant. However, nuclear localization of Notch4 was significantly lower in TNBC malignant cells and stroma.

Present data further confirm previous findings showing that TNBC cells in culture (MDA-MB231) secreted more leptin (approximately four-fold) than ER+ breast cancer cells (MCF-7) [34]. Notch-induced transcriptional activity was not previously correlated with Notch receptor levels in breast cancer cell cultures, but Notch-induced gene transcription was highest in TNBC cells [2]. The global biological relevance of these findings is unclear. Nevertheless, present findings might indicate that TNBC could greatly depend on leptin's actions, which could underline the role of NILCO in this breast cancer type.

Notch1 expression was also associated with the cell proliferation marker, Ki67. This marker was detected in approximately 60% of breast cancer independently of the expression of hormone receptors. It was previously reported that Ki67 is found in 90% of TNBC [12] and its expression correlates to Notch4, which is induced by Notch1 in breast cancer samples [2].

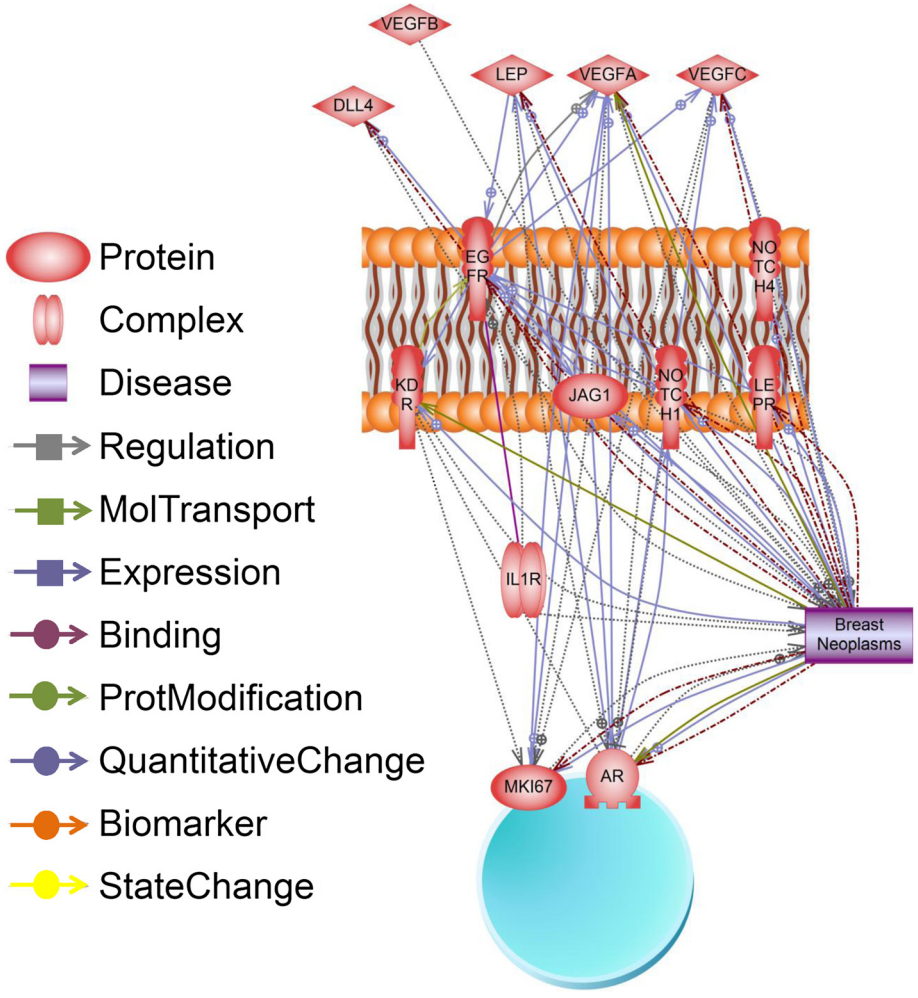

Figure 2 Processes and outcomes involving leptin, OB-R, Notch1, Notch4, IL-1R tI, JAG1, DLL4, VEGF, VEGFR2 (KDR), EGFR, AR and Ki67 (MKi67 gene) in the development of breast neoplasms. Breast cancers showed a quantitative increase in the expression of leptin, OB-R, Notch1, Notch4, DLL4, JAG1, IL-1R tI, VEGF, VEGFR2, EGFR, AR and Ki67. Increased expression of leptin, Notch1, Notch4, JAG1, and VEGF upregulated the development of breast neoplasms. Elevated levels of leptin, OB-R, Notch1, DLL4, VEGF, AR, EGFR and Ki67 proteins are reported to be biomarkers for breast cancers (data obtained from 1064 references; see Additional file 1; Pathway Studio9, Ariadine Genomics).

The transformation of normal breast epithelial cells by increased Notch signaling was previously linked to the repression of apoptosis *in vitro* [9]. Notch NICD1 interacted and mediated p53 inactivation through phosphorylation *in vitro* [37]. Additionally, it was also suggested that Notch signaling regulated apoptosis specifically caused by p53-induced expression of Puma and Noxa *in vitro* [6]. However, our present data suggest that p53 was not associated with the expression of NILCO and its targets, and is independent of hormonal receptor status. This data may also suggest that Notch-induced apoptosis in breast cancer *in vivo* may not always be p53 dependent.

TNBC and ER- breast cancers are not responsive to steroid hormones, but are highly aggressive tumors that respond to several other growth factor-related signals [5]. TNBC frequently show EGFR expression and resistance to EGFR drugs that could be driven by the Notch pathway [38]. In these cancers, Notch, leptin and OB-R could further contribute to tumor growth via increased the survival of breast cancer stem cells [24]. Indeed, the abrogation of Notch can negatively affect stem cells [10,27]. Moreover, inhibition of OB-R significantly reduces the expression of several stem cell self-renewal transcription factors (NANOG, SOX2, and OCT4), and induces a mesenchymal-to-epithelial transition in TNBC cells [39]. Our present investigations show that EGFR expression was found in more ER- and TNBC (five-fold) than ER+ tumors. Additionally, EGFR was associated with higher expression of Notch1. Interestingly, leptin-induced activation of EGFR was suggested as a potential mechanism that promotes metastasis as well as invasion and, migration of breast cancer [33].

Obesity could affect breast carcinogenesis by autocrine and paracrine actions mediated by two major adipokines: leptin and adiponectin [24]. Obese breast cancer patients show poor prognosis, higher aggressiveness, and drug resistance [40-42]. Accumulated evidence suggests that obesity could induce Notch signaling. Indeed, an intact leptin-Notch axis could be involved in obesity-related breast cancer [18,19]. However, diverse factors from adipose and other organs could also influence breast carcinogenesis and tumor growth. Therefore, more investigations are necessary to understand obesity-related breast cancer causes and mechanisms [43,44]. All tissue samples used in this investigation were from Asiatic women (mean age of approximately 50 years). Obesity in China is currently a health problem. In 2002, the prevalence of obesity in China was relatively low (overweight prevalence at about 22.8% and for obesity, 7.1%) compared with Western countries, but the rapid increase in obesity is alarming [45]. Unfortunately, body weight and obesity data were not available for the breast cancer tissues used in this study.

Conclusions

For the first time, we are reporting on a comprehensive data analysis on protein levels of NILCO and targets in three major groups of breast cancer: TNBC, ER-, and ER+. TNBC showed distinctive patterns of expression and localization of NILCO, which suggests these molecules may be useful as markers for disease progression and aggressiveness. It is known that inhibition of Notch [9] and leptin signaling [17,21,34,39] can revert the transformed phenotype of human breast cancer cell lines. Thus, treatments aimed to abrogate NILCO could provide the development of novel therapeutic interventions. More research is needed to establish the biomarker and potential therapeutic values of NILCO, and target expression in breast cancer, particularly in obese contexts.

Additional file

Additional file 1: List of references reporting relationships between NILCO and its targets in breast cancer.

Abbreviations

OB-R: Leptin receptor; IL-1R tI: Interleukin 1 receptor type I; NILCO: Notch interleukin 1 leptin crosstalk outcome; EGFR: Epidermal growth factor receptor; NANOG: A homeobox protein and transcription factor; SOX2: Sex determining region Y-box 2 protein; OCT4: Octamer-binding transcription factor 4; IGF-1R: Insulin like growth factor receptor 1; ER: Estrogen receptor; AR: Androgen receptor; PR: Progesterone receptor; HER2: Epidermal growth factor receptor 2; DLL4: Delta like notch ligand 4; JAG1: Jagged 1 protein (a Notch ligand); VEGF: Vascular endothelial growth factor; VEGFR2: Vascular endothelial growth factor receptor 2 or KDR; TNBC: Triple negative breast cancer cells; HSCORE: A semi-quantitative analysis of intensity staining.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RRGP designed and wrote up the current study. LSC was involved in the study design, immunohistochemistry assays and HSCORE determinations of NILCO and targets. GN developed the *in silico* analysis of NILCO relationships using Pathway Studio9 Program and contributed to writing and editing the manuscript. KW, CG and TD developed immunohistochemistry assays and HSCORE determinations of NILCO, and targets. GO, performed immunohistochemical and pathological analyses of tissue samples. SK and YL performed the statistical analysis and interpretation of data. All authors read and approved the final manuscript.

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Molecular cues on obesity signals, tumor markers and endometrial cancer

Abstract: Tumor markers are important tools for early diagnosis, prognosis, therapy response and endometrial cancer monitoring. A large number of molecular and pathologic markers have been described in types I and II endometrial cancers, which has served to define the main oncogenic, epidemiological, genetic, clinical and histopathological features. Ongoing attempts to stratify biological markers of endometrial cancer are presented. However, data on changes in tumor marker profiles in obesity-related endometrial cancer are scarce. Obesity is a pandemic in Western countries that has an important impact on endometrial cancers, albeit through not very well-defined mechanisms. Although endometrial cancer is more common in Caucasian women, higher mortality is found in African Americans who also show higher incidence of obesity. Here, we describe how obesity signals (estrogen, leptin, leptin induced-molecules, Notch; cytokines and growth factors) could affect endometrial cancer. Leptin signaling and its crosstalk may be associated to the more aggressive and poor prognosis type II endometrial cancer, which affects more postmenopausal and African-American women. In this regard, studies on expression of novel molecular markers (Notch, interleukin-1 and leptin crosstalk outcome) may provide essential clues for detection, prevention, treatment and prognosis.

Keywords: endometrial cancer; leptin; NILCO; obesity; tumor markers.

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Introduction

Endometrial cancer (EmCa) is an adenocarcinoma of the endometrium and the most common cancer of the female reproductive tract in developed countries and the seventh most common cancer in women worldwide [1].

The development of cancer is characterized by self-sufficiency in growth signals, insensitivity to growth inhibition, evasion of apoptosis, angiogenesis, invasion and metastasis. All these features are unique in EmCa and have given origin to two major divisions. Type I, associated with endometrioid histology, unopposed estrogen exposure and often preceded by precancerous disease, occurs primarily in pre- and peri-menopausal women and is most common in White population usually carrying a good prognosis. In contrast, type II EmCa, with no endometrioid histology and no association with hormonal risk, occurs most commonly in postmenopausal women and are mostly seen in African-American women (AAW), with an aggressive clinical course and generally with poorer prognosis [2].

The incidence of EmCa is higher in well-developed countries and countries with high obesity rates [2]. As of 2013, there were 49,560 new cases of EmCa reported and 8190 deaths reported in the USA. The American Cancer Society has estimated that 810,320 American women will suffer from cancer and 275,710 will die in the USA in 2014. From these, 6% will present uterine cancer. Cancer lifetime probabilities show that 2.69% (1 in 37) of American women will present with uterine cancer, which places it as the fourth most frequent cancer among women behind breast, lung and colorectal cancers. These figures have been almost steady from 1975 to date in the USA [3].

Caucasian women (CSW) are at a higher risk of developing EmCa when compared to AAW in the USA. However, AAW are more likely to develop the more aggressive form of EmCa and are more likely to die from this disease [4].

The incidence rate of EmCa in CSW is 24.8 per 100,000 women, whereas in AAW it is 20.9 per 100,000 women [5]. The reason(s) for this disparity is unknown [3].

Mainly modifiable factors impacting the rise of cancer incidence are the consumption of highly caloric diets and low physical activity. EmCa is associated with obesity, diabetes and excessive estrogen exposure. Indeed, in comparison with all obesity-related cancers, EmCa incidence and death are associated most with increasing body mass index (BMI) [5].

Several tumor markers have been described for EmCa, which are mainly related to classification, treatment outcome and epidemiology of EmCa. High levels of leptin characterize obesity. Leptin is an adipokine with proliferative, pro-angiogenic and pro-inflammatory effects on many cancer types [6]. Leptin crosstalks to oncogenic signaling molecules, i.e., Notch and IL-1 could play important roles in the incidence and progression of EmCa, particularly in obese patients. However, currently, data on the expression patterns and effects of such leptin signaling crosstalk in EmCa and its relation to obesity are scarce.

Endometrial cancer

EmCa is classified into two main types: type I, which is estrogen dependent, and type II, which is usually associated with endometrial atrophy and is estrogen independent [2]. Type II is the more aggressive form with a poor prognosis. Approximately 85% of all EmCas are type I, low grade with an excellent prognosis. In contrast, type II is usually high-grade tumors with some patients exhibiting early metastasis. Type II tumors are associated with high recurrences. Approximately half of all recurrences occur in patients with type II tumors [2] (Figure 1).

The common pathological type I is an endometrioid adenocarcinoma with some resemblance to endometrial morphology. Type I EmCa is more differentiated and dependent on hormonal cues and has a better prognosis (Figure 1A and B). In contrast, type II represents 10% of EmCa and has serous or clear cell appearance (Figure 1C and D). Type II EmCa is less differentiated, independent of sex hormones stimuli, more aggressive and shows poor prognosis. There are other less abundant EmCa types that include the mixed mesenchymal Mullerian malignant tumor (MMMT) or carcinosarcoma, mucinous, clear cell, squamous cell, mixed and undifferentiated. Additionally, uterine sarcoma is a more rare type and forms in the muscles or other tissues of the uterus [7].

Type I EmCa is probably derived from atypical hyperplasia, and its progression is closely related to unopposed estrogenic stimulation. Type I EmCa shows few p53 mutations but numerous mutations in other tumor suppressors and oncogenes (i.e., PTEN, PI-3K and KRAS) and CTNNB1 or β-catenin [8]. Type II develops from atrophic endometrium. Type II EmCa had extensive copy number alterations, few DNA methylation changes, low estrogen receptor (ER)/progesterone receptor levels and frequent p53 mutation. Additionally, type II serous EmCa have extensive mutations in DNA polymerase ε (POLE) exonuclease domain. However, about 10% of type I EmCa also shows high number of POLE mutations [9].

Remarkably, type II EmCa shares genomic features with basal-like breast cancer [8], which shows low levels or absence of ER, progesterone and EGFR2 (Her2) receptors. Basal-like breast carcinomas (triple negative) are very aggressive and have poor prognosis. Type II EmCa are also very aggressive, have poor prognosis and no targeted therapies.

EmCa stage is an important feature related to progression of tumor and invasion of surrounding tissues, which is currently based on the tumor-node-metastases (TNM) system 7 and FIGO (International Federation of Gynecology and Obstetrics) staging [3]. The FIGO system and the American Joint Committee on Cancer TNM staging system are basically the same. They both classify EmCa on the basis of three factors: the extent of the tumor (T), whether the cancer has spread to lymph nodes (N) and whether it has spread to distant sites (M). The American Cancer Society classifies EmCa by grades: grade 1 present ≥95% of the cancerous tissue forming glands; grade 2 tumors show 50% to 94% of tissue forming glands and grade 3 <50% of tissue forming glands. Grade 3 tumors are more aggressive and show poor prognosis [3].

Endometrial cancer and obesity

Obesity is a pandemic, particularly in developed countries. It is predicted that in 2015 about 700 million people will be obese worldwide [10]. Obesity, mainly due to unhealthy diets and lifestyles, is a proven factor contributing to higher risk and poor prognosis of cancer [11]. Currently, obesity is considered the second major risk factor for several cancer types, only surpassed by smoking. Interestingly, smoking is inversely correlated to EmCa. That could be associated to the transformation of estrogen via 2-hydroxylation, which produces 2-hydroxyestradiol and

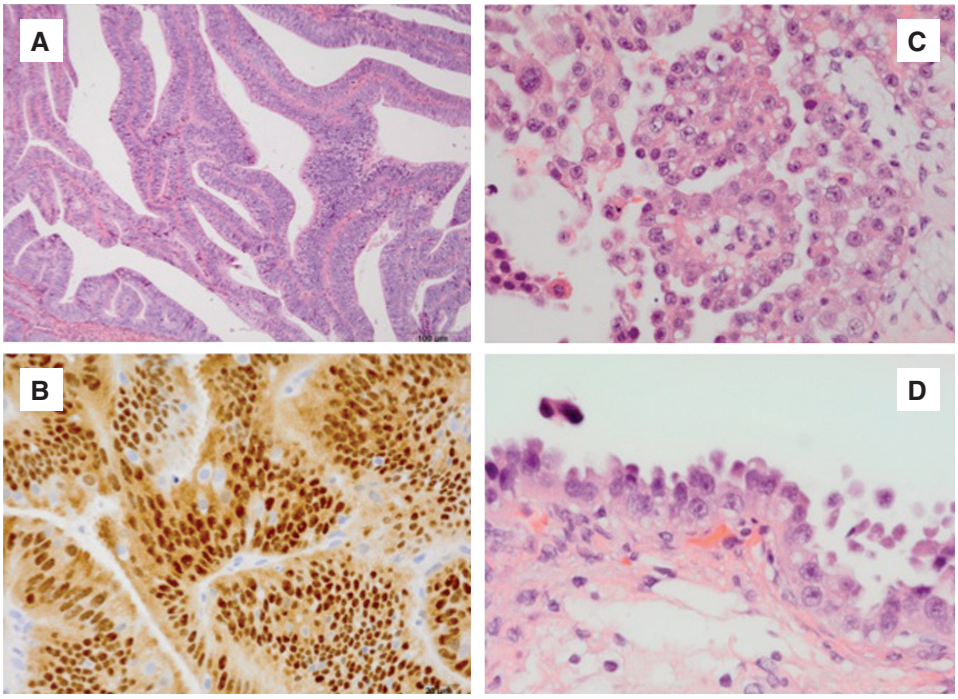

Figure 1: Type I EmCa (A, B) and type II EmCa (C, D).
(A) Endometrioid carcinoma containing glandular features that resemble glands of the benign endometrium (HE, 10×). (B) Estrogen receptor positive tumor (HE, 40×). (C) Serous carcinoma of the endometrium (HE, 40×). (D) Endometrial carcinoma in situ (HE, 60×).

its methoxy derivative that are not proliferative factors for EmCa but anti-apoptotic and can inhibit inflammatory cytokine actions [12, 13]. However, the precise causes for the protective role of smoking on EmCa are not completely understood.

Accumulated evidence supports the notion that obesity is a risk factor for EmCa. Approximately 40% of EmCa cases are related to obesity [14]. An increase in EmCa incidence in the last 30 years is believed to be due to the increasing number of elderly people and increasing rates of obesity. Indeed, a 5 kg/m^2 increment of BMI correlated to a significant increase of EmCa [RR: 1.50 (1.42–1.59)] [15].

Several studies have found significant associations between obesity and increase of EmCa mortality. In a large prospective epidemiological study including approximately half a million women followed for 16 years, it was demonstrated that obesity increased EmCa mortality [11]. Obesity was associated with earlier age at diagnosis of endometrioid-type EmCa. Similar associations were not, however, observed with non-endometrioid cancers, consistent with different pathways of tumorigenesis [11]. An association of BMI with age at diagnosis was found in 985 cases of EmCa. Age at diagnosis was inversely proportional to BMI only in patients suffering from type I EmCa ($y=67.89–1.86x$, $R=0.049$, $p<0.001$) but not with type II EmCa [16]. In vitro studies show the influence of adipose-derived factors on EmCa. Conditioned media from adipose-derived stem cells increased proliferation and secretion of vascular endothelial growth factor (VEGF) by Ishikawa cells (type I EmCa), which demonstrates that adipose tissue secretes factors inducing EmCa cell growth [17]. However, the specific mechanisms involved in obesity-related cancer incidence are still not completely understood [18, 19].

Potential players involved in the relationships between EmCa incidence and progression, and obesity are the elevated levels of estrogens (unopposed estrogen stimulus), insulin, insulin growth factor-1 (IGF-1), adipokines (leptin, resistin) and cytokines [20–22].

Obesity is characterized by altered profiles of several cytokines and, therefore, is considered a mild inflammatory condition [21, 22]. Adipose tissue secretes several cytokines including leptin, VEGF, interleukin-1 (IL-1), interleukin-6 (IL-6), hepatocyte growth factor (HGF) and tumor necrosis factor-alpha (TNF-α). Several of these cytokines can induce tumor angiogenesis contributing to the growth of solid tumors [6, 23–27]. Abnormal patterns of these factors are associated to obesity-induced changes in tumor and stroma cells [28, 29]. High levels of colony stimulator factor-1 (CSF-1) and its receptor, CSFR, have

long been associated with poor-prognosis uterine cancer, among others [30].

Recruitment of inflammatory cells significantly contributes to adipose neovascularization and breast cancer inflammation and angiogenesis [31]. In addition, inflammatory cells and cytokines found in tumors are more likely to contribute to tumor growth, progression, and immunosuppression than they are to mount an effective host antitumor response [32]. It has been proposed earlier that chronic inflammation is a principal mechanism inducing EmCa, through the induction of mitosis, mutations and defective DNA repair [33, 34]. Higher levels of VEGF were detected in type I EmCa and increased CSF-1 and TNF-α in tumor with deep myometrium invasion. Many cytokines induce NFkB signaling pathway, which is involved in cancer cell survival. Also, inflammatory cytokines and cells induce estrogen production [33].

High levels of estrogen are produced by adipose tissue via overexpression of aromatase (that converts androgens into estradiol and estrone) after the sharp decline of ovarian estrogen production in postmenopausal women. Obesity induces high levels of estrogen, which in turn increase the growth of endocrine responsive of cancer, in particular, type I EmCa. In addition, skeletal and vascular tissues and the brain are important sites of postmenopausal estrogen production. Furthermore, obesity leads to the development of metabolic syndrome, which is generally characterized by insulin resistance [16], which is a risk factor for EmCa [35].

Insulin is produced mainly by the pancreatic β cells and is a proliferation factor for EmCa, which expresses insulin receptor (IR). Activation of IR induces direct and indirect effects that contribute to the development of EmCa [36]. Insulin/IR directly promotes EmCa cell proliferation and survival via PI-3K/Akt and Ras/MAPK signaling pathways. Additionally, IGF-1 mainly synthesized in the liver is also a proliferation factor for endometrial cells. Insulin crosstalk to IGF-1 contributes to the progression of EmCa [37]. IGF-1 is mainly regulated by the actions of the growth hormone (GH). Insulin can increase IGF-1 through the upregulation of GH receptors (GHR) and crosstalk to GH/GHR signaling [38]. Furthermore, hyperinsulinemia reduces the levels of IGF binding proteins that increase IGF availability [39]. Insulin induces changes of sex hormone levels that further increase the levels of estrogen in obese individuals. ERs can also crosstalk to IGF-1 signals inducing proliferation and survival changes in EmCa cells [40].

Metformin, a common drug used in diabetes type II patients, was earlier shown to inhibit aromatase expression in adipocytes [41]. In addition to normalizing insulin levels, metformin has been shown to downregulate IGF-1R and upregulate IGF binding protein-1 in EmCa cell lines, and inhibit IGF-1 pathway in type I EmCa [42]. A recent report from a 10-year retrospective cohort study of diabetic patients suffering from type II EmCa (non-endometrioid EmCa) and treated with metformin showed an increase of overall survival compared with a similar cohort of non-diabetic and EmCa patients not treated with metformin. The association was significant (hazard ratio=0.54, 95% CI: 0.30–0.97, $p<0.04$) after adjusting several variables (age, clinical state, grade and treatment). However, no association between the use of metformin and overall survival in diabetics with endometrioid histology (type I EmCa) was observed [43]. Another recent report shows that non-diabetic EmCa patients that did not use metformin had worst free (95% CI: 1.1–2.9, $p=0.02$) and overall survivals (95% CI: 1.3–4.2, $p=0.005$) compared to EmCa diabetic and obese patients treated with metformin after adjusting for age, stage, grade, histology and adjuvant treatment [44].

Leptin and endometrial cancer

Leptin is a small non-glycosylated protein (16 kDa; 167-amino acid non-glycosylated) secreted by adipose tissue that is coded by the obese (*ob*) gene. It is the most studied adipokine since this protein was first cloned in 1994 [45]. Leptin is a pleotropic cytokine that regulates energy intake and energy expenditure. The role of leptin involves the regulation of glucose homeostasis, growth response, reproduction and immune response [6]. Leptin is secreted by adipocytes as well as cancer cells, and its circulating levels are proportional to total body fat. Obese individuals exhibit high circulating levels of leptin in the body due to what is known as leptin resistance [6].

Leptin exists as a unique protein with pro-inflammatory functions that belongs to the family of helical cytokines. Leptin is structurally similar to IL-6, IL-12, IL-15, prolactin, GH, granulocyte CSF (G-CSF) and oncostatin M. The N-terminal region (94 amino acids) of leptin is essential for both the biological and the receptor binding activities [46].

The *ob* gene is preserved in mammals providing a high sequence identity for leptin. A nonsense mutation in codon 105 (*ob/ob*) causes the lack of protein synthesis resulting in morbid obesity, hyperphagia, hypothermia, insulin resistance and infertility [45]. Leptin functions as a long-term signal from adipose tissue that regulates appetite and energy balance. Exogenous administration of leptin on mutant obese mice recovered the normal lean

phenotype in C576J mice, which early demonstrated leptin's role in energy balance [47].

Leptin receptor [OB-R, the product of the diabetic (*db*) gene], in contrast to leptin, shows at least six alternative spliced isoforms: a long isoform (OB-RL, OB-Rb or LEPR) with full intracellular signaling capabilities and shorter isoforms with less biological activity (OB-Rs or OB-Ra) [48] and a soluble leptin isoform (OB-Re or sOB-R) [49]. The large extracellular domain of OB-R (816 amino acids) is common to all OB-R forms, and the variable length cytoplasmatic tail (300 amino acid residues) distinguishes the several isoforms [50]. OB-R has a helical structure that is similar to those of gp130, the common signal-transducing receptor component for the IL-6 family of cytokines, G-CSF (granulocyte colony stimulating factor) and LIF (leukemia inhibitory factor) receptor. OB-R is related to class I cytokine receptors, which includes the receptors of IL-1, IL-2, IL-6 and GH. This super-family of receptors lacks auto-phosphorylation capabilities and needs auxiliary kinases for activation [51].

Leptin binding to OB-R seems to be very specific and triggers several canonical (i.e., JAK2/STAT3; MAPK; PI-3K/AKT1) and non-canonical signaling pathways (p38MAK; JNK and AMPK). OB-RL and membrane-bound shorter isoforms of OB-R have a cytoplasmatic motif (Box 1) required for JAK (Janus kinases)-related activation of PI-3K (phosphatidylinositol-3-kinase) and MAPK (mitogen-activated protein kinase) pathways. A second docking site (cytoplasmatic tail motif; Box 2) is essential for the activation of the JAK-STAT (signal transducers and activators of transcription) pathway. Induced mutations of the OB-Rb intracellular domain showed that Tyr 113s controls STAT3 [52]. In addition, the SH2 domains of SOCS (suppressor of cytokine signaling) bind the phosphorylated tyrosine residues on JAK2 regulating OB-Rb [53]. SOCS-3 plays an important role as a negative regulator of leptin signaling. It seems that SOCS-3 is activated by a feedback induced by leptin. The over-expression of SOCS-3 inhibits leptin-induced tyrosine phosphorylation of JAK2 and ERK activation by binding to phosphorylated Tyr 985 of OB-Rb [54].

Although the primary source of leptin is adipose tissue, several other tissues have been found to synthesize leptin, however, in lower quantities (i.e., stomach, skeletal muscle, brain, placenta and endometrium at the time of embryo implantation) [6, 55]. Leptin levels are higher in women compared to males (pre-menopausal females>post-menopausal females>males) even after correction by body weight [56]. These gender differences in leptin levels could be related to subcutaneous synthesis and estrogen and androgen regulations [57]. Leptin/OB-R seems to be involved in early embryo implantation [55, 58]. An active crosstalk between leptin secreted by embryos and OB-R expressed by decidual tissue has been described. Moreover, it seems that leptins/OB-R function as essential upstream events in the embryo implantation process [58].

Remarkably, leptin exhibits low or undetectable expression levels in normal endometrial cells, but it is synthesized by uterine cancer cells [59]. Additionally, several other cancer types express leptin including breast, ovarian, prostate, melanoma, esophagus, thyroid, brain, lung and colon [6]. Leptin expressed by adipose or EmCa can promote in a paracrine or autocrine manner the proliferation of cancer cells. However, the correlation between leptin and OB-R expressions in the EmCa clinicopathology is still unclear [60].

We earlier reported that EmCa cell lines expressed higher levels of OB-R (full-length OB-Rb and short isoforms) in contrast to benign primary non-malignant endometrial cells [61]. Leptin and OB-R are found in EmCa tissue. Positive staining for STAT3, HIF-1, leptin and OB-R was detected in 75%, 79%, 60% and 31% of endometroid adenocarcinomas (type I), respectively [59]. Leptin and OB-R overexpression correlated to ER expression, tumor invasion, metastasis and poor prognosis (3-year survival rate) [60].

Leptin signaling crosstalk in endometrial cancer

Elevated lifetime estrogen exposure is a major risk factor for EmCa. This is also true for other hormone-dependent cancers (i.e., breast and ovarian) [62]. ER signaling regulates an elevated number of genes affecting cancer proliferation and vascular function. Antiestrogens could also stimulate the synthesis and release of leptin in the adipocytes [63].

A complex crosstalk between leptin and pro-angiogenic, inflammatory and mitogenic factors occur in breast cancer, which could also be present in EmCa. Leptin actions would provide a link between pro-inflammatory and pro-angiogenic actions of IL-1, VEGF and macrophages in cancer progression [6]. However, the individual contributions of these factors to obesity-related cancers, including EmCa, are not well understood. We earlier reported that leptin signaling was associated to several pro-angiogenic factors in EmCa cell lines. In malignant endometrial epithelial cells (An3Ca, SK-UT2 and Ishikawa) leptin regulated in a dose-dependent manner VEGF, IL-1β, LIF and their respective receptors, VEGFR2, IL-1R tI and LIFR. However, IL-1β was only increased by leptin in benign primary endometrial cells [61]. Additional factors involved in leptin crosstalk are TNFα, IL-6 and resistin [6].

Additionally, vitamin D3 and microRNA signaling regulate the effects of leptin signaling on EmCa growth. Molecular analyses showed that leptin increased human telomerase reverse transcriptase (hTERT) mRNA expression and cell growth through ERα activation. Real-time polymerase chain reaction analyses revealed an inverse correlation between hTERT mRNA and miR-498 in response to vitamin D3 (1,25(OH)2D3) in estrogen-sensitive cancers, including EmCa. The studies suggest that miR-498-mediated hTERT downregulation is a key event mediating the anti-leptin activity of 1,25(OH)2D3 in estrogen-sensitive EmCa [64].

Notch-system signaling

Notch signaling is a complex transduction process initiated by the binding of a membrane-bound ligand (in adjacent cells) to a membrane-bound receptor in the target cells. Notch affects processes such as proliferation, apoptosis, cell survival, epithelial-mesenchymal transition, differentiation and angiogenesis. Currently, four Notch receptors have been identified in mammals (Notch 1–4). Each receptor consists of an extracellular domain, which is involved in ligand binding and a cytoplasmic domain involved in signal transduction. Five ligands for Notch have been identified: Jagged (JAG1 and JAG2) and Delta-like (DLL1, DLL3 and DLL4). Once the ligand binds to its receptor, the Notch receptor is proteolytically cleaved at the extracellular domain by an α-secretase (ADAM10), which is subsequently followed by cleavage of the receptor's intracellular domain by γ-secretase, resulting in the formation of the intracellular domain of Notch (NICD or Notch-IC). The Notch canonical pathway is characterized by the translocation of the cleaved NICD to the nucleus to initiate transcription of target genes. Inside the nucleus, NICD interacts with a transcription factor CSL (RBPjk), converting CSL from a transcriptional repressor to a transcriptional activator [65]. Simultaneously, coactivators (CoA) are recruited and form a transcription-activating complex with CSL to modulate the expression of the genes HES and HERP (hairy/enhancer of split and hairy/enhancer of split-related protein), respectively [66, 67]. Polyubiquitination and degradation of NICD can occur in a proteasome-dependent manner [65, 66]. However, Notch signaling can be regulated by canonical (CSL, ADAM) and non-canonical pathways (Wnt/β-catenin) [68].

An uncommon characteristic of the Notch signaling pathway is the lack of an amplification step during the canonic signal transduction process. Amplification usually involves phosphorylation of multiple core proteins within the pathway to augment the signaling process. Additionally, Notch signaling exhibits a 1:1 ratio of signaling input and output in each reaction, wherein the Notch receptor is consumed yielding only one NICD. Therefore, to generate an appropriate cellular response, signal strength is an important factor [69]. Hence, the Notch signaling pathway can be extremely sensitive to deviations in gene expression [70].

Aberrant activation of Notch signaling can lead to various pathological conditions such as cancer [71]. Therefore, Notch is a hallmark for many cancers [65]. In tumorigenesis, irregular Notch activation can be initiated through the abnormal expression of Notch ligands, receptors and target genes, all of which have been reported in many solid tumors, including breast, prostate and pancreatic tumors [70, 71]. The Notch signaling pathway exhibits oncogenic properties in some tumors and suppressive properties in others, which suggests a dual role in carcinogenesis [71]. In salivary gland carcinomas, the Notch pathway acts as an oncogene via the translocation of a Notch transcriptional co-activator (Mastermind-like gene). In contrast, Notch functions as a tumor suppressor in skin carcinomas through Wnt and Hedgehog signaling pathways [72]. Hence, Notch signaling is cell and context dependent [65].

Expression of Notch in endometrial cancer

Notch signaling has been studied in many gynecologic cancers, including ovarian cancer and cervical cancer [73]. A role for Notch signaling molecules has been suggested in normal and malignant endometria. The function of Notch signaling in EmCa is poorly understood. However, there are only few reports of Notch signaling in EmCa. Moreover, the role of Notch signaling in EmCa is controversial [70, 74, 75].

Notch1 expression was significantly higher in the proliferative phase of the endometrium when compared to the other phases, which may suggest that Notch signaling is necessary for cell proliferation [74]. Additionally, the expression of Notch signaling molecules was higher in EmCa when compared to normal endometrium [75]. Notably, Notch1 expression was increased in tumors with invasive properties. Consequently, Notch1 expression was positively correlated with high FIGO staging and lower survival rates for patients. Moreover, high expression levels of JAG1/Notch1 was associated with a poor prognosis [75]. In contrast, it has been suggested that Notch

signaling could function as a tumor suppressor in EmCa due to the down-regulated expression of certain Notch ligands and receptors [70]. Notch receptors (Notch 1–4), ligands (JAG1, JAG2 and DLL1) and target (HES1) had an overall decreased expression of mRNA in EmCa when compared to the normal endometrium [70]. Therefore, more studies must be conducted to identify the specific role Notch plays in EmCa.

Notch crosstalk to many signaling pathways in endometrial cancer

An active crosstalk occurs between Notch, Wnt and Hedgehog signaling pathways. Remarkably, Notch, Wnt and Hedgehog are involved in embryonic development. Abnormal activation of these pathways is linked to many cancers. Indeed, the proliferation of EmCa cells involves the aberrant activation of the Hedgehog signaling pathway [76, 77]. However, there are few reports of Wnt signaling in EmCa. An essential player of Wnt signaling, β-catenin, was abnormally expressed in type I EmCa. Deregulation of the Wnt/β-catenin signaling pathway by inactivating β-catenin mutations was found in approximately 10%–45% of EmCa [77]. In contrast, no significant difference in Wnt1, FZD1 and Wnt5 expression between EmCa and normal endometrium was found [78]. Additionally, Wnt and Hedgehog expressions were lower in EmCa compared to normal endometrium, which may suggest that Notch acts a tumor suppressor in these pathways [70, 79].

Notch, IL-1 and leptin crosstalk in endometrial cancer

Leptin is an important pro-inflammatory, pro-angiogenic and mitogenic factor [5, 80]. Studies have shown that high leptin levels are linked to poor cancer prognosis [5, 80]. Leptin produced by cancer cells act in an autocrine and paracrine manner to promote tumor cell proliferation, migration and invasion, pro-inflammation and angiogenesis [5, 81, 82]. Obesity and leptin significantly alter the profiles of numerous proteins linked to cellular processes in cancerous tissues such as Notch and IL-1 [6, 65, 83]. OB-R short isoforms are higher expressed than OB-R long isoform in EmCa [81]. High levels of leptin and OB-R are associated with metastasis and decreased survival rates in breast cancer patients [80]. However, it is unknown whether this association is found in EmCa.

Leptin induces IL-1 system in endometrial and breast cancer cells [61, 82, 84, 85]. The IL-1 system is composed of ligands (IL-1α and IL-1β), receptors (IL-1R tI and IL-1R tII) and an antagonist (IL-1Ra). IL-1β is the more abundant ligand that preferably binds IL-1R tI in normal and cancer cells. The IL-1 system is involved in various roles in both physiological and pathological states [84]. In cancer cells, IL-1 promotes angiogenesis, tumor growth and metastasis [85]. IL-1 is known to be up-regulated in many tumor types. Indeed, the presence of IL-1 in some human cancers is associated with aggressive tumor biology [86]. IL-1 has been shown to up-regulate leptin levels in some cancer cells. Overexpression of IL-1 is seen in breast cancer and linked to proliferation of breast cancer cells [84]. However, we previously showed that leptin up-regulates the IL-1 system in endometrial cells [61]. Moreover, we have shown that IL-1R tI levels were increased in EmCa cells in the presence of leptin, which was related to the activation of JAK2/STAT3, MAPK/ERK1/2 and mTOR pathways [61].

An active crosstalk exists between Notch, IL-1 and leptin in breast cancer termed NILCO [83, 84, 87, 88]. Notch, IL-1, and leptin crosstalk outcome (NILCO) is involved in the induction of breast cancer cell proliferation and migration. In these cells, leptin up-regulates Notch ligands, receptors and target genes. Additionally, leptin up-regulates IL-1 [85, 89]. Remarkably, the blockade of IL-1R tI abrogated leptin up-regulation of Notch [84]. Interestingly, IL-1/IL-1R tI signaling has been shown to mediate leptin up-regulation of VEGF/VEGFR-2 in breast cancer [89]. Leptin can directly induce VEGF/VEGFR-2 up-regulation and indirectly up-regulates VEGF/VEGFR-2 through IL-1 and Notch [83, 84, 88–90]. However, this crosstalk has not been investigated in EmCa.

Currently, our lab is investigating NILCO in EmCa. We have shown that NILCO is expressed significantly higher in type II EmCa, the more aggressive non-hormonal form of EmCa [91]. Paraffin sections were analyzed using malignant and surrounding benign tissue biopsies from obese AAW (n=21). The patient samples were classified by a histopathologist as either type I or type II EmCa. Patients had well-annotated clinicopathological data (including race, age, parity, body weight and pathology). Institutional Review Board approval was obtained from Morehouse School of Medicine and Grady Memorial Hospital, Atlanta, GA. Expression levels of NILCO components (Notch 1–4, JAG1 and DLL4) and targets (survivin, Hey2, IL-1R tI and OB-R) were determined via immunohistochemistry (IHC) [91]. Staining intensity were assigned using semi-quantitative H-score [∑pi (i+1), where "i" is the intensity with a value of 0, 1, 2 or 3 (negative, weak, moderate or strong, respectively) and "pi" is the percentage of stained cells for

each intensity] calculated by two independent observers in three different fields (100 cells/each) [87]. Preliminary immunohistochemistry results showed that Notch1 and 4 (receptors), JAG1 and DLL4 (ligands), survivin, OB-R and IL-1R tI were expressed higher in type II EmCa (Figure 2).

These results were assessed in preliminary studies using tissue microarray from type II EmCa from Chinese patients (data not shown). Preliminary data suggested that the more aggressive and non-hormonal form of EmCa (type II) could be dependent on Notch signaling. The results also might suggest that an active crosstalk between obesity signals (leptin) and Notch occurs in EmCa. Therefore, NILCO expression in EmCa may serve as a new tumor marker. Moreover, novel therapy strategies targeting Notch and leptin signaling could be a new way to treat type II EmCa. Identification of the role of NILCO is a current research challenge that could provide information to better understand the mechanisms involved in obesity-induced effects on EmCa.

Endometrial cancer markers

Tumor markers are molecules found in malignant cells or body fluids. These molecules are produced by the cancer or host normal cells in response to the presence of cancer. Tumor markers can be used to differentiate malignant from normal tissue. These molecules include proteins and genes (i.e., cell receptors, growth and angiogenic factors, cytokines, extracellular and cell adhesion molecules, serum proteins and tumor suppressor genes among others). Since the description of first tumor marker by Henry Bence-Jone in 1846, several tumor markers have been reported. Tumor markers are useful to identify the risk, screen for early cancer, establish diagnosis, monitor disease progression and response to therapy, detect recurrence and estimate cancer prognosis [92].

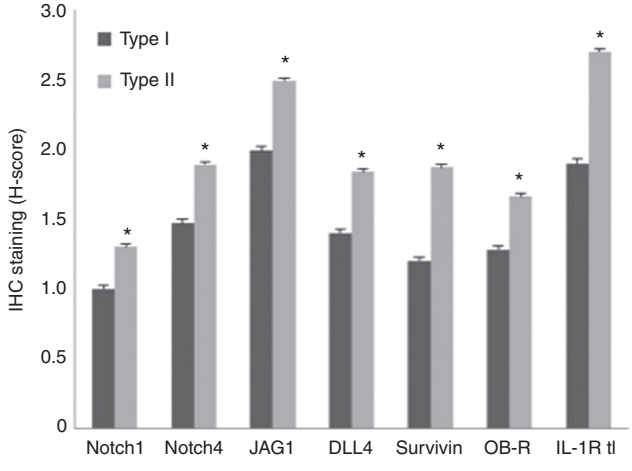

Figure 2: H-scores from immunohistochemistry staining of NILCO components and targets in type I and type II EmCa from AAW. H-scores were significantly higher in type II versus type I EmCa for Notch1 (p=0.0044), Notch4 (p=0.0081), JAG1 (p=0.0116), DLL4 (p=0.0042), survivin (p=0.0144), OB-R (p=0.0008) and IL-1R tI (p=0.0028).

Mismatch repair genes (MMR) and microsatellite instability (MSI)

DNA repair and the mismatch repair system (MMR) play crucial roles in promoting genetic stability. Microsatellites are simple repetitive DNA sequences in the genome susceptible to replication errors. Microsatellite instability (MSI) is due to inactivation of intranuclear proteins, which comprises the MMR, resulting in accumulation of structural mutations during DNA replication [93]. MSI is a molecular phenotype found in approximately 20% of sporadic endometrioid EmCas (type I EmCa) of all grades. MHL1 inactivation (MutL homolog 1, a component of MMR) is the most common altered mechanism of DNA mismatch repair in the endometrium, which is accomplished by hypermethylation of CpG islands in gene promoters (epigenetic silencing). MSI may specifically target for inactivation of those genes which contain susceptible repeat elements, such as transforming growth factor beta receptor II (TGF-RII), BAX, insulin growth factor receptor (IGFIIR) and hMSH3 resulting in secondary tumor subclones with altered capacity to invade and metastasize. However, MSI is rare (<5%) in type II EmCa, where the primary genetic defect is in the p53 gene [94].

Tumor suppressor genes

Tumor suppressor genes code for proteins that inhibit tumor growth. When mutated, they become inactive and tumor growth is allowed. Phosphatase and tensin homolog (PTEN) is a tumor suppressor gene encoding a phospholipid phosphatase, which acts to maintain G1 arrest and enable apoptosis, antagonizing the P13K/AKT pathway. Inactivation of PTEN tumor suppressor gene is the most common genetic defect in type I EmCa. PTEN mutations are found in endometrial hyperplasia suggesting that it is an early event in carcinogenesis and is involved in the

early phases of endometrial tumorigenesis [95, 96]. Discrete premalignant phase of EmCa precedes the inactivation of PTEN in up to 83% of endometrial abnormalities. PTEN inactivation may be caused by mutations, deletions or promoter hypermethylation, and decreased PTEN activity causes increased cell proliferation, cell survival and angiogenesis [92].

The serine/threonine kinase Akt (protein kinase B), a major downstream effector of PI-3K and PTEN, is very frequent activated. This occurs in human cancers not only by inactivation of PTEN but also through activation of Ras (a small GTPase family of kinases), PI3K and growth factor receptors [95, 97]. The deficiency of Akt1 has been shown to reduce the development of tumors in endometrium induced by PTEN inactivation in mouse models [98]. Thus, inhibition or ablation of Akt activity could be an approach of prognostic value [99].

The *p53* gene regulates cell cycle, apoptosis and differentiation [95]. It can initiate cell cycle arrest as a response to DNA damage, by increasing the cyclin-dependent kinase inhibitor p21. *p53* mutations have been found in 10%–20% of endometrial carcinomas [97, 98]. However, p53 protein overexpression is more frequent in serous papillary (type II) than endometrioid tumors (type I) [99] and has been associated with higher FIGO stage. Furthermore, overexpression of p53 (detected by IHC) is associated in several retrospective studies with an unfavorable prognosis of EmCa [100, 101].

Additionally, the inactivation of the *p21* gene, a downstream effector in the p53 pathway of cell growth control, may potentially lead to EmCa progression. Low expression of p21 protein has been associated with significantly decreased survival of EmCa patients, including those without p53 alterations [102].

Cell cycle regulation, proliferation and apoptosis

Ki67 (proliferation marker) is increased in serous papillary and high-grade tumors and at the invasive front of EmCas [103, 104]. Ki67 expression has been correlated with clinical stage and histological grade in a series of primary untreated EmCa patients [105], which could be a strong prognostic indicator of EmCa recurrence. Moreover, Ki67 detection is related to the efficacy of endocrine treatments [106]. Additionally, a few other studies estimating cell proliferation rate by determining cells in S phase have associated cell proliferation with significant prognostic outcome in EmCa [106].

Mutations in *Bax*, an apoptosis inducer gene, induces a loss of Bax protein expression in endometrial carcinomas [107, 108]. In contrast, Bcl-2, an inhibitor of apoptosis, is highly expressed in endometrial hyperplasia but shows decreased expression in EmCa [109].

Cables is a cell cycle regulatory protein and tumor suppressor that is up-regulated by progesterone and down-regulated by estrogen actions in the endometrium. Cables expression is lost in type II EmCa and more that 80% of type I EmCa. Nuclear immunostaining for Cables is lost in a high percentage of cases of endometrial hyperplasia and EmCa, which are likely the product of unopposed estrogen. Thus, loss or suppression of Cables may be an early step in the development of endometrial cancer [110].

Steroid receptors

The presence of hormone receptors has been relevant for the phenotype classification and targeted treatment in EmCa [100]. Activated ER can suppress the expression of Bax by upregulating a group of microRNAs including hsa-let-7 family members and hsa-miR-27a. Therefore, ER promotes the increase of Bcl2/Bax ratio as well as enhanced survival and proliferation of endometrial cells. ER-regulated hsa-let-7 microRNAs can be detected in most hyperplastic endometria, suggesting their potential utility as indicators of estrogen over-exposure [107].

Protein kinase C-α (PKC-α) is aberrantly expressed in endometrioid tumors and is an important mediator of EmCa cell survival, proliferation, and invasion. PKC-α signaling, via PI-3K/Akt, may be a critical element of the hyperestrogenic environment and activation of ER. This signaling crosstalk is thought to trigger the development of estrogen-dependent endometrial hyperplasia and malignancy [92].

Progesterone receptor (PR) expression has been correlated to EmCa grade, histology, adnexal spread and recurrence [111]. However, EmCa recurrence occurs more in PR-negative tumors [112]. In recent years, the expression of PR-A and PR-B isoforms in endometrial adenocarcinoma has been significantly associated with increasing tumor differentiation [101].

Markers of invasion and metastasis

Genetic alterations in cellular adhesion molecules, such as catenins and cadherins, are important for tumor stroma and tumor vascular interactions. It was found

that several features of type I EmCa occur significantly more often in tumors expressing nuclear β-catenin. These results suggest that abnormal Wnt/beta-catenin signaling pathway could be a molecular feature of a subset of type I EmCa [113]. Mutations in the β-catenin gene have been associated with a low metastatic potential [114].

CD146 (cell surface glycoprotein MUC 18) is a cell adhesion molecule found overexpressed in various cancers including breast and ovarian cancers. CD146 promotes tumor growth, angiogenesis and metastasis, and its levels were higher in EmCa and positively correlated with histological grade and myometrial invasion [114, 115].

DNA ploidy

Aneuploidy tumors are present in 20%–30% of endometrial carcinomas associated with a high grade, non-endometrioid subtype, deep myometrial invasion and high FIGO stage. DNA ploidy was the strongest independent predictor of poor outcome in series of EmCa and was correlated to recurrence and survival patterns [116–118].

Serum tumor markers

Elevated pre-diagnostic concentrations of tumor necrosis factor-alpha (TNF-α) and its soluble receptors TNFR1 and TNFR2 were related to a higher risk and advanced EmCa stage [119, 120]. However, lower levels of TNF-α were found in endometrial hyperplasia compared to normal controls. Estrogen-stimulated TNF-α expression from EmCa cells induced the stromal expression of HGF that could be targeted with NK4 (HGF-antagonist/angiogenesis inhibitor) [120].

Serum human epididymis protein 4 levels correlated with an aggressive tumor phenotype and may constitute an independent prognostic factor for poorly differentiated EmCa [121].

YKL-40 (human cartilage glycoprotein-39) was elevated in 76% of EmCa patients, and its pre-operative serum level may predict worse clinical outcome [100]. YKL-40 correlated to VEGF overexpression and co-activation of syndecan-1 (S1), integrin αvβ3 and focal adhesion and MAP kinases [122].

Higher levels of M-SCF (stem cell factor) were detected in 25%–73% of EmCa cases and were predictive of aggressive clinical course [111, 114]. Elevated levels of serum sFas (Fas ligand, a pro-apoptotic molecule) were found in endometrioid adenocarcinoma ($p<0.0001$). Additionally, human serum amyloid A protein was overexpressed and actively secreted by grade-3 endometrioid adenocarcinoma and serous papillary carcinoma [112].

Elevated serum CA 125 (cancer antigen 125 or MUC16) levels have been detected in 11%–43% of EmCa and related to disease stage, myometrial invasion, peritoneal cytology and lymph node metastasis. CA 125 serum cutoff of 20 U/mL had a sensitivity of 69.0%, specificity of 74.1%, positive predictive value of 58.8% and negative predictive value of 81.6% for assessment of myometrial infiltration. Serum CA 125 level usually parallels the clinical course of the disease [123]. Other tumor serum markers, CA 15.3 and CA 72.4 were found in 47% of EmCa patients with occult stage III compared to 18% of those with stages I and II [123].

Low levels of serum taurine (an organic acid) are found in EmCa patients [124]. Apolipoprotein A, pre-albumin and transferrin levels were found higher in early and late-stage EmCa (71/88% sensitivity and 82/86% specificity) [125].

Despite the abundant literature on EmCa markers, there are scarce data on relationships between these molecular markers and obesity cues.

Treatment of endometrial cancers

High risk of EmCa recurrence correlates to deeply tumor invasion, type II and advanced age. For advanced stage EmCa, hysterectomy is the first therapy choice. Chemotherapy reduces the mortality due to recurrent EmCa by a quarter and also reduces the risk of developing the first recurrence outside the pelvis. Radiotherapy is commonly used in conjunction with chemotherapy and surgery to treat EmCa [3].

Initial management of early EmCa is surgical staging with total hysterectomy, bilateral salpingo-oophorectomy, bilateral pelvic and para-aortic lymph node dissection and pelvic washings. Minimally invasive approach, such as laparoscopy or robotic assistance, is preferred in certain situations over laparotomy due to similar outcomes and decreased postoperative adverse events. Vaginal hysterectomy may be appropriate for patients with increased risk of morbidity, however does not allow lymphadenectomy [126].

Lymphadenectomy is useful in triaging need for adjuvant therapy but can be eliminated for patients identified as low risk by the Mayo criteria, with grade 1–2 of type I endometrioid tumors, <50% myometrial invasion and tumor of 2 cm or less. Sentinel node dissection may further clarify patients who need nodal dissection while minimizing morbidity. Vaginal brachytherapy is the adjuvant therapy of choice for patients with early stage EmCa.

For type II EmCa with high risk of recurrence, adjuvant therapy in addition to brachytherapy is often considered, and there is a Gynecologic Oncology Group study ongoing; however, no prospective data are currently available [126]. For advanced or recurrent EmCa, aggressive surgical cytoreduction including exenteration have been shown to improve progression-free and overall survival. Adjuvant therapy is given as combination of chemotherapy with paclitaxel and carboplatin, along with radiation. Protocols often studied employ the sandwich technique with three chemotherapy cycles, radiation and then additional three chemotherapy cycles. Patients who are not candidates for surgery may be treated with primary radiation therapy with adjuvant chemotherapy [127].

Conservative management with hormonal agents has been studied in women who are poor surgical candidates or who desire fertility-sparing treatment. Most often studied are medroxyprogesterone acetate and megestrol acetate. Other regimen studies include diverse progestins, oral contraceptives, tamoxifen and intrauterine device containing levonorgestrel. Failure or recurrence may occur. If medical therapy fails or childbearing has been completed, definitive surgical therapy may be recommended [127].

Oncogenes and targeted treatment of EmCa

Oncogene overactivation stimulates cell division. However, few oncogenes have been found over-activated in EmCa. Among them, mutations of *K-Ras* (a proto-oncogene involved in growth control and differentiation) could be related to the progression of several cancers. K-Ras mutations occur in 10%–30% of EmCa, predominantly in type I as well as endometrial hyperplasia. These data suggest that K-Ras mutation is an early event in the development of type I EmCa [128].

Her2 overexpression was found in approximately 20% endometrioid and serous carcinomas. Her2 was associated with EmCa aggressive phenotype and poor survival. However, trastuzumab therapy (anti-Her2 antibody) as a single agent did not demonstrate activity against EmCa with Her2 overexpression or gene amplification [129].

Somatic mutations in the fibroblast growth factor receptor 2 (FGFR) were found in 12% of the EmCa. Altered ligand specificity and constitutively activated FGFR2 mutations have oncogenic roles in EmCa cell lines [130]. Dovitinib, a FGFR2 inhibitor induced complete EmCa regressions in a long-term in vivo study in FGFR2 wild-type EmCa xenograft models. mTOR pathway in concert with oncogenic FGFR2 may drive EmCa growth [131]. Indeed, Ridaforolimus, a selective inhibitor of mTOR, reduced EmCa growth in vivo [132]. Additionally, amplifications of PI-K3CA, a catalytic subunit of PI-3K, correlated to PI-3K activation and PTEN mutations in EmCa. This suggests that these molecules are potential targets for therapy [133].

Increased levels of VEGF and angiogenic markers are associated with poor outcome and high grade in type I EmCa [134, 135]. Detection of VEGF and its receptor type 1, VEGFR-1, could be useful markers for predicting 5-year disease-free survival in endometrioid EmCa [136]. Avastin, a humanized antibody against VEGF-A, retarded tumor growth in athymic mice. Interestingly, c-Jun oncogene was detected in bevacizumab-treated EmCa that suggests that c-Jun-mediated pathway(s) contributes to bevacizumab resistance [137].

Cancer stem cells

Accumulating evidence has revealed that there are rare populations of cells that display adult stem cell properties of self-renewal and differentiation in both epithelium and stroma of the human endometrium. These cells are probably responsible for the regenerative capacity of the endometrium. Epithelial stem cells might be located in the basal layer of the endometrium [138]. Stem cells can be classified as embryonic stem cells [139], germ stem cells [140], fetal stem cells [141], cancer stem cells (CSCS) and EmCa side population cells (ECSP) as well as somatic or adult stem cells [142, 143].

Embryonic stem cells are pluripotent cells derived from the inner cell mass of the blastocyst. Germ stem cells are defined as pluripotent stem cells having derived from germ cells. Fetal stem cells are responsible for the initial development of all tissues before birth; they can be isolated not only from fetal blood and hemopoetic organs but also from fetal organs, amniotic fluid and placental membranes. Adult stem cells are found in several tissues, and it has also been suggested that stem-like cells exist in cancerous tissues [143].

The human endometrium contains rare epithelial and stromal cells able to produce colony-forming units: endometrial epithelial stem cell-like (also referred to as side population) and endometrial mesenchymal stem cell-like (MSC). MSC are found in perivascular and basalis and functionalis endometrial layers [144]. MSC express CD146+ (MCAM), PDGF-Rβ+ (CD140B), ITGB1 (9CD29), CD44, NT5E (CD730), THY1 (CD90) and ENG (CD105) but not endothelial or hematopoietic markers [145]. Similar populations of cells, called EmCa stem cells (CSCS), have been described.

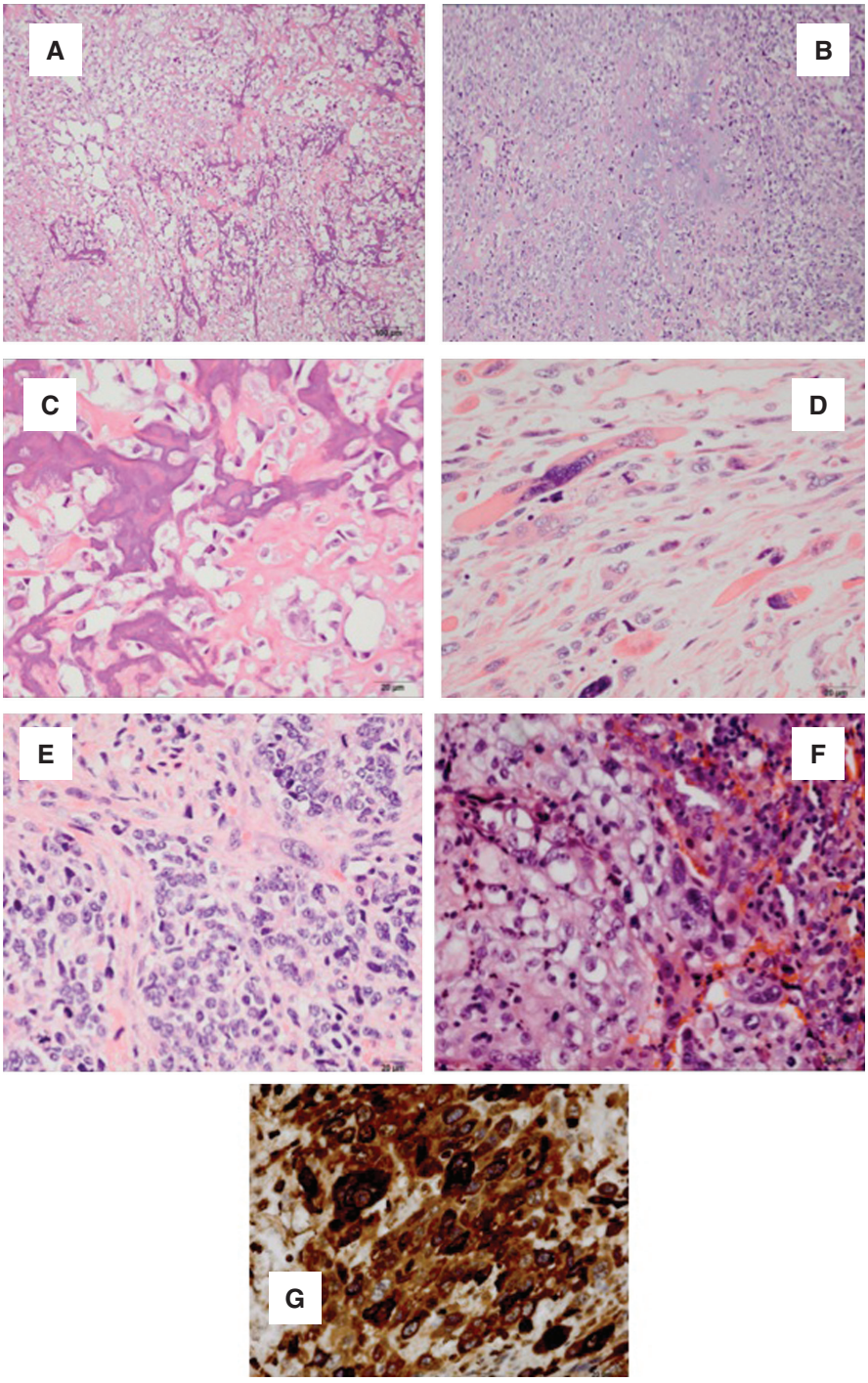

Figure 3: EmCa derived from EmCa stem cells (ECSC). (A, B) Osteosarcomas; (C, D) chondrosarcomas; (E) rhabdosarcoma; (F) epithelial sarcomatous and (G) choriocarcinoma differentiation.
(A) Sarcomatous stromal cells produce osteoid. Tumoral osteoid represented by amorphous fibrillary eosinophilic deposits. Early osteoid deposition forms a lace-like pattern around tumor cells, and advanced osteoid shows evidence of mineralization (darker pink-purple color) (HE, 10×). (B) High magnification shows highly malignant cells with high nuclear-to-cytoplasmic ratio, anaplastic hyperchromatic nuclei or clearing of the chromation and conspicuous, cherry-red nucleoli (HE, 40×). (C) Sarcomatous stromal cells produce chondroid matrix (HE, 10×). (D) Malignant nuclear anaplastic features (HE, 40×). (E) Anaplastic rhabdomyosarcoma showing large strap cells with abundant cytoplasm and striations. The cells are mononuclear or multinucleated. Numerous mitoses are identified (HE, 40×). (F) Epithelial and sarcomatous component blend in this tumor. The malignant epithelial component is composed of cells with high nuclear-to-cytoplasm ratios that show numerous mitoses. Spindle cells with large anaplastic nuclei and prominent nucleoli representing the sarcomatous component are immediately adjacent to the epithelial component (HE, 40×). (G) Malignant polygonal/round cells with single nucleus are reminiscent of the cytotrophoblast. Few multinucleated cells reminiscent of the syncytiotrophoblast are also present. Hemorrhagic background and necrosis areas are present in this tumor (HE, 40×).

CSCS have unlimited proliferative potential, show reduced level of differentiation markers, resistance to conventional chemotherapeutic agents and high DNA repair capacity [138]. ECSP isolated and characterized from EmCa cell line express CD133, CD44, CD146, PDGFRβ and aldehyde dehydrogenase-1 markers. These cells may be identified as label-retaining cells using their property to retain DNA synthesis label bromo-deoxyuridine (BrdU) [146]. ICD133 (+) EmCa cells and/or ECSP cells can initiate tumor formation and recapitulate the phenotype of the original tumor (Figure 3) [145].

Musashi-1, an evolutionary conserved marker for neural stem cells, was co-expressed with Notch1 in a subpopulation of endometrial cells. Additionally, Musashi-1 and telomerase expressing cells were found significantly increased in proliferative endometrium, endometriosis and EmCa [147]. Also, Notch1 pathway was increased in EmCa and endometriosis suggesting the concept of a stem cell origin of EmCa and endometriosis [147].

ECSP possess the following characteristics: (i) reduced expression levels of differentiation markers, (ii) long-term repopulating properties, (iii) self-renewal capacity, (iv) enhanced migration and podia formation, (v) enhanced tumorigenicity and (vi) bi-potential development (tumor cells and stroma-like cells), suggesting that they have cancer stem-like cell features. Recently, sodium butyrate, a histone deacetylase inhibitor, was shown to inhibit the self-renewal capacity of ECSP by inducing a DNA damage response [148].

Race and endometrial carcinomas

The lifetime risk of being diagnosed with EmCa is as follows: CSW>AAW>Hispanics>Asian Pacific islanders>Native American women. However, there is a large disparity in the death rate. AAW have a 12% decrease in incidence rate and an 86% increase in death rate in comparison with CSW [148].

The 5-year survival rate of AAW is lower than in CSW for every stage of disease at the time of diagnosis. AAW have a higher grade and more aggressive tumor types (serous, clear cell type and carcinosarcomas) [149]. However, divergent data on race factor impact on survival have been shown [149, 150]. CSW were more likely to have PTEN mutation, present in type I EmCa histology and associated with better prognosis [151]. In contrast, AAW suffering from EmCa have more p53 mutations and Her2 expression in cancer tissue, and show poor treatment response, which are associated with type II Emca [4, 152, 153]. Additionally, incidence of obesity is significantly higher in AAW than in CSW. Obesity contributes to EmCa morbidity, progression, recurrence and mortality [14]. These data suggest that incidence of types I and II EmCa, obesity and socio-economic factors may impact on the AAW survival differences [4, 154, 155].

Expert opinion

In spite of the important role suggested for tumor markers and CSCS in EmCa pathology, recurrence and response to treatments, there are not comprehensive data available on how obesity and race could impact on these factors. Moreover, whether health disparity in AAW could be influenced by obesity-induced changes in CSCS and EmCa markers is unknown. There is a need to better understand the mechanisms involved in obesity-related EmCa. NILCO markers may provide additional information for obesity-related type II EmCa. These important questions on EmCa biology, detection, potential prevention, treatment and recurrence warrant further investigations.

Outlook

Determination of NILCO and other obesity-related molecules together with traditional tumor markers may be used as additional tools to predict the impact of obesity on EmCa prevention, treatment and recurrence.

Highlights

EmCa is a multifactorial disease classified in two major types: type I and type II.

Type I EmCa is responsive to steroid hormonal medium, more differentiated and shows better prognosis.

Type II EmCa is unresponsive to steroid hormones, less differentiated and shows poor prognosis.

CSW show higher incidence of EmCa, but AAW show higher grade and poor prognosis.

Obesity correlates to higher incidence and poor prognosis of EmCa.

Race, obesity and socioeconomic factors could be related to higher incidence of type II EmCa in AAW. Frequent overexpression of p53 has been reported in type II EmCa from AAW. More studies are needed to better understand how race and obesity could impact on EmCa health disparity.

Several tumor and serum markers are being used to detect and predict treatment response in EmCa. However,

no data are available on relationships between EmCa markers, CSCS profiles and obesity cues.

NILCO may serve as novel biomarker for obesity-related type II EmCa.

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