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Oncogenic role and therapeutic target of leptin signaling in breast cancer and cancer stem cells

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Abstract

Significant correlations between obesity and incidence of various cancers have been reported. Obesity, considered a mild inflammatory process, is characterized by a high level of secretion of several cytokines from adipose tissue. These molecules have disparate effects, which could be relevant to cancer development. Among the inflammatory molecules, leptin, mainly produced by adipose tissue and overexpressed with its receptor (Ob-R) in cancer cells is the most studied adipokine. Mutations of leptin or Ob-R genes associated with obesity or cancer are rarely found. However, leptin is an anti-apoptotic molecule in many cell types, and its central roles in obesity-related cancers are based on its pro-angiogenic, pro-inflammatory and mitogenic actions. Notably, these leptin actions are commonly reinforced through entangled crosstalk with multiple oncogenes, cytokines and growth factors. Leptin-induced signals comprise several pathways commonly triggered by many cytokines (i.e, canonical: JAK2/STAT; MAPK/ERK1/2 and PI-3K/AKT1 and, non-canonical signaling pathways: PKC, JNK and p38 MAP kinase). Each of these leptin-induced signals is essential to its biological effects on food intake, energy balance, adiposity, immune and endocrine systems, as well as oncogenesis. This review is mainly focused on the current knowledge of the oncogenic role of leptin in breast cancer. Additionally, leptin pro-angiogenic molecular mechanisms and its potential role in breast cancer stem cells will be reviewed. Strict biunivocal binding-affinity and activation of leptin/Ob-R complex makes it a unique molecular target for prevention and treatment of breast cancer, particularly in obesity contexts.

Keywords

Leptin; Breast cancer; Tumor angiogenesis; Breast cancer stem cells; Leptin antagonist; Leptin signaling

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1. Introduction

Obesity and overweight conditions are prevalent in the world. The World Health Organization (WHO) reported that more than 400 million people are obese, with a predicted increase to possibly reach 700 million by 2015 worldwide [1]. In the United States, the current epidemic of obesity in adults is 30%–35%, posing a major public health challenge [2–5]. Obesity or overweight conditions are associated with a significantly increased risk of development of various diseases, particularly cardiovascular disease [6], type 2 diabetes [7], hypertension [8], dyslipidemia [9], liver disease [10], as well as cancer [11–12]. Compelling evidence indicates over 13 different cancers including breast, cervical, colon or rectal, esophageal, gall bladder, kidney, liver, ovarian, pancreatic, stomach, uterine cancer, as well as multiple myeloma, non-Hodgkin lymphoma are associated with obesity [11–14].

How obesity is connected to cancer incidence is still an unexplainable or unanswered question. However, accumulated evidence shows that these two conditions have intertwined inflammatory patterns. In obesity, deregulated secretion of pro-inflammatory cytokines, chemokines and adipokines such as TNF-α, plasminogen activator inhibitor-1 (PAI-1), IL-1, IL-6, adiponectin and leptin from the expanding adipose tissue and inflammatory cells could make a clinically relevant contribution to the onset and progression of cancer [15–17]. However, the individual contributions of these factors to obesity-related cancers are often contradictory and not well understood in diverse scenarios. Among the above mentioned molecules, leptin has been the most studied adipokine since this protein was first cloned in 1994 [18].

The identification of spontaneous mutations in the leptin (ob or LEP) and Ob-R (db or LEPR) genes in mice opened up a new field in obesity research. Although allelic frequencies of ob and db polymorphisms show ethnic variation, systematic search for mutations showed low penetration and scarce number of affected individuals. The data suggests the lack of association between the genes under study and obesity. The lack of association could be due to the complex pathogenesis of obesity, which involves a number of genetic and environmental factors [19]. High leptin levels in obesity and overweight individuals or populations are clearly correlated with body fat and adipocyte size. Under these conditions leptin is unable to regulate appetite/size of fat deposits leading to a “leptin resistance status”, which could induce deregulated peripheral actions in many Ob-R expressing tissues. The molecular mechanisms underlying how obesity causes an increased risk of cancer are poorly understood, but compelling evidence shows that pandemic obesity and cancer incidence are connected [20]. A retrospective study on a large cohort of women (n=495,477) in the US reported by Calle et al., shows a significant correlation between increasing risk and higher body-mass-index values and death from breast cancer in obese/overweight women [21]. Other studies have also found that women with a higher percentage of adipose tissue/leptin levels have higher incidence of breast cancer [11, 22–23].

In light of the increasing reported role of leptin in several types of cancer [24–28], in this review we wish to focus on the role of leptin in breast cancer, highlighting the leptin-mediated signaling pathways and its potential as a drug target. Additionally, we will review and discuss recently identified molecular mechanisms of leptin in breast cancer, including its potential role in breast cancer stem cells (BCSC), tumor angiogenesis, as well as its crosstalk with other oncogenic signaling pathways.

2. Structure and function of leptin and leptin receptor

Leptin is a small 167-amino acid non-glycosilated protein with a molecular weight of 16 kDa, coded by the LEP gene, whose name is derived from the Greek word “leptos,” which means “thin.” The LEP gene is preserved in mammals providing a high sequence identity for

leptin. Indeed, human leptin and mouse leptin share 84 percent sequence homology. The cDNA sequence encoding for leptin was identified on the mouse ob (obese) gene. A nonsense mutation in codon 105 (ob/ob) causes the lack of protein synthesis resulting in morbid obesity, hyperphagia, hypothermia, insulin resistance, and infertility [18]. The biological role of leptin in energy homeostasis was demonstrated by normalization of hyperphagy and obese phenotypes with recombinant leptin administration in rodents and humans [29–30]. In addition, leptin plays critical roles in the regulation of glucose homeostasis, reproduction, growth and the immune response [31–33]. It is currently believed that leptin binding to Ob-R induces the extracellular domains of Ob-R to form a homodimer which constitutes the functional unit responsible for leptin-mediated signals. Analysis of highly conserved regions in the leptin sequence and studies of a homology-based model of the leptin-Ob-R complex suggest that two helices of leptin (H1 and H3) contain the most important receptor binding sites [34]. The N-terminal region (94 amino acids) of leptin is essential for both the biological and the receptor binding activities [35].

The leptin receptor (Ob-R) belongs to a member of the class I cytokine receptor super-family, which includes the receptors of IL-1, IL-2, IL-6 and the growth hormone [36]. This super-family of receptors lacks autophosphorylation capabilities and needs auxiliary kinases for activation. Ob-R binding to leptin induces conformational changes that recruit JAKs (Janus kinases), which in turn phosphorylates Ob-R and activates STATs (signal transducers and activators of transcription). Six leptin receptor isoforms have been discovered so far, and are generated by mRNA alternative splicing [37]: the long isoform (OB-RL or OB-Rb) with full intracellular signaling capabilities and shorter isoforms with less biological activity (OB-RS) [36]. 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 [38–39]. Soluble leptin receptor has also been reported in humans [37]. Leptin binding to Ob-R induces canonical (JAK2/STATs; MAPK/ERK 1/2, PI-3K/AKT) and non-canonical signaling pathways (PKC, JNK, p38 MAPK and AMPK) in diverse cell types. Among the leptin receptor isoforms, only Ob-Rb, which contains an intact intracellular domain, has the ability to activate the intracellular JAK-STAT pathway on ligand binding [40]. Tyr 1138 of Ob-Rb is required for leptin-mediated STAT3 signaling [41–43]. However, Ob-Ra, which contains a truncated intracellular domain, and unable to activate the STAT pathway [41] may still transduce signals by way of activation of JAK2, IRS-1 or extracellular factor-regulated kinases (ERKS) including MAPKs (mitogen-activated protein kinases) [42]. In addition, the SH2 domains of SOCS (suppressor of cytokine signaling) bind the phosphorylated tyrosine residues on the JAK2s regulating OB-Rb. SOCS-3 expression is leptin-induced and is a negative regulator of leptin signaling. 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 [44]. Although, leptin-induced signaling occurs in normal peripheral tissues, high levels of leptin in obese women could lead to increased leptin signaling, which is a hallmark of the development of breast and other cancers.

3. Leptin signaling in normal breast development

Human placenta was earlier identified as an important source of leptin for fetal and neonatal growth [45]. Further, the role of mammary glands in providing leptin to the newborn was reported [46]. Leptin produced by human mammary epithelial cells was revealed by RT/PCR analysis and immunohistochemical staining of breast tissue, cultured mammary epithelial cells, and secretory epithelial cells present in human milk [46]. Analyses of micrographs of whole-mount preparations of mammary tissues for $Lep^{ob}Lep^{ob}$ and $Lepr^{db}Lepr^{db}$ leptin-deficient and leptin receptor-deficient mice, respectively, demonstrated a notable lack of mammary duct formation in both strains of genetically obese mice,

compared with their respective lean counterparts, whose ductal branching was prominent [47]. These results indicate that both leptin and Ob-R are necessary for normal mammary gland development. In line with this finding, leptin expression was also found in epithelial cells from normal human breast tissues [48]. Adipose tissue is a component of normal breast tissue. Adipocytes account for more than 90% of human breast volume and secrete adipocytokines, including leptin. Leptin derived from breast adipose tissue can act as a paracrine positive stimulus for breast cancer growth and may also have pro-oncogenic actions on the stroma-vessel components. Many inflammatory compounds are secreted by the breast adipose tissue that can also induce the recruitment of macrophages [20]. Thereby, derived-adipose factors can increase tumor angiogenesis and growth. In fact, adipose tissue can act as an effector organ and influence both breast cancer risk and tumor behavior [12, 49–50].

4. Expression of leptin and Ob-R in human breast cancer and other cancer types

Data from reverse transcription-polymerase chain reaction, immunoblotting, and immunohistochemistry techniques show that leptin and Ob-R are weakly expressed in normal tissues or cells, but these molecules are overexpressed in various cancers of the lung, colon, uterus, ovary, and breast. In contrast, Ob-R was downregulated in bladder cancer [51]. However, no supporting or conflicting data on these earlier results on bladder cancer have been published to date. In the case of human breast cancer [52], ovarian cancer [53], prostate cancer [54] and skin melanoma [55], leptin was overexpressed in more than 80% cases of malignant tissues determined by immunohistochemistry techniques. In brain [56], breast [52], esophagus cancers [57], Ob-R was also overexpressed in more than 80% cases of malignant tissues according to immunohistochemistry results. There are two reports about Ob-R in thyroid papillary carcinoma, which showed different positive rates, 80% and 51%, respectively, probably due to different antibodies used in these studies [58–59]. To the best of our knowledge, there are no reports about either leptin or Ob-R expression in human pancreatic cancer. In recent groundbreaking studies conducted by Ishikawa et al, immunohistochemical analysis of normal breast tissues resulted in weaker staining levels of leptin in normal epithelial cells compared to adipocytes in adjacent adipose tissue. In sharp contrast, leptin production was enhanced in the majority of breast cancers. Their results also showed that compared to leptin, none of the normal mammary glands exhibited significant immunoreactivity to Ob-R, whereas Ob-R was expressed in most of the carcinoma cells [52].

Table I shows the relative levels of expression of leptin and Ob-R in different cancer types as evidenced via immunohistochemistry. Notice that the percentages of leptin or Ob-R proteins represent positive staining of tissues in which only cancer cells were evaluated for antigen expression, but the expression of leptin or Ob-R in stroma and endothelial cells was not determined (see Table I).

5. Leptin signaling and breast cancer stem cells

Stem cells (SC) are defined by their ability to undergo self-renewal, as well as multilineage differentiation. Classical models of carcinogenesis propose that any cell can be transformed by accumulation of oncogenic mutations in a random way, producing a tumor. According to this model, most of the cells in a fully developed tumor are equally malignant. In contrast, the SC theory of cancer proposes that tumors contain cancer cells that retain key SC properties [60]. These cancer stem cells (CSCs) initiate and drive carcinogenesis and differentiation contributing to tumor cellular heterogeneity through deregulation of the self-

renewal process [61]. Thus, the population of CSC may be a risk factor for carcinogenesis [60].

Many human cancers including breast cancer possess an enhanced tumor-initiating capacity, can undergo self-renewal, partially recreate the cellular heterogeneity of the parental tumor, and seem to be generally more resistant than other cancer cells to conventional anticancer therapeutics. Over the last decade, studies have identified CSC in solid tumors including breast cancer that are responsible for tumor initiation, growth and metastasis [62–63]. Breast CSCs (BCSCs) are characterized by the expression of molecular markers (phenotype CD44+CD24−/ALDH+) [60]. Several factors could be linked to self-renewal of BCSC. Wnt, FGF (fibroblast growth factor), Notch, Hedgehog, and TGF-β/bone morphogenetic proteins (BMP) signaling cascades constitute the stem-cell signaling network, which plays a key role in the maintenance or homeostasis of pluripotent stem cells and CSC. Human embryonic stem cells (ESCs) are supported by FGF and TGF-β signals, whereas mouse ESCs by LIF (leukemia inhibitory factor) and canonical Wnt signals [64]. Associations between birth weight, maternal levels of Insulin-like growth factor 1(IGF-1), breast cancer risk in progeny and BCSC have also been reported [65]. Recently, several reports have revealed that leptin regulates or activates certain oncogenes, such as HER2 [66–67], Akt [68] as well as transcriptional factors, such as STAT3 [69], NF-κB [70] and Notch [71–72] which are critically associated with BCSC. In addition, leptin also activates the Notch signaling pathway [71, 73] that is important for the maintenance of SC or progenitors through the inhibition of differentiation [74–76] (See Fig 1). It is known that breast cancer cell lines contain BCSC. Although the percentage of CD44+/CD24− cells within cell lines does not correlate with tumorigenicity, these cells can form tumors and preferentially survive chemotherapy [77]. Interestingly, we have found leptin induces the expression of CD44 and ALDH1 in several cancer cell lines [78]. In a recent report, leptin deficiency in murine mammary tumor virus (MMTV)-Wnt-1 transgenic mice results in functional depletion of BCSCs leading to less tumor outgrowth [79]. More recently, Feldman et al also demonstrated that robust and selective expression of Ob-R is a characteristic feature of CSCs and of a broad array of embryonic and induced pluripotent stem cells [80]. CSCs exhibit sensitized responses to leptin, including the phosphorylation and activation of STAT3 and induction of Oct4 and Sox2, thereby establishing a self-reinforcing signaling module [80].

Triple-negative breast cancer (TNBC) is characterized by the lack of expression of ER, PgR, and HER-2. This difficult-to-treat form of breast cancer shows an undesirable tendency to overcome drug effectiveness. BCSCs are thought to be responsible for the development of drug-resistance and relapse of TNBC [77]. Basal-like breast cancer (BLBC) frequently expresses a CD44+/CD24− phenotype, which has been associated with a ‘stem-cell’ phenotype. These cells show resistance to conventional treatment and allow repopulation of the cancer. BCSC show some specific molecular alterations including activation of the Hedgehog and Notch pathway [81]. Leptin signaling reduces cisplatin effects on TNBC in vitro. Indeed, MDA-MB-231 (TNBC) cells treated with cisplatin (3.3 μg/ml) showed a significant reduction of cell proliferation/viability (≤ 30%) as determined by MTT assay. However, when these cells were co-incubated with cisplatin plus leptin an impressive recovery of cell proliferation (up to 150%) compared to basal conditions was observed [82]. The connection between leptin signaling and BCSC, as well as its crosstalk with many oncogenic pathways, including Notch, suggest that leptin signaling may be a viable drug target in the treatment of TNBC.

6. Leptin pro-angiogenic signature in breast cancer

Angiogenesis is critical for the growth of solid tumors [64]. The stimulation of this process in response to continuous pro-angiogenic factors by tumor cells results in a state of angiogenesis not normally present in tissues. Thus, anti-angiogenesis agents provide an important therapeutic option for prevention and treatment of breast cancer. Leptin, a regulator of energy homeostasis, is also a mitogenic and angiogenic cytokine that promotes anchorage, proliferation of microvessel and hematopoiesis, as well as increases the levels of several factors including cell cycle regulators [83–86]. Leptin induces the expression of several molecules (i.e., MMP-2, MMP-9, αvβ3 integrin, LIF, IL-1 and VEGF) in breast cancer cells that could further increase tumor angiogenesis. Leptin is as potent as VEGF in promoting tumor angiogenesis. In addition leptin phosphorylates VEGFR-2 independent of VEGF and upregulates Notch in endothelial and breast cancer cells. Moreover, leptin also acts on other stromal cells: fibroblasts and macrophages, which can secrete pro-angiogenic factors. Thus, leptin pro-angiogenic actions in breast cancer likely proceeds by mechanisms involving tumor endothelial (rapid response: leptin/Ob-R mediated pVEGFR2 and mid-term response: leptin-induced Notch), breast cancer (leptin induces VEGF expression, which induces tumor angiogenesis) and stromal cells (leptin induces the secretion of pro-angiogenic factors) (See Fig 1). However, leptin pro-angiogenic actions in breast cancer are mediated by poorly characterized mechanisms.

6.1. Metalloproteases and Integrins

The degradation of connective tissue/basement membrane proteins by MMPs is an important event for tumor angiogenesis [87]. Likewise, the interaction between integrin αvβ3 (vitronectin receptor) and the extracellular matrix is also crucial for angiogenesis and is involved in the VEGF-mediated activation of VEGFR2 [88–89] and metastasis of breast cancer cells [90]. We earlier reported that phenotypic changes of integrin and metalloproteinase secretion of the invasive human cytotrophoblast (whose invasive potential closely resembles cancer cells) were upregulated by leptin, IL-1, IL-6 and TGF-β [91]. Leptin induced some of the changes that cytotrophoblasts undergo to achieve a more invasive phenotype: upregulated α5 and α6 integrins and MMP-9 activity [91]. Moreover, leptin and IL-1β increased the expression of β3 integrin in Ishikawa cells (human epithelial endometrial cancer cell) [92]. In vivo, stimulation of circulating angiogenic cells with leptin increased their capacity to promote new vessel formation in the chorioallantoic membrane of chicken embryos and to improve neovascularization of ischemic murine hind limbs. These effects were linked to leptin/Ob-R-induced activation of JAK2 and phospholipase C (PLC) γ-mediated, which in turn induced Src kinase phosphorylation of αvβ5 integrins [93]. Furthermore, leptin induced the activation of αvβ5 and α4 integrins in human endothelial progenitor cells (EPCs) through STAT3 mechanisms. Leptin's effects modulated the vascular remodeling and neointima formation in vitro and in vivo by increasing the number of EPCs adhering to vitronectin. In addition, leptin- enhanced the ability of EPCs to incorporate into a monolayer of human endothelial cells and the adherence of these cells to activated platelets. These findings suggest that leptin specifically modulates the adhesive properties and the homing potential of EPCs, thus enhancing their capacity to promote vascular regeneration in vivo [94]. Additionally, leptin increased the migration of human androgen-dependent (LnCaP) and independent (PC3 and DU145) prostate cancer cells and the expression of αvβ3 integrin. These leptin-induced effects were related to leptin activation of IRS-1, PI3K, Akt, and NF-κB signaling cascades [95]. In line with these findings, the expression of αvβ3 integrin was upregulated by leptin in human chondrosarcoma cells through the same signaling pathways [96].

6.2. Regulation of LIF and IL-1

LIF (leukaemia inhibitory factor) and leptin have several similar features. Both of them are down-regulated by the suppressor of cytokine signaling-3 (SOCS-3). LIF signals through its receptor LIF-Rα (also called LIF-Rβ and gp130) by activating similar signaling pathways that those canonical pathways induced by leptin (JAK/STAT3, PI-3K/Akt and SHP2 [SH2 (Src homology 2) domain-containing tyrosine phosphatase 2]/MAPK) [97]. LIF is a key cytokine for maintaining self-renewal and pluripotency of embryonic stem cells. LIF is also an essential factor for embryo implantation and is involved in inflammation/angiogenesis inducing disparate biological responses in different cell types. Moreover, the angiogenic effects of LIF reported have been contradicting [86, 98–99]. LIF induces chemotaxis of mouse peritoneal macrophages in vitro and infiltration of macrophages in CNS lesions is delayed in LIF knock-out mice [100]. Leptin exerts differential effects on LIF regulation in diverse cell types. Leptin also induced opposing effects by increasing or repressing the secretion of LIF in monocytes and T cells, respectively. These results suggest leptin-induced LIF may modulate macrophage function [101]. In addition, leptin treatment to endometrial cancer cells (Ishikawa cells) increased the levels of phosphorylated STAT3, LIFR, and LIF. Moreover, treatment of Ishikawa cells with IL-1β also resulted in elevated levels of LIFR [102]. Furthermore, leptin induction of LIF/LIFR in human cancer endometrial cells (An3Ca, SK-UT2 and Ishikawa) involved JAK2, MAPK and mTOR (mammalian target of Rapamycin) activation [103].

IL-1 family belongs to pro-inflammatory/-angiogenesis cytokines, which is represented by two ligands: IL-1α, IL-1β, an antagonist: interleukin-1 receptor antagonist (IL-1Ra) and two receptors: IL-1R tI (type I receptor) and IL-1R tII (type II receptor) [104]. IL-1 plays a key role in the onset and development of the host reaction to invasion, being an important factor in the initiation of the inflammatory response and immune functions. IL-1 system is overexpressed in cancer cells and inflammatory cells invading tumor tissues. IL-1 is abundant at tumor sites, where it may affect carcinogenesis, tumor growth and invasiveness, and the patterns of tumor-host interactions and tumor angiogenesis [105]. Furthermore, IL-1 family, Notch and leptin crosstalk represents a major link among obesity, inflammation, angiogenesis and breast cancer progression [71, 106–108].

The IL-1 family and leptin are associated in several pathological situations, i.e., cachexia, cancer, reumathoid arthritis, and endogenous Cushing's syndrome [106, 109–112], suggesting an interaction between them. In endometrial cancer cells, leptin induces the expression of IL-1 system [103]. Kinase inhibition studies showed that leptin increase of IL-1β levels in primary-EEC was mediated by JAK2/STAT3, PI-3K/AKT1 and mTOR signaling pathways (see Fig 2). In contrast, in benign immortalized HES and cancer-EEC, leptin-mediated increase in IL-1R tI involved the activation of JAK2/STAT3, MAPK/ERK1/2 and mTOR but PI-3K/AKT1 phosphorylation did not show a clear pattern. Notably, the highest levels of IL-1 R tI induced by leptin were seen in SK-UT2 cancer cells [103]. In MCF-7 xenograft model of breast cancer, MCF-7 cells produce a high level of IL-1α, which in turn induces leptin expression in stromal cells or in infiltrating immune cells recruited in the tumor microenvironnement [109]. We have recently reported that leptin increased the protein and mRNA levels of all components of the IL-1 system in breast cancer cells [113]. Leptin-induced canonical and non-canonical signaling pathways were all associated with IL-1 up-regulation. Furthermore, as it was shown for Notch, VEGF and VEGFR2, leptin upregulation of IL-1α promoter also involved the activation of SP1 and NF-κB transcription factors. Interestingly, blockade of IL-1R tI inhibited leptin-induced VEGF/VEGFR-2 expression. These data suggest that leptin pro-angiogenic signature is related to IL-1 [71]. Moreover, inhibition of IL-1 signaling by specific anti IL-1R tI antibodies also abrogated leptin-induced Notch, Hey2 and Survivin [71]. Then, a complex crosstalk between leptin and pro-angiogenic, inflammatory and mitogenic factors occurs in breast cancer (See Fig 2).

6.3. Leptin impact on VEGF/VEGFR-2 signaling

Signals triggered by VEGF (a major angiogenic factor) binding to its receptor, VEGFR-2 is required for normal and pathological proliferation, migration and differentiation of endothelial cells [114–115]. While VEGF and VEGFR-2 are not oncogenes, their expression is enhanced by several signals triggered in angiogenic tumors including breast cancer [114–115]. VEGFR-2, receptor type 2 (KDR or flk-1) is generally recognized to have a principal role in mediating VEGF-induced responses, and is considered as the earliest marker for endothelial cell development (Review see [89]). Moreover, VEGFR-2 directly regulates tumor angiogenesis [89, 115–116]. In addition to its angiogenic actions in endothelial cells the VEGF/VEGFR-2 paracrine-autocrine loop functions as an important survival mechanism in cancer cells [89].

Recently, leptin was reported to upregulate VEGF-A and VEGFR-2 in cancer cells [103, 117–118]. Leptin increased VEGFR-2 expression in endometrial [103] and breast cancer cells in vitro [69] and in vivo [117–118]. Interestingly, leptin upregulation of VEGF-A/VEGFR-2 was partially mediated by IL-1 system [113]. A comprehensive mechanism for leptin regulation of VEGF in breast cancer cells through activation of several canonical and non-canonical signaling pathways and NFκB and SP1 was recently reported [119] (see Fig. 2). Moreover, leptin induced rapid phosphorylation of VEGFR-2 (Y^{1175}) in absence of VEGF in endothelial cells [120]. These leptin effects positively impacted on endothelial cell proliferation, survival and migration. Leptin-VEGFR-2 actions in endothelial cells involved a crosstalk between p38 kinase/Akt1 and COX-2 [120]. We have also found leptin induces pY^{951} VEGFR-2 in 4T1 mouse breast cancer cells. However, the biological significance of this finding needs further investigation.

Leptin is a known mitogenic, inflammatory and angiogenic factor for many tissues [84] and increases VEGF/VEGFR-2 expression in cancer cells [103, 117–118]. Therefore, leptin from corporal, mammary adipose or tumor tissues could affect cancer cells through autocrine and paracrine actions. Findings from our laboratory suggest that mouse (4T1, ER +) [117] and human breast cancer cells (MCF-7, ER+ and MDA-MB-231, ER-) express VEGFR-2 in vitro and in vivo [118]. Leptin increased the proliferation of 4T1 cells, but treatment with anti-VEGFR-2 antibody resulted in a further increase in leptin-induced VEGF concentrations in the medium. This suggests a feedback loop between VEGF expression and VEGFR-2 linked to cancer cell survival/proliferation that was later assessed by Aesoy et al in MCF-7 cells [121]. Remarkably, in mice hosting breast tumors either derived from 4T1 [117] or MCF-7 and MDA-MB231 cells [118], the inhibition of leptin signaling mediated an impressive reduction of tumor growth that paralleled a significant reduction of VEGF and VEGFR-2 levels [117–118]. However, the effects of inhibition of leptin signaling in these mouse models were more marked in MCF-7 ER+ cells [118]. We have also found that leptin activated NFκB, Sp1 and HIF-1α increasing the expression of Notch [71, 119] and VEGF mRNA and protein [119] in breast cancer cells under normoxic conditions. Furthermore, leptin-mediated activation of NFκB increased VEGFR-2 expression. Additionally, leptin increased promoter activities of VEGFR-2-Luc transfected-cells through several signaling pathways. Interestingly, the effects of leptin on VEGFR-2 were abrogated by a γ-secretase inhibitor. These data imply that a crosstalk between Notch and leptin is required for regulation of VEGFR-2 expression in breast cancer. Indeed, we have shown that the Notch, IL-1 and leptin crosstalk outcome (NILCO) is essential for leptin regulation of VEGF/VEGFR-2 in breast cancer [71]. Moreover, VEGFR-2 transcription and expression were heavily dependent upon VEGFR-2 gene methylation and histone acetylation that could be linked to leptin and Notch effects (Guo and Gonzalez-Perez, unpublished data). However, the molecular mechanisms of how leptin signaling regulates VEGFR-2 are largely unknown [122–124].

6.4. Leptin, macrophages, tumor stroma and breast cancer progression

It has long been recognized that once a critical mass of tumor cells is achieved, then the cancer cells initiate a colossal transformation of the microenvironment by secreting several factors. Many of these factors trigger angiogenesis needed for the tumor maintenance and growth. Accumulating data suggest that angiogenesis is controlled by opposing actions of an increasing number of pro- and anti-angiogenic factors. It is known that tumor stroma (noncancerous cells; that is, fibroblasts, immune and endothelial cells) could play a role in promoting tumor growth. Interestingly, data from SCID mice hosting human breast cancer cell xenografts suggested that stroma from MCF-7 breast cancer had higher levels of mouse VEGF than those from MDA-MB-231. These data were in contrast to the higher levels of human VEGF secreted by MDA-MB-231 in comparison with MCF-7 breast cancer cells. Thus, in addition to secrete VEGF the cancer cells (ie., MCF-7) can secrete factors may further promote angiogenesis via mouse tumor-stroma, consequently, contributing to tumor growth [118].

Recent evidence supports important roles for macrophages and adipocytes in breast cancer progression. Macrophage density (tumor associated macrophages, TAM) in breast cancer is specifically associated with poor tumor prognosis. These immune cells express diverse receptors that enable an active crosstalk with cancer and adipose cells [i.e., Ob-R, TLR4 (toll-like receptor 4, a lipopolysaccharide receptor also activated by fatty acids); CCR2 [(inflammatory chemokine (CCL2)/ chemoattractant protein -1 (MCP1) receptor] and ER] [125–128]. Macrophages respond to leptin stimulation by secreting pro-inflammatory and pro-angiogenic cytokines, i.e. IL-1, TNFα, IL-1, IL-6, IL-12 and nitric oxide, and thus may modulate macrophage phenotype: M1 (Th1 immunity-proinflammatory/cytotoxic) and M2 (suppress Th1/anti-inflammation/pro-angiogenesis) activation profiles [129]. Polarized M1 macrophages also colonize adipose tissue, particularly in obese individuals. These findings reinforce the idea that obesity is a state of chronic inflammation [130–131].

IL-1 secreted by macrophages, cancer and adipose cells all contribute to trigger signaling pathways that are shared by the TLR4 pathway [132]. Remarkably, in endometrial and breast cancer cells leptin-induced IL-1, which could further impact angiogenesis through intracellular and extracellular loops [102–103, 113]. Leptin and IL-1 synergic actions can activate NFκB that increase VEGF through an intracellular loop. It would appear that leptin-induced levels of IL-1 could enhance the synthesis of macrophage MCP-1 by breast cancer cells via an extracellular loop leading to the induction of angiogenesis upon the rise of recruitment and activation of infiltrating TAM. These actions would provide a link between pro-inflammatory and pro-angiogenic actions of leptin, IL-1, VEGF and macrophages in breast cancer progression.

Moreover, leptin can increase aromatase expression and ERα activation in macrophages, further contributing to macrophage recruitment [133]. After menopause, when the ovaries cease to produce estrogen, adipose tissue is the main source of estrogen. Moreover, breast adipose tissue may have enlarged or modified composition in postmenopausal-obese women, thus, locally providing higher amounts of estrogen, pro-inflammatory cytokines and adipokines such as leptin. Interestingly, it is known that leptin upregulates Ob-R receptor in tumor cells [134], however, we have found leptin downregulates Ob-R in TAM from murine breast cancer [Torroella-Kouri M, unpublished]. These data suggest that regulation of the leptin system may be different in macrophages and tumor cells. The biologic significance of these findings requests further investigation.

STAT3 constitutively activated in TAM is associated with the expression of several tumor-promoting genes [135]. Leptin signaling may also induce STAT3-mediated VEGF expression in macrophages [136]. TAM show activated oncogenic signaling pathways such

as Notch and Wnt-β–catenin [137–138]. Probably, these signaling pathways are related to the TAM interplay with CSC. Indeed, glioma CSC induced M2-macrophages. In addition, macrophages induced tumorigenicity, self-renewal capacities and drug resistance in colon and lung CSC [139]. Furthermore, TAM regulates in a TGF-β dependent manner the epithelial-mesenchymal transition (EMT), which is associated with increased aggressiveness, invasive and metastatic potential of basal-like phenotype of tumor cells [140].

All the above data suggest that unexplored mechanisms are involved in an active crosstalk between macrophages-adipose and breast cancer cells. Investigations aimed to clarify these poorly understood relationships could lead to the discovery of novel ways to prevent and treat breast cancer.

7. Crosstalk between leptin signaling and oncogenic pathways in breast cancer

Leptin is a pleitropic adipocytokine that exerts mitogenic, pro-inflammatory and pro-angiogenic effects promoting anchorage, proliferation of breast cancer cells, microvessel and hematopoiesis and increases the levels of several factors including cell cycle regulators [117–118, 141]. As discussed before, leptin induces several canonical and non-canonical signaling pathways. These signals are essential for leptin to exert its biological effects in food intake, energy balance, and adiposity as well as in the immune and endocrine systems [142–143]. Many of the effects of leptin are attributable to effects in the central nervous system, particularly in the hypothalamus, a site of high Ob-Rb mRNA expression. Potentially important roles for membrane bound short-form receptors include the endocytosis and transport of leptin across the blood-brain barrier. Alternative splicing and proteolytic cleavage events also produce a circulating extracellular domain of Ob-R, which may affect the stability and/or availability of circulating leptin [32]. High levels of leptin - found in blood of obese individuals- fail to suppress feeding and decrease body weight/adiposity. This failure to prevent or mitigate obesity suggests a relative resistance to the catabolic effects of leptin action in obesity. Proposed mechanisms of leptin resistance include alterations in the transport of leptin across the blood-brain-barrier, alterations in cellular Ob-Rb signaling, perturbations in developmental programming, and others [144]. However, high levels of circulating leptin could over regulate signaling and expression of active Ob-R in peripheral tissues. These phenomena lead to the deregulation of leptin signaling, which contributes to breast cancer progression through its crosstalk with multiple oncogenic pathways (Fig 3).

7.1. Developmental signaling pathways

7.1.1 Wnt signaling pathway—Wnt signaling is a developmental signaling pathway that plays a critical role in regulating the normal development of the mammary gland. Deregulation of Wnt signaling is critical for breast cancer [145–146]. Fenton et al reported that genes regulating the Wnt/β-catenin-mediated pathway including Mdm2, Pik3r1, and Rb1 were upregulated by leptin inducing the survival and proliferation of colon epithelial cells possessing an Apc mutation but not normal cells [147]. These leptin effects were linked to IGF-I metabolism as in colon epithelial APC-mutated cells IGFBP-6 and IGF-1expression was upregulated, while IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5, and nephroblastoma overexpressed gene-protein (NOV) expression was downregulated by leptin treatment [147]. Interestingly, *HES1*and *HES5*, which are the downstream transducers of Notch signaling, could be activated by NOV, thus reducing cell differentiation [91]. Therefore, inhibition of leptin signaling may be an effective strategy for therapy and prevention of colonic adenoma and cancer, which shows activation of Wnt signaling [148]. More recent data suggest that

leptin could also regulate the Wnt pathway contributing to breast cancer development. Indeed, leptin deficiency suppressed MMTV-Wnt-1 mammary tumor growth in obese mice, and abrogated tumor initiating cell survival [79]. We also found that leptin increased the levels of β-catenin and Notch in breast cancer cell cultures. The addition of Wnt-1 agonist to these cells also affected Notch levels. Likewise, the inhibition of leptin signaling decreased both Wnt and Notch expression in mammary tumors induced by DMBA in diet-induced-obesity mice (DIO) [149].

7.1.2. Notch signaling pathway—Notch is a family of mammalian transmembrane proteins that function as receptors for membrane bound ligands. There are four mammalian Notch genes, Notch1–Notch4, and five ligands, Jagged1, Jagged2 (homologs of Drosophila Serrate-like proteins), Delta-like 1 (DLL1), DLL3, and DLL4. Notch receptors have an extracellular domain made up of multiple epidermal growth factor domains (EGF), but the Notch intracellular domain is made up of many domain types [150]. The Notch proteins have been proven to affect diverse cell programs including angiogenesis, proliferation, differentiation, and apoptosis [72, 151]. Consequently, Notch influences organogenesis and morphogenesis [74]. The activation of a Notch receptor is triggered by ligands expressed on adjacent Jagged and Delta cells. In an earlier report, it was shown that leptin may regulate Jagged1 and Notch4 in human cord blood $CD34^+$ cells and early differentiated human endothelial cells (HUVEC) possibly promoting cell differentiation [152]. We recently observed that leptin was able to upregulate Notch (ligands, receptors and targets) in HUVEC cells [153]. Moreover, leptin activated Notch signaling pathway (Notch1,3,4 NICD and CSL) in mouse (4T1, EMT6 and MMT) and human breast cancer cell lines (MCF-7 and MDA-MB-231) under normoxic conditions [71–72]. Leptin was demonstrated to increase the expression of both Notch receptors and ligands [71–72]. In these cells, leptin also up-regulated Notch-target genes, Hey2 and Survivin [89, 107]. Leptin-induced non-canonical signaling pathways (PKC, p38 and JNK) differentially impacted on CSL promoter activity and on the expression of IL-1 system [108]. Interestingly, leptin upregulatory effects on pro-angiogenic factors IL-1, VEGF/VEGFR-2, Notch, as well as OB-R were significantly abrogated by a γ-secretase inhibitor, DAPT as well as siRNA against CSL [71].

7.2. Growth factors

7.2.1. HER/ErbB—HER/ErbB (HER1/EGFR, HER3 and HER4) receptor tyrosine kinases bind several ligands (epidermal EGF; amphiregulin; heregulin or *neu*-mouse and TGF-α). HER2/*neu* (HER2 in humans and *neu* in mice) is an orphan receptor without a known ligand. The HER2/*neu* proto-oncogene (also known as *c-erbB-2*) encodes a 185 kDa transmembrane glycoprotein receptor known as HER2/*neu* or $p185^{HER2}$ that has partial homology with EGFR and shares with that receptor intrinsic tyrosine kinase activity. It consists of three domains: a cysteine-rich extracellular domain, a transmembrane domain, and a short cytoplasmic domain. The HER2/*neu* gene has been implicated in cancer with special emphasis in breast cancer [154–155]. Amplification of HER2 occurs in 20%–25% of breast cancers and is also associated with an aggressive tumor phenotype and poor prognosis [156].

Importantly, HER2/*neu* could be transactivated by leptin/Ob-R signaling [92]. Furthermore, Ray et al [157] showed that leptin-induced proliferation of ER-positive (T47-D and MDA-MB-361) and ER-negative (MDA-MB-231 and SK-BR-3) breast cancer cell lines was HER2/*neu* dependant. Soma et al [158] further observed that leptin induced rapid-tyrosine phosphorylation of HER2 in SK-BR-3 cells that was partially reduced by an EGFR inhibitor, AG1478, or a JAK inhibitor, AG490. In these cells, leptin also induced pERK 1/2, which was mostly abrogated by a HER2 tyrosine kinase inhibitor, AG825. Therefore, leptin can transactivate HER2 through both EGFR and JAK 2 activation. In line with these

findings, Ob-R and HER2 were also observed to physically interact and be coexpressed in several breast cancer cell lines; further suggesting leptin may contribute to enhanced HER2 activity [159]. Therefore, above emerging evidence suggests the existence of crosstalk between HER2/*neu* and Ob-R signaling pathways in breast cancer.

7.2.2. IGF signaling—Obesity correlated with high IGF-I [160] and leptin [161] levels in women without breast cancer. High expression levels of leptin [162] and IGF-I [163] were also positively associated with increased breast cancer risk. Both leptin and IGF-I are components of the metabolic system [164–165] and their levels are influenced by body mass index (BMI) [162, 166]. In addition, leptin can be regulated by other obesity-related stimuli such as insulin, estrogens, and hypoxic conditions [167]. In contrast, inconsistent data on leptin-induced regulation of IGF-I have been shown [168–169]. Interestingly, a bidirectional crosstalk between leptin and IGF-I signaling through EGFR signals were related to the invasion and migration of breast cancer cells *in vitro* (i.e., ER+, MCF-7 and TNBC, MDA-MB-231 and MDA-MB-468) [169]. This study further suggested that phosphorylation of Ob-R and IGF-IR subsequently activated downstream signaling molecules Akt, ERK, IRS-1, and IRS-2. EGF inhibitor erlotinib and dual tyrosine kinase inhibitor lapatinib inhibit leptin- and IGF-I-induced invasion and migration of breast cancer cells [169]. These data were later corroborated by using p- on Tyr1141, which is required for Ob-R to bind to STAT3 and subsequently activation by JAK2 [168]. However, these studies suggested leptin-IGF-I crosstalk is unidirectional, as leptin was not able to activate IGF-IR on Tyr 1131 or 1135/1136, which are critical for its mitogenic and transforming activity [168]. Conspicuously, the cell lines used in the study highly expressed HER2 (MCF-7, BT-474 and SKBR3), which may affect the crosstalk between leptin and IGF-I signaling. On the other hand, E2 is known to enhance both leptin/Ob-R and IGF-I/IGF-I receptor pathways. ERα actions on leptin/Ob-R pathway were suggested to be mediated by IGF-1 signaling pathway [170–171]. In summary, interactions between leptin/Ob-R and IGF-I/IGF-IR could contribute to the initiation and progression of breast cancer. Further research in this field is necessary to address these challenges.

7.3. Oncogenic kinases and Transcription factors

7.3. 1. PI-3K/Akt signaling pathway—PI-3K/Akt pathway plays a central role in a variety of cellular processes including cell growth, proliferation, motility, survival and angiogenesis in tumor cells including breast cancer [172–173]. The PI-3K/Akt pathway is also instrumental in EMT during carcinogenesis [174]. Moreover, many of the transforming events in breast cancer are a result of enhanced signaling of the PI3-K/Akt pathway [173, 175]. A number of studies have already established the central role of leptin-induced regulation of the PI-3K/Akt signaling pathway in several type of cancer [53, 56, 59, 176–182].

7.3.2. mTOR Signaling—The mammalian Target of Rapamycin (mTOR), a key protein kinase, controls signal transduction from various growth factors and upstream proteins to the level of mRNA translation and ribosome biogenesis [183–184]. mTOR has been intensely studied for over a decade as a central regulator of cell growth, proliferation, differentiation, autophagy, angiogenesis and survival [183, 185–186]. mTOR signaling has been reported to crosstalk with the leptin signaling pathway in several types of malignant cells [71, 103, 113, 187]. IGF-I induced phosporylation of Ob-R leucine could activate leptin expression via mTOR in adipocytes [93]. A recent review has provided an excellent overview of the detailed relationships between leptin and mTOR signaling [188].

mTOR is a ser/threo kinase that is often a downstream effector of PI-3K/Akt signaling pathway in breast and other types of cancer cells. Leptin-induced IL-1/IL-1Ra and VEGF in

endometrial cancer cells involved PI-3K/Akt phosphorylation. Activated PI-3K/Akt was in turn strongly linked to mTOR-mediated activation of its downstream targets, S6K1 and 4BEP-1 [113]. However, MAPK pathway was identified as the preferential upstream regulator of mTOR in the induction of inflammatory/pro-angiogenic molecules in endometrial cancer cells [103]. mTOR can also phosphorylate Akt [189]. Aberrant activation of mTOR pathway was found in many types of cancer and thus may play a major role in breast cancer cell proliferation and resistance to anti-cancer drugs [190–192].

7.3.3. NF-κB signaling pathway—The family of NF-κB transcription factors is involved in the expression of key genes for innate and adaptive immunity, cell proliferation and survival, and lymphoid organ development. NF-κB, linked to tumor angiogenesis [119], is activated in a variety of cancers, including breast cancer [193–194]. NF-κB family, RelA (p65), RelB, c-Rel, p105/p50 and p100/p52 are evolutionarily conserved molecules that form hetero- or homodimers. The p65/p50 heterodimer, the most abundant form of NF-κB is regulated by the so-called canonical pathway [193, 195]. NF-κB is activated by many divergent stimuli, including pro-inflammatory cytokines such as interleukin-1β (IL-1β), epidermal growth factor (EGF), T- and B-cell mitogens, bacteria and lipopolysaccharides (LPS), viruses, viral proteins, double-stranded RNA, and physical and chemical stressors [194, 196]. Cellular stressors such as ionizing radiation and chemotherapeutic agents also activate NF-κB [197].

It is not surprising that NF-κB has been found to be activated by leptin in many tumors, such as glioma [198–199], chondrosarcoma [96], colon cancer [200–201], prostate cancer [95], as well as breast cancer [113, 119]. Leptin enhances migration, invasion and proliferation of malignant cells, possibly through activation of NF-κB [96, 199–200]. Our previously work strongly suggests that leptin regulates pro-angiogenic, pro-inflammatory and anti-apoptotic actions in breast cancer cells through activation of NF-κB [113, 117–119].

7.3.4. STAT3 signaling pathway—Signal transducer and activator of transcription (STAT3) has been placed at a central node in the development, progression, and maintenance of many human tumors [202–203]. STAT3 modulates the transcription of a variety of genes involved in the regulation of critical functions, including cell differentiation, proliferation, apoptosis, angiogenesis, metastasis, and immune responses. STAT3 protein is located in the cytoplasm in an inactive form and is activated via phosphorylation (pSTAT3) by the Janus tyrosine kinases (JAKs). The active form of STAT3 dimerizes and quickly translocates to the nucleus. For many cancers, elevated levels of activated STAT3 have been associated with poor prognosis, suggesting that STAT3 could be an attractive molecular target for the development of novel cancer therapeutics [204–205].

The STAT3 pathway is critical for mediating leptin actions on food intake, glucose metabolism, and weight gain, but it does not influence fertility [206]. A number of studies have also demonstrated that the STAT3 pathway is involved in leptin actions in cell migration [182, 207], proliferation [208–213], anti-apoptosis [179, 214–215] of malignant cells. STAT3 as a transcriptional factor regulates genes including cyclin D1[208, 210], cyclooxygenase (COX)-2 [180], VEGF [119, 216], leptin [216], human telomerase reverse transcriptase (hTERT) [217] and Survivin [218]. In addition, STAT3 could also directly or indirectly regulate IL-1, Notch [71, 113], as well as cancer stem cell markers, CD44 and ALDH1 (Guo and Gonzalez-Perez, unpublished).

7.3.5. HIF signaling pathway—Hypoxia-inducible factors (HIFs), key molecules overexpressed in response to oxygen deficiency, are master regulators of genes involved in tissue reoxygenation [219]. HIFs facilitate cancer progression by promoting tumor neoangiogenesis, cell motility, and invasion [220]. HIF-1 is a heterodimer consisting of a

constitutively expressed HIF-1β subunit and an oxygen-regulated, unstable HIF-1α subunit. HIF-1 interactions with DNA are mediated through Hypoxia-Responsive Elements (HRE) [219]. Several studies have demonstrated that HIF-1 plays important roles in the development and progression of cancer or CSC through activation of various genes involved in angiogenesis, energy metabolism, vasomotor function, erythropoiesis, and cell survival [221–222].

Correlation between leptin and HIFs is bi-directional. HIFs can upregulate leptin signaling in several cell types. In trophoblast-derived BeWo cells exogenous HIF-1α markedly increased leptin-promoter luciferase-reporter activity [223]. Moreover, HIF-1α induced aberrant expression of leptin in ectopic endometriotic stromal cells, which may have a role in the etiology of endometriosis [224]. In endometrial cancer, the expression of HIF-1α was positively correlated with leptin ($P < 0.0001$, $r = 0.573$) and Ob-R ($P = 0.020$, $r = 0.299$) [225]. Furthermore, in human colorectal cancer HIF-1α and leptin signaling were also positively correlated [226]. A recent report employing reverse chromatin precipitation in MCF-7 breast cancer cells further demonstrated that loading of HIF-1α on the proximal leptin promoter concurred with the recruitment of p300, the major HIF coactivator, suggesting that the HIF/p300 complex was involved in leptin transcription [227]. On the other hand, leptin could also upregulate HIF-1. We recently reported that leptin induced the increase of HIF-1α DNA binding activity to VEGF promoter [119]. Moreover, deletion analyses of VEGF promoter suggest that HRE binding sites are critical for leptin regulation of VEGF gene in mouse mammary tumor (MT) cells. Treatment of MT with HIF-1α shRNA completely abrogated leptin-mediated induction of VEGF protein, mRNA, promoter-luciferase activity and HIF-1α accumulation in MT nuclei [119]. These results suggest that leptin-induced VEGF transcription involves the enhanced ability of HIF-1α to bind HRE within the VEGF promoter in MT.

7.4. Other crosstalk signaling

7.4.1. ER signaling—Elevated lifetime estrogen exposure has been shown to be a major risk factor for cancer in hormone-dependent organs, particularly in breast and endometrium [228–229]. Estrogens are also important regulators of the development and progression of ER+ breast carcinoma [230–231]. ERs are known to regulate a huge number of genes affecting cancer proliferation and vascular function [232–233].

Earlier studies suggest that estrogen may influence leptin synthesis in a tissue- and cell type-specific fashion [234]. Jarde et al [199] examined the expression of leptin, Ob-R and ER in human primary breast cancer and adjacent non-cancerous tissue. Their findings suggest that Ob-R and ER are co-expressed in breast cancer indicating a possible interaction between leptin and estrogen systems to promote breast carcinogenesis [235]. Antiestrogens may stimulate the synthesis and release of leptin in the adipocytes. Moreover, tamoxifen therapy commonly used in ER+ breast cancer induces the levels of leptin. This effect of antiestrogens resembles that of the estrogen and consequently stimulation of leptin production can be added to the agonistic effects of some antiestrogens [236]. In addition, leptin can transactivate ER [237] and increase the expression of aromatase in MCF-7 cells [238], potentially contributing to the proliferation and progression of breast cancer. These leptin effects represent additional links between obesity and estrogen signaling that promotes the development of estrogen-responsive breast cancer.

7.4.2. Survivin—Survivin, a member of the inhibitor of apoptosis (IAP) family of proteins, regulates two essential cellular processes for cancer growth: inhibition of apoptosis and stimulation of cell proliferation. Survivin is highly expressed during fetal development and cancer development, but it is rarely found in normal healthy adult tissues [239–240].

Recently, Survivin, was identified as a novel target of Notch [239–240] and CD44 genes [241]. Notch directly activates at least one RPB-Jκ site in the Survivin promoter [242]. Activation of Notch resulted in direct transcriptional up-regulation of Survivin in ER–breast cancer cells [243–244]. This Notch-Survivin signaling axis may contribute to worse clinical outcome of basal-like breast cancer (TNBC) patients, and may open new prospects for individualized therapy of these recurrence-prone patients.

Leptin upregulated Survivin mRNA and protein expression in MCF-7 breast cancer cells [218, 245]. It seems leptin-induced pSTAT3 is involved in these effects as siRNA-STAT3 knockdown blocked leptin-induced upregulation of Survivin [218]. Moreover, leptin-induced transactivation of EGFR was also involved in leptin-mediated Notch1 and Survivin upregulation [73]. We recently reported that in breast cancer cells, leptin up-regulated Notch1-4/JAG1/Dll-4, Notch target genes: Hey2 and Survivin, together with IL-1 and VEGF/VEGFR-2 [71]. Blockade of Notch or IL-1R tI signaling inhibited leptin-induced Survivin as well as VEGF/VEGFR-2 expression. These data suggest leptin induction of Survivin is related to NILCO crosstalk [71].

7.4.3. Insulin and Adiponectin—Obesity and insulin resistance are risk factors for breast cancer and are associated with late-stage disease and poor prognosis. Thus, leptin actions may provide a biological mechanism by which obesity and insulin resistance are causally associated with breast cancer risk and poor prognosis [246]. Hyperinsulinemia could induce breast cancer progression through leptin-dependent mechanisms. In MDA-MB-231 cells, insulin up-regulates leptin by induction of Sp1- and HIF-1α mediated mechanisms. This process is partially regulated by the PI-3K and ERK1/2 pathways [247]. In contrast, adiponectin, the most abundant gene product in adipose tissue is a known anti-angiogenic and anti-breast cancer adipokine. Adiponectin levels are inversely correlated with body fat percentage in adults. This adipokine seems to have a protective role in the maintenance of insulin sensitivity and it is down-regulated by insulin [248]. Low serum adiponectin levels are significantly associated with an increased risk for breast cancer; tumors arising in women with low serum adiponectin levels are more likely to show a biologically aggressive phenotype [249]. Adiponectin and leptin have opposite roles in breast cancer cells and on their gene regulation. Adiponectin decreased leptin and Ob-Rt mRNA levels. In contrast, leptin significantly reduced the adiponectin receptor (AdipoR1) mRNA level. Similar contrasting results were found on the proliferation of MCF-7 cells (ER +). Moreover, microarray analyses showed that adiponectin and leptin have opposite effects on the expression of genes involved in cell cycle regulation [250].

8. Clinical Significance of leptin signaling in human breast cancer

8.1. Tumor marker and prognostic value of leptin signaling in breast cancer

In an earlier study, Ishikawa et al evaluated the expression of Ob-R, leptin, and clinicopathological features of breast cancer [52]. Protein expression of leptin and Ob-R was determined in 76 invasive ductal carcinomas and 32 samples of corresponding normal mammary glands. Distant metastasis was detected in 21 (34%) of 61 Ob-R+/leptin-overexpressed tumors, but was not detected in Ob-R−/non-leptin expressed tumors. (n=15, 100%; $P < 0.05$). In agreement with these data, leptin and Ob-R were found significantly overexpressed in primary (n=148) and metastatic breast cancer (n= 66) when compared to benign mammary lesions (n= 90) [167]. Moreover, high mRNA expression levels of leptin (99%) and leptin receptor (100%; Ob-Rb and ObRs) were found in breast cancer patients (n=322). Patients with elevated ObR-S expression had longer relapse-free survival ($P = 0.008$), whereas high ObR-L/ObR-S was associated with a shorter relapse-free survival ($P = 0.05$). These results indicate that leptin/Ob-R are biological markers of a more differentiated phenotype; and that ObR-S could be an independent prognostic factor [251]. Taken together,

these data suggest that leptin and Ob-R might be good makers of breast cancer and may have potential value for prognosis. In contrast, the analysis of a large cohort of breast cancer patients (n=517) showed positive cytoplasmic immunoreactivity for leptin (39%) and Ob-R (79%) proteins that correlated with a high Ki-67 labeling index (P=0.019). Although there were no statistical significance, the rate of a leptin expression was higher for TNBC (P=0.08), no p53 overexpression (P=0.08) and no bcl-2 expression (P=0.38). No significant difference in overall survival was noted between the leptin/Ob-R expressing and non-expressing breast cancer patients [252]. However, the authors did not differentiate breast cancer expressing specific Ob-R isoforms as the antibody used (M-18; Santa Cruz Biotech.Inc.) can detect both Ob-Rb and Ob-Ra. Nevertheless, to date it is unknown if leptin/Ob-R markers would have independent prognostic value.

8.2. Leptin/Ob-R signaling as a therapeutic drug target

Extracellular activation of Ob-R is only achieved upon leptin binding to its extracellular region. Moreover, leptin only binds to Ob-R. Additionally, this family of receptors is only capable to bind leptin or leptin-modified peptides. These data highlight the potential use of leptin antagonists to block leptin binding to Ob-R and thereby, abrogating Ob-R signals [34].

We have previously demonstrated that inhibition of leptin signaling resulted in decreased growth of mammary tumors derived from mouse and humans [117–118]. A pegylated leptin peptide receptor antagonist (PEG-LPrA2) markedly reduced tumor growth and the expression of VEGF-A/VEGFR-2 in orthotopic mouse models of syngeneic and human breast cancer xenografts responsive or unresponsive (MDA-MB-231 cells; TNBC) to estrogens [117–118]. Interestingly, LPrA2-induced effects were more pronounced in vivo than in vitro. These results suggested that paracrine actions in stromal, endothelial, and/or inflammatory cells that may impact the growth of breast cancer. The relevance of these observations was underscored by the use of an orthotopic-syngeneic 4T1 breast cancer cell-model [117]. The mice treated with PEG-LPrA2 had diminished expression of VEGF-A/VEGFR-2, Ob-R, leptin, IL-1R tI, PCNA and cyclin D1 [118]. PEG-LPrA2 was not toxic and did not change body weight (BW) or appetite of a large number of normal lean (male and female) CD-1 and BALB/c mice during a several months. Similar data were later reported by another group using the same orthotropic xenograft model, (MDA-MB-231) but with a different leptin antagonist (Allo-aca) [253]. Subcutaneous delivery of this antagonist significantly extended mouse survival time for 1–2 weeks. While Allo-aca did not show systemic toxicity when tested for toxicity effects in normal CD-1 mice, it induced 6–10% body weight increase [253]. We further hypothesized that inhibition of leptin signaling could also be used in the prevention of human breast cancer. This hypothesis was tested in a 7,12-dimethylbenz[a]anthracene (DMBA).-induced breast cancer mouse model using lean or diet induced obesity (DIO)-mice fed with high-fat diet (54% Kcal from fat) [254]. Mice were treated with either one or two PEG-LPrA2 dose/weekly (i.v.; 50 μl/0.1 mM) one week before DMBA challenge. Breast cancer was only detected in control DIO-mice (21%; 3 of 14) receiving saline injections. High levels of leptin-targeted molecules: OB-R, IL-1R tI, VEGF/VEGFR-2, anti-apoptotic protein B-cell lymphoma 2 (Bcl-2), HIF-1α, NFκB and Notch (receptors, ligand and targeted genes) were found in DMBA-breast cancer tissues. Remarkably, PEG-LPrA2 (1 or 2 doses) completely prevented DMBA-breast cancer in DIO-mice (0%; 0 of 14) and reduced aryl hydrocarbon receptor (AhR) and Notch expression in mammary glands. Body weight and food intake of lean and DIO-mice treated for 32 weeks with PEG-LPrA2 were not affected. However, two-dose treatment decreased BW in DIO-mice [254]. These data suggest that inhibition of leptin signaling may serve as a novel adjuvant for prevention and treatment of breast cancer. The alarming increase of obesity incidence in the Western countries underscores the importance of these findings [243–244].

9. Conclusion and overall perspectives

High leptin level is a hallmark of obesity which has been correlated to incidence of several cancers including breast cancer. Leptin is an adipocyte-derived cytokine co-expressed with Ob-R by breast cancer cells. Leptin/Ob-R expression pattern correlates with metastasis and lower survival rate of breast cancer patients. *In vitro* and *in vivo* studies clearly demonstrated a role of leptin in mammary tumor development. Furthermore, leptin signaling and its crosstalks with many signaling pathways play critical roles in breast cancer cell growth, migration, invasion, angiogenesis and metastasis (see Fig 3). Importantly, inhibition of leptin signaling could be relevant for breast cancer prevention, especially for obese populations showing higher levels of leptin and incidence of breast cancer.

Better understanding of expression regulation, redundant functions and intracellular signaling pathway targets, as well as the complex crosstalk of leptin/Ob-R with other oncogenic signals in breast cancer cells will be essential to ensure rational use of treatment and development of new combinatory therapeutic possibilities. There are still many gaps to fill in the field of leptin signaling in breast cancer, especially the molecular mechanisms of leptin signaling-mediated regulation of BCSC. Novel opportunities could emerge from the discovery of leptin crosstalk with inflammatory and angiogenic cytokines (i.e., NILCO) and their links to obesity-related cancers and BCSC.

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Glossary

4T1 cells	mouse mammary cancer cell line
Akt	protein kinase B
ALDH	aldehyde dehydrogenase
BALB/c	an albino, laboratory-bred strain of the house mouse
Bcl-2	B-cell lymphoma 2
Bcl-xl	B-cell lymphoma-extra large
BCSC	breast cancer stem cells
BMP	bone morphogenetic protein
CSC	cancer stem cell
CSL	CBF1/Su(H)/Lag-1
Cyclin D1	kinase and regulator of cell cycle D1
DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
DLL-1	Delta-like 1
DSL	Delta/Serrate/LAG-2
E2	17β-estradiol
EC	endothelial cells

EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transformation
EMT6, MMT	mouse mammary adenocarcinoma cell lines
ER	estrogen receptor
ERK 1/2	extracellular regulated kinase 1 and 2
HIF-1α	hypoxia regulated factor-1 α
HUVECs	human umbilical vein endothelial cells
IGF-1	insulin like growth factor-1
IL-1	interleukin-1
IL-1R tI	interleukin-1 type I receptor
JAK2	Janus kinase 2
MAPK	mitogen activated protein kinase
MCF-7	ER positive human breast cancer cell line
MDA-MB-231	ER negative human breast cancer cell line
mTOR	mammalian target of rapamycin
NF-κB	eukaryotic nuclear transcription factor kappa B
NICD	Notch intracellular domain
NILCO	Notch, IL-1 and leptin crosstalk outcome
LIF/LIFR	leukemia inhibitory factor and its receptor
Ob-R	leptin receptor
PEG-LPrA2	pegylated leptin peptide receptor antagonist 2
PI-3K	phosphoinositide 3-kinase
STAT3	signal transducer and activator of transcription 3
VEGF	Vascular endothelial growth factor
VEGFR-2	Vascular endothelial growth factor receptor 2 or KDR or Flk-1

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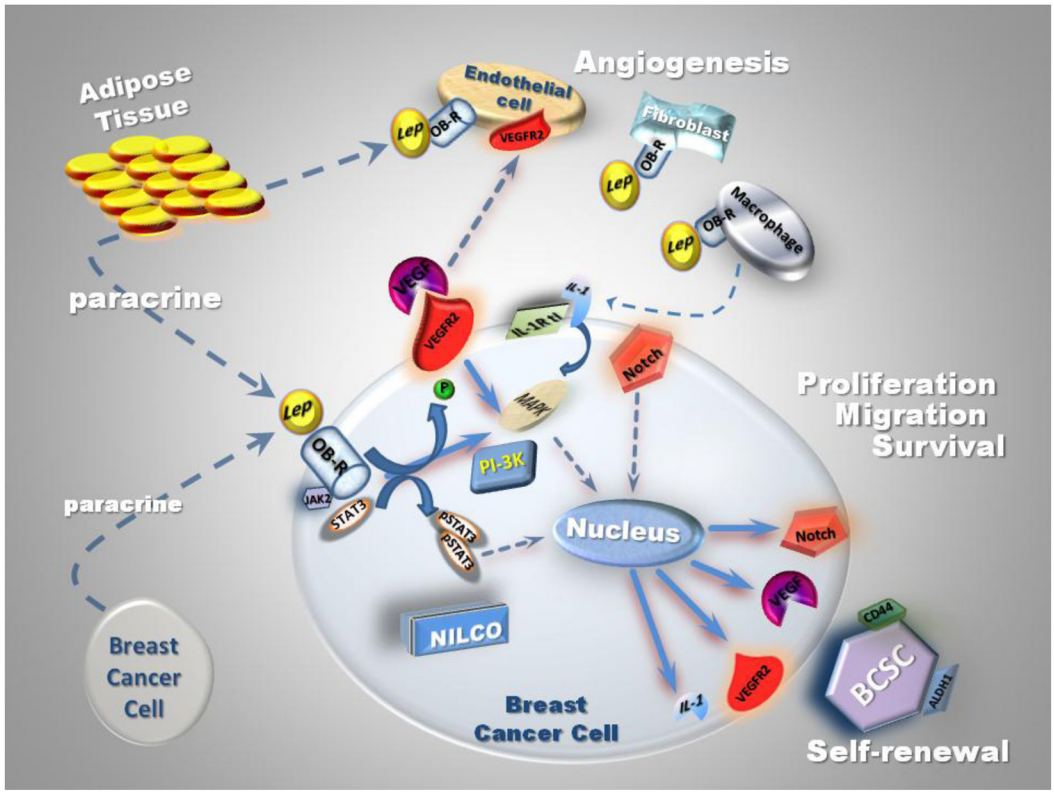

Fig. 1.

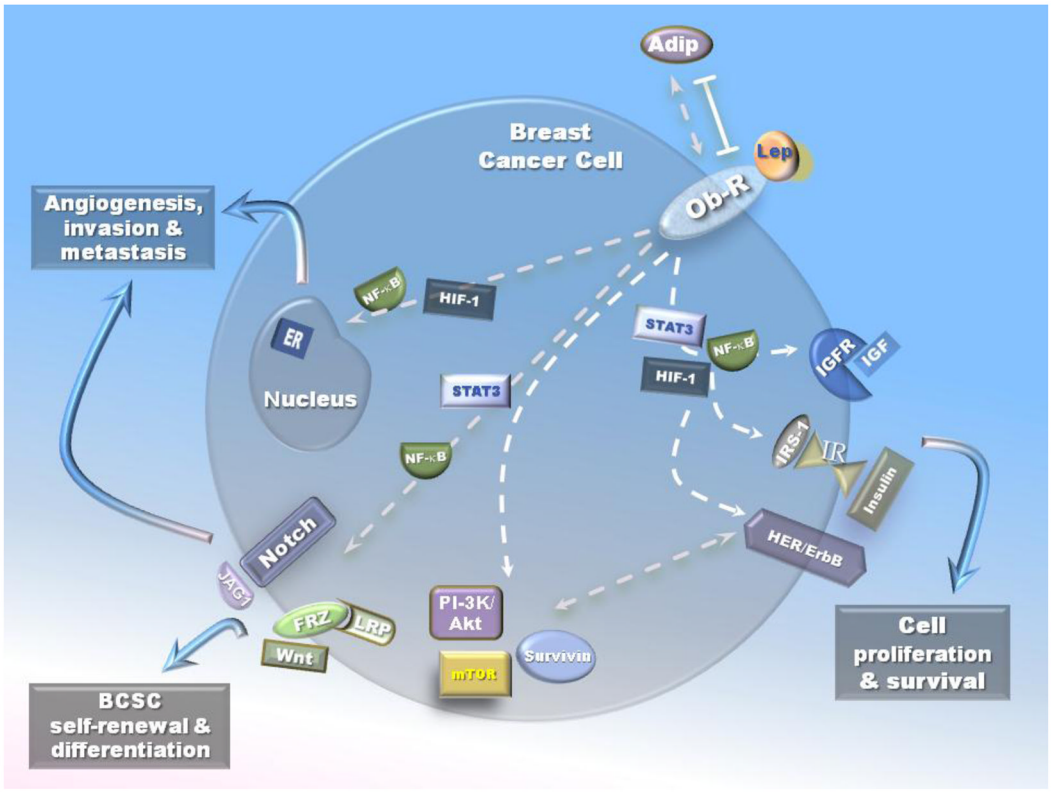

Fig. 2.

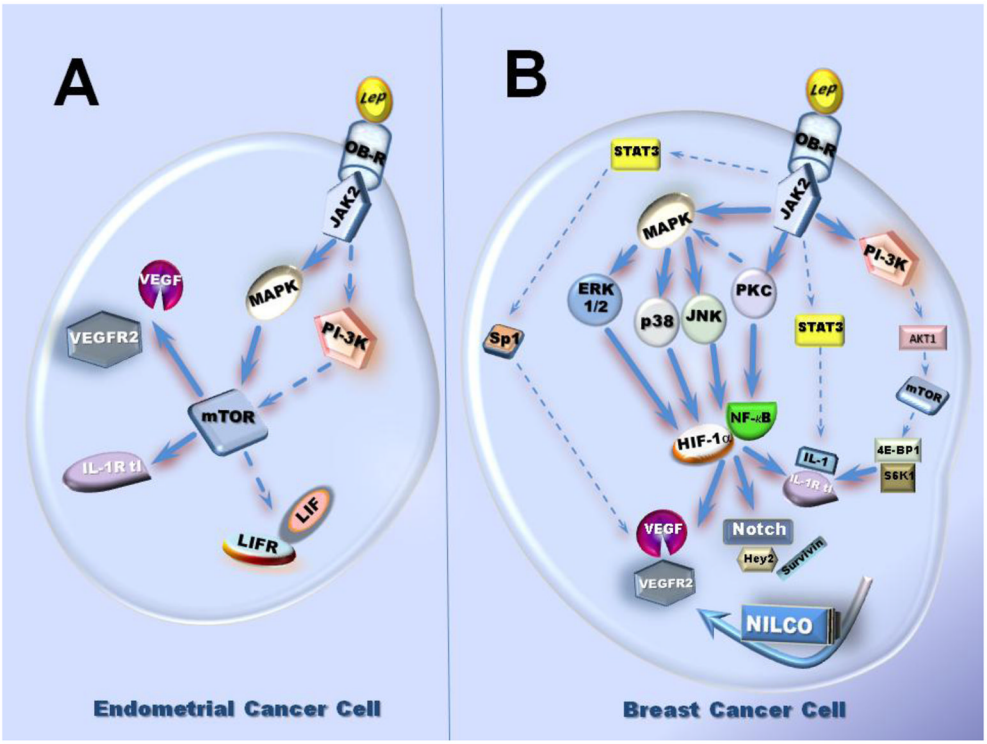

Fig. 3.

Table I

Leptin and Ob-R expression in human cancer tissues by immunohistochemical staining

Tissue	Subtype	Leptin	Ob-R	References
B-cell	Lymphoma	ND	39.8%	[177]
Bladder	Carcinoma	ND	↓↓	[51]
Brain	Glioma	55.2%	80.5%	[56]
Breast	Carcinoma	92%	83%	[52]
Colon	Carcinoma	73.5%	72.5%	[255–256]
Endometrioid	Adenocarcinomas	60%	31%	[257]
Esophagus	Carcinoma	ND	91%	[57]
Kidney	Carcinoma	↑↑	38.6%	[258]
Liver	Carcinnoma	60.3%	53%	[259–260]
Lung	Adenocarcinoma	69.0%	ND	[261]
Ovary	Carcinoma	89.5%	59.2%	[53]
Prostate	Cancer	96.7%	63.3%	[54]
Skin	Melanoma	90.9%	43.8%	[55]
Thyroid	Carcinoma	ND	80%	[59]
Thyroid	Carcinoma	37%	51%,	[58]

ND: Not determined; ↑↑, High serum leptin in cancer patients; ↓↓, low Ob-R expression