

Adipokines as endocrine, paracrine, and autocrine factors in breast cancer risk and progression

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Abstract

Adipokines (leptin, adiponectin, and hepatocyte growth factor (HGF)) secreted from adipose tissue have come to be recognized for their contribution to the mechanisms by which obesity and related metabolic disorders influence breast cancer risk. In this review, we discuss the direct and indirect effects of these protein factors on the biological and clinical aspects of breast cancer biology, and emphasize their distinctive modes of action through endocrine-, paracrine-, and autocrine-mediated pathways. The stimulatory effects of leptin on breast cancer growth were considered to occur primarily via activation of the estrogen receptor; however, new evidence suggests that leptin may be acting on downstream cell signaling pathways in both estrogen-dependent and -independent cell types. Another secretory adipokine, HGF, may act largely not only to promote tumor cell invasion, but also to enhance tumor growth indirectly by stimulating angiogenesis. In contrast, adiponectin, an endogenous insulin sensitizer, exerts a direct growth-inhibitory effect on tumor cells by downregulating cell proliferation and upregulating apoptosis, and also inhibits tumor-related angiogenesis.

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Introduction

A proportion of breast cancers require the presence of estrogens for their growth and progression to an invasive and metastatic phenotype. This hormone dependence derives from the capacity to respond to *endocrine* signaling in which the estrogen is secreted from its production sites in the ovaries or adipose tissue and carried via the blood to the target tumor cells where it interacts with the specific estrogen receptors (ER). In premenopausal women, the need for estrogens acting in an endocrine manner in order to sustain tumor growth is illustrated by the regression of ER-positive breast cancers which occurs in response to treatment by bilateral oophorectomy (Jensen & Jordan 2003).

After the menopause, aromatization of the C19 steroid androstenedione in adipose tissue is the source of the circulating estrone and estradiol, the endocrine activities of which are considered to be major contributors to obesity-related postmenopausal breast cancer risk and progression (Key *et al.* 2003). However, there is an alternative mode of estrogen

action which applies to this situation: the dense layer of adipose tissue fibroblasts which forms the capsule surrounding a premalignant or cancerous breast lesion may possess particularly high levels of aromatase activity, and in consequence enhance estrogen biosynthesis, so that the local conditions favor estrogenic stimulation of breast cancer cells by a paracrine mechanism in which the steroid diffuses from its site of origin to interact with the ER of the nearby tumor cells (Bulun *et al.* 1994). In addition, some breast cancers themselves possess aromatase activity, and the production of estrogens by ER-positive breast cancers presents the opportunity for growth stimulation by an *autocrine* mechanism (reviewed by de Jong *et al.* 2001, Sasano *et al.* 2006).

There is an extensive literature in which the relationships between hormones and growth factors and breast cancer risk, stage at diagnosis, and prognosis have been investigated using assays of their concentrations in plasma or serum samples. Examples of the success of this approach are the

Table 1 Production of adipokines by the individual components of adipose tissue

Adipokine	Principal synthesizing cell type	Other cell sources
Leptin	Preadipocytes and adipocytes Simons <i>et al.</i> (2005)	Fibroblasts and keratinocytes Murad <i>et al.</i> (2003)
Adiponectin	Mature adipocytes (Simons <i>et al.</i> 2005, Komer <i>et al.</i> 2005)	None
Tumor necrosis factor- α	Macrophages Weisberg <i>et al.</i> (2003)	Preadipocytes and adipocytes Meng <i>et al.</i> (2001) and Wang <i>et al.</i> (2005)
Interleukin-6	Macrophages (Weisberg <i>et al.</i> 2003)	Stromal cells Crichton <i>et al.</i> (1996) adipocytes Wang <i>et al.</i> (2005)
Hepatic growth factor	Stromal cells Rehman <i>et al.</i> (2004)	Adipocytes Rahimi <i>et al.</i> (1994)
Vascular endothelial growth factor + HB-EGF	Stromal cells Rehman <i>et al.</i> (2004) Adipocytes Matsumoto <i>et al.</i> (2002)	Adipocytes Mick <i>et al.</i> (2002) Vascular smooth muscle cells Mick <i>et al.</i> (2002)

+ , vascular endothelial growth factor release from stromal cells and adipocytes is of similar degree (Mick *et al.* 2002). HB-EGF, heparin-binding epidermal growth factor-like growth factor.

demonstration of a positive association between high plasma estrogens and total and biologically active estradiol concentrations and postmenopausal breast cancer risk (Toniolo *et al.* 1995), and a positive correlation between fasting serum insulin levels and breast cancer risk (Del Giudice *et al.* 1998, Hirose *et al.* 2003, Lawlor *et al.* 2004) and prognosis (Goodwin *et al.* 2002).

More recently, there has been considerable interest in the potential role of the adipokines, a group of proteins synthesized in adipose tissue, in the development of cancers for which obesity has been established as a risk factor: these include the endocrine-related cancers of the breast (Harvie *et al.* 2003, Stephenson & Rose 2003, Rose *et al.* 2004) and endometrium (Petridou *et al.* 2002, 2003, Cymbaluk *et al.* 2007). The adipose tissue cells that produce the adipokines include the fat cells (adipocytes) and the stromal cells (fibroblasts) which have the potential for differentiation to mature adipocytes, and the macrophages that infiltrate the adipose cell mass, and do so to an increased degree in obesity (Table 1). These macrophages are an important source of tumor necrosis factor- α (TNF- α) and responsible for the significant increase in local TNF- α synthesis which occurs in

obese individuals (Weisberg *et al.* 2003). Fain *et al.* (2004) found that with the exception of leptin and adiponectin over 90% of the adipokines released by adipose tissue cultured *in vitro* were produced by nonfat cells. In addition to exerting local biological effects, adipokines circulate in the plasma, where the concentrations of most, including leptin, TNF- α , interleukin-6 (IL-6), hepatocyte growth factor (HGF), and heparin-binding epidermal growth factor-like growth factor (HB-EGF) are positively correlated with the body mass index (BMI); one exception is adiponectin, the relationship of which with the BMI is a negative one (reviewed by Rose *et al.* 2004).

Two adipokines in particular, leptin and adiponectin, have come to be recognized for their influence on breast cancer risk and tumor biology. Their physiological and pathological relationships are largely in opposition to each other, as are their biological effects on breast cancer cells (Table 2). The third adipokine, HGF or ‘scatter factor’, may have a positive effect on tumor growth as a result of its angiogenic properties, but it is recognized primarily for its capacity to promote cell invasion.

In this review, we will consider those adipokines, leptin, adiponectin, and HGF, which are currently

Table 2 Comparison of leptin and adiponectin: pathophysiological relationships and effects on breast cancer biology

	Leptin	Adiponectin
Obesity	Plasma levels elevated	Plasma levels suppressed
Insulin activity	Reduced sensitivity	Reduced resistance
Type 2 diabetes	Promotes development	Reduces risk
Metabolic syndrome	Hyperleptinemia	Hypo adiponectinemia
Breast cancer risk	Plasma levels equivocal relationship	Increased risk with low plasma levels
Breast cancer cell growth	Mitogenic	Antimitogenic
Tumor cell apoptosis	Reduced	Enhanced
Tumor angiogenesis	Stimulated	Inhibited

recognized as contributing to breast cancer cell biology, notably with effects on tumor cell mitosis and apoptosis, invasive capacity and metastasis, and the associated process of tumor-mediated angiogenesis. Our emphasis will be on the relative significance of their roles as endocrine, paracrine, and autocrine agents with the postulate that only by studying the individual adipokines in each of these separate roles can a full understanding be achieved of their contribution to the biological and clinical aspects of breast cancer.

Adipokines as circulating hormones in breast cancer

Leptin

The identification of leptin as a product of the obese (*ob*) gene (MacDougald *et al.* 1995) and the demonstration that its circulating concentrations correlate positively with the BMI were quickly followed by attempts to relate the levels of leptin in serum or plasma to breast cancer risk. These have produced conflicting results: at present, there are three published reports of case–control studies which found that relatively high serum leptin concentrations were associated with an increased risk of breast cancer (Tessitore *et al.* 2000, Han *et al.* 2005, Chen *et al.* 2006a), but another six of straightforward case–control design (Petridou *et al.* 2000, Ozet *et al.* 2001, Coskun *et al.* 2003, Sauter *et al.* 2004, Miyoshi *et al.* 2006, Woo *et al.* 2006), and a seventh with a case–control nested within a prospective study (Stattin *et al.* 2004), all gave negative results (Table 3).

The study by Tessitore *et al.* (2000) involved only 23 patients and 103 controls and found that the plasma leptin concentrations were elevated in breast cancer and correlated positively with the plasma insulin, but not the BMI. There were corresponding elevations in leptin mRNA expression in adipose tissue. Han *et al.* (2005) assayed the serum leptin concentrations in 90 breast cancer patients, 40 women with benign breast disease, and 103 healthy controls. The menopausal status of these Chinese women was not described, but the mean values and the standard deviations of their ages indicate that they included both premenopausal and postmenopausal women in all three diagnostic categories. In addition to the hyperleptinemia, the breast cancer patients had elevated serum insulin and triglyceride levels and reductions in the serum high-density lipoprotein cholesterol, all features of the metabolic syndrome.

A carefully detailed study by Chen *et al.* (2006a) involved 100 newly diagnosed Taiwanese premenopausal and postmenopausal breast cancer patients who

were studied after surgery, but before the institution of any form of adjuvant therapy, and 100 healthy control women. The serum leptin concentrations were not related to menopausal status and were significantly higher in the breast cancer group. It is of interest that hyperleptinemia occurred in these patients in the absence of obesity, which is an uncommon condition in the Taiwanese female population.

Stattin *et al.* (2004) studied a relatively large number of breast cancer cases and controls, all of whom were matched for age (Table 3). Their experimental design was unique in that it was the only one of the nine studies in which a case–control comparison was made which was nested in a large prospective study. In consequence, the plasma samples were collected prior to the diagnosis of breast cancer, at which point all of the women were beyond the menopause. The observed lack of an association between the plasma leptin levels and breast cancer risk was unaffected by adjustment for the BMI or plasma insulin.

Miyoshi *et al.* (2006) reported that in their case–control study, there was no association between the serum leptin levels and breast cancer risk, but that there was a correlation between the serum leptin levels and intratumoral leptin mRNA expression. However, they did not consider that the tumor leptin synthesis would be sufficient to account for the serum leptin concentrations; rather, the source was likely to be the much higher levels of leptin production in the corresponding adipose tissue samples as reflected in their leptin mRNA expression.

The study performed by Ozet *et al.* (2001) included 30 breast cancer patients who had been receiving tamoxifen and 20 who were not on antiestrogen therapy; only the tamoxifen-treated patients showed plasma leptin concentrations which were significantly higher than those of the control women. An elevation in the serum leptin levels of tamoxifen-treated postmenopausal breast cancer patients had been reported previously and was considered to represent the well-recognized estrogen-like systemic effects of the antiestrogen (Marttunen *et al.* 2000). However, in general, the patients included in Table 3 were investigated before the initiation of postsurgical treatment and a confounding effect of tamoxifen adjuvant therapy does not account for the observed discrepancies between the results of the individual studies.

Most of the studies summarized in Table 3, and discussed earlier, assayed the leptin concentrations in the serum of blood collected from fasting patients (Petridou *et al.* 2000, Ozet *et al.* 2001, Coskun *et al.* 2003, Sauter *et al.* 2004, Han *et al.* 2005, Chen *et al.* 2006a, Woo *et al.* 2006), but in two instances

Table 3 Reports of plasma and serum leptin concentrations and breast cancer risk

Reference	Number of cases Age of women	Number of controls Age of women	Menopausal status	Results
Tessitore <i>et al.</i> (2000)	23 64.4 ± 12.3	103 42.7 ± 8.7	NA ^a	Plasma leptin higher in breast cancer patients than in controls
Han <i>et al.</i> (2005)	90 45.9 ± 9.2	103 46.6 ± 9.6	Serum leptin levels not related to menopausal status	Serum leptin higher in breast cancer than in benign breast disease or in healthy controls
Chen <i>et al.</i> (2006a,b)	100 49.9 ± 1.0	100 48.9 ± 1.6	Pre- and postmenopausal No significant effect of age or menopausal status on leptin	Serum leptin higher in breast cancer; <i>P</i> = 0.025
Petridou <i>et al.</i> (2000)	75 < 55 (<i>n</i> = 18) > 55 (<i>n</i> = 57)	75 < 55 (<i>n</i> = 18) > 55 (<i>n</i> = 57)	NA	No association in postmenopausal women Significant inverse association premenopausally
Ozet <i>et al.</i> (2001)	58 Pre (<i>n</i> = 15) 38.7 ± 1.3 Post (<i>n</i> = 43) 56.7 ± 1.2	25 51.8 ± 1.2	Pre- and postmenopausal	No association
Coskun <i>et al.</i> (2003)	85 Remission (<i>n</i> = 55) 51.2 ± 11.1 Metastatic (<i>n</i> = 30) 48.5 ± 13.5	25 44.5 ± 11.6	NA	No association
Sauter <i>et al.</i> (2004)	28 Median 47 (23–81)	54 Median 47 (23–81)	Pre- and postmenopausal No effect of menopausal status on leptin levels	No association
Stattin <i>et al.</i> (2004)	149 59.8 (50.1–68.7)	258 60.1 (50.1–68.8)	Postmenopausal	No association
Woo <i>et al.</i> (2006)	45 Pre (<i>n</i> = 30), Post (<i>n</i> = 30) < 40 (5), 40–60 (33), ≥ 60 (7)	45 Pre (<i>n</i> = 26), Post (<i>n</i> = 19) < 40 (5), 40–60 (33), ≥ 60 (7)	Pre- and postmenopausal No effect of menopausal status on leptin levels	No association
Miyoshi <i>et al.</i> (2006)	104 NA	104 NA	NA	No association

^aNA, not available.

(Tessitore *et al.* 2000, Stattin *et al.* 2004), fasting plasma samples were used. Miyoshi *et al.* (2006) assayed serum, but did not state the fasting status of the patients. Whether the use of serum or plasma for leptin studies is of practical significance is unclear, but it has been reported that the concentrations determined are lower in sera, and have a greater coefficient of variation, than in the EDTA or lithium heparinate-anticoagulated plasmas prepared from the same blood samples (Groschl *et al.* 2000).

The majority of the reported studies of circulating leptin concentrations in breast cancer involved only small groups of patients, and some included both premenopausal and postmenopausal women without subgroup analysis. This may have introduced hormone-related confounding factors because some investigators have reported that the leptin concentrations are higher in blood from premenopausal women than in postmenopausal women who are not

receiving estrogen replacement therapy (Centro *et al.* 1999, Hayase *et al.* 2002). In premenopausal women, it may be necessary to take into account of the stage in the menstrual cycle at which the blood samples are collected, as the levels of leptin have been found to be higher in the late follicular and luteal phases of the normal cycle (Riad-Gabriel *et al.* 1998, Cella *et al.* 2000, Geithoviel *et al.* 2004).

Further studies of circulating leptin concentrations in relation to breast cancer risk are obviously needed, and in addition to including larger numbers of women, these should ideally be of prospective design. Consideration needs to be given to the possible presence of other common clinical conditions which are known to be associated with an increased breast cancer risk, such as type 2 diabetes and the metabolic syndrome (Rose *et al.* 2004), and which may also be associated with elevated serum leptin levels in the absence of obesity (Fischer *et al.* 2002, Franks *et al.* 2005).

We reviewed elsewhere the biological actions of leptin which suggested that this adipokine would prove to be associated with aggressive breast cancers expressing an invasive/metastatic phenotype and having a poor prognosis (Rose *et al.* 2002). Later, Goodwin *et al.* (2005) evaluated plasma leptin levels as a prognostic indicator in breast cancer patients, but although they did observe an association between relatively high concentrations and both higher tumor stage and grade, and negative steroid hormone receptor status, recognized predictors of a poor prognosis, there was no clear-cut relationship to disease outcome in this patient population. In the study from Taiwan, Chen *et al.* (2006a) found no relationship between the serum leptin levels and either tumor grade or regional lymph node involvement, but the concentrations were positively correlated with tumor size. There is clearly a need for additional studies of this issue with larger numbers of patients.

Adiponectin

Adiponectin is present in serum as three molecular species: a trimer, a hexamer, and a high molecular weight isoform (Kobayashi *et al.* 2004). The biological functions of adiponectin and the individual isoforms are currently the subject of intense investigation, with emerging roles in both protection from the consequences of insulin resistance, and the modulation of endothelial function.

In contrast to leptin, the three reported epidemiological studies of adiponectin have all demonstrated an inverse association between the serum adiponectin levels and breast cancer risk (Miyoshi *et al.* 2003, Mantzoros *et al.* 2004, Chen *et al.* 2006a,b). One of these (Miyoshi *et al.* 2003) also found that low serum adiponectin concentrations were associated with large tumors and tumors of high histologic grade, a result consistent with hypoadiponectinemia being a marker of an aggressive phenotype; there was no relationship between the adiponectin levels and ER status. Serum adiponectin concentrations were also assayed in the study of Taiwanese women referred to earlier which was performed by Chen *et al.* (2006a). Here too, the adiponectin levels were significantly lower in the breast cancer patients when compared with the control women. As in the case of the leptin results obtained by these investigators, the serum adiponectin levels were not related to ER status, or to tumor grade or stage; however, there was a particularly strong positive correlation between the leptin:adiponectin ratio and tumor size.

Hepatocyte growth factor (HGF)

HGF is produced at multiple sites, but the demonstration that it is synthesized and secreted by both adipocytes and adipose stromal cells (Rahimi *et al.* 1994, Rehman *et al.* 2004, Bell *et al.* 2006) qualifies it as an adipokine. Moreover, in support of adipose tissue as a major source of circulating HGF, it was shown that serum HGF levels are elevated in obese women and men; there is a positive correlation between the serum HGF concentration and BMI (Rehman *et al.* 2003). In addition, there was a reduction in serum HGF after weight loss accompanied by a decrease in the secretion of this adipokine by isolated adipocytes (Bell *et al.* 2006).

Several investigations have shown that the serum concentrations of HGF are often elevated in breast cancer patients, and particularly so in those with advanced disease. Toi *et al.* (1998) reported that in a study of 200 breast cancer patients, high serum HGF levels were associated with large primary tumors and regional lymph node involvement, and in a multivariate analysis, the HGF concentration functioned as an independent prognostic indicator. These observations were consistent with those described by Sheen-Chen *et al.* (2005) who found that high preoperative serum HGF levels in 124 breast cancer patients, compared with 35 patients with benign breast disease, occurred particularly in those with ER-negative and poorly differentiated tumors and those with high-stage disease. Taniguchi *et al.* (1995) also found a relationship between preoperative serum HGF concentrations and disease stage, but what makes this study of particular interest is that the HGF levels were reduced after the removal of primary tumor, suggesting that a major portion of the circulating levels was produced by the tumor itself and/or the closely associated, and excised, adipose tissue.

Adipokines as paracrine factors: ligand and receptor

Physiological functions

Adipokines circulating in the blood can exert their biological actions on target cells by classical endocrine mechanisms, but in addition, like the inflammatory cytokines, they also operate at the local level through paracrine pathways (Fig. 1). For example, leptin enters the bloodstream from its principal site of production in the adipose tissue and as a circulating hormone is responsible for the regulation of energy balance through effects at the level of the hypothalamus (Blundell *et al.* 2001). However, it also performs as a paracrine factor in a number of its physiological activities, including its important role in normal

placental function (Bajoria *et al.* 2002), its expression by myofibroblasts, keratinocytes, and s.c. adipocytes at sites of wound healing, where it contributes to the repair process by exerting paracrine stimulation of angiogenesis (Murad *et al.* 2003), and its local production in the anterior pituitary gland where it modulates thyrotropin secretion (Jin *et al.* 2000).

The physiological actions of adiponectin appear to be largely hormonal; for example, it acts as a circulating endocrine factor to enhance insulin sensitivity and protect against the risk of type 2 diabetes and inflammatory processes (Matsuzawa 2005). There are, however, physiological activities which are clearly paracrine in nature, such as the capacity of adipocyte-secreted adiponectin to block leptin-induced production of TNF- α by macrophages (Zhao *et al.* 2005).

Adiponectin and leptin are both synthesized by adipocytes (Korner *et al.* 2005, Simons *et al.* 2005), and so may strictly be designated 'adipocytokines', a term which was applied more loosely by Funahashi *et al.* (1999) when they introduced it to include all of the growth factors and cytokines produced within the adipose tissue. Leptin, but not adiponectin, is also synthesized by preadipocytes and especially when these are stimulated in a paracrine manner by the pro-inflammatory cytokines, TNF- α and IL-1 β (IL-1 β) which are themselves secreted by adipose tissue-infiltrating macrophages (Simons *et al.* 2005).

Adiponectin biosynthesis by adipocytes is down-regulated in obesity and so the circulating concentrations are reduced (Kern *et al.* 2003). The mechanisms responsible for the downregulation are unclear, but in cell culture experiments, an unidentified component of serum was found to suppress adiponectin production, with serum from obese individuals having a somewhat greater effect (Korner *et al.* 2005). In other co-culture experiments, another paracrine loop between adipocytes and macrophages was demonstrated which this time resulted in the downregulation of adiponectin production, with the involvement of macrophage-secreted TNF- α (Suganami *et al.* 2005).

Paracrine activity in breast cancer

The structural and cellular features of the breast that favor the estrogenic stimulation of tumor cells by a paracrine mechanism also apply to the adipokines. Their production by the components of the adipose tissue and the infiltrating macrophages provides a microenvironment with opportunities for multiple and interactive paracrine activities between the tumor cells and the cellular components of local fat deposits.

Manabe *et al.* (2003) performed an instructive experiment which demonstrated the ability of adipose tissue to stimulate breast cancer growth by a paracrine mechanism. They used a three-dimensional collagen gel matrix co-culture technique to show that mature adipocytes secrete a factor(s) which can promote the

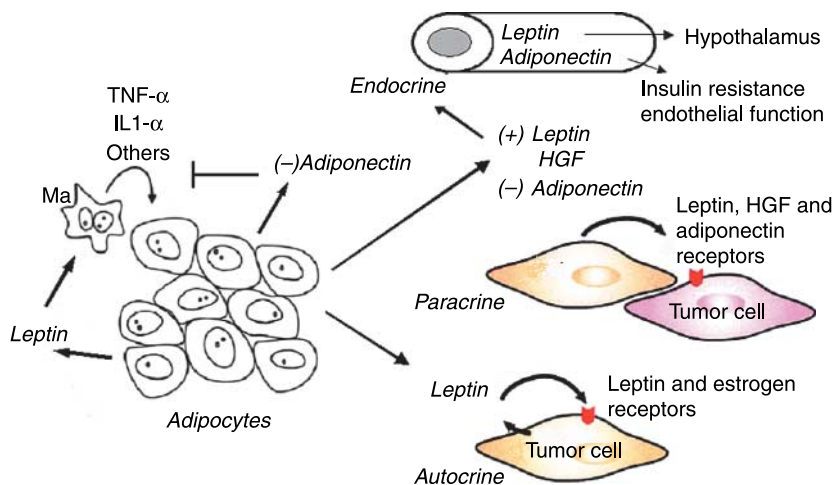


Figure 1 In obesity and breast cancer, adipokines (leptin, adiponectin, and HGF) circulate in the plasma to interact with preneoplastic or cancerous breast epithelium. Endocrine-, paracrine-, and autocrine-mediated relationships exist between leptin and the cellular microenvironment to support the growth of tumor cells via leptin and estrogen receptor activation. A paracrine relationship exists between HGF-synthesizing adipocytes and nearby mammary tumor cells to stimulate growth. Adiponectin exerts a direct growth-inhibitory effect on the tumor cells, blocks leptin secretion from surrounding breast adipose tissue, and prevents macrophages from producing inflammatory cytokines (TNF- α and IL-1 β). HGF, hepatocyte growth factor; TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin-1 β ; Ma, macrophage.

growth of ER-positive human breast cancer cell lines, as reflected in an enhanced uptake of bromodeoxyuridine: ER-negative cell lines were not included in this study. *Iyengar et al. (2003)* have taken a systemic approach using the powerful techniques of microarray analysis and luciferase reporter assays to study adipocyte-secreted factors which synergistically promote mammary tumorigenesis. They detected the presence of unidentified factors which induced NF- κ B and cyclin D1 production in MCF-7 cells, and promoted cell motility, migration, and angiogenesis.

Celis et al. (2005) performed a detailed proteomic analysis of breast adipose tissue collected from sites remote from ER-negative, high grade, tumors which were present in young premenopausal women, and who in consequence were at a high risk for recurrence. Histological examination of these cancers had shown how the tumor cells interdigitated with and spread through the adipose tissue providing the proximity necessary for maximally effective paracrine activity; samples for analysis were taken away from the tumor mass to avoid erroneous inclusion of autocrine factors secreted by the cancer cells. Among the 359 proteins that were identified were signaling molecules, hormones, cytokines and growth factors, including leptin, TNF- α , IL-6, HGF, and vascular endothelial growth factor (VEGF). A similar analysis of the interstitial fluid that bathes the breast cancer cells and provides their immediate microenvironment revealed the presence of the same adipokines among over nearly 100 different cytokines and growth factors.

Leptin

Leptin is a mitogen for various cell types, including myelocytic and primitive hematopoietic progenitor cells (*Bennett et al. 1996*), normal and transformed breast epithelial cells (*Hu et al. 2002, Somasundar et al. 2003*), and vascular endothelial cells (*Park et al. 2001, Artwohl et al. 2002*). It can also exert antiapoptotic effects (*Artwohl et al. 2002, Rouet-Benzineb et al. 2004*), so further promoting cell proliferation. The critical role of angiogenesis in breast cancer and the angiogenic properties of leptin and VEGF were reviewed by *Rose et al. (2002)*; the two angiogenic factors act synergistically, and so their co-production in adipose tissue has added significance both for wound healing and for breast cancer progression.

The completion of a paracrine loop requires the presence of a specific ligand-binding receptor on the target cell. The leptin receptor occurs in multiple forms, only one of which, the long form (LRb or

OBRI) has an intracellular domain of sufficient length to provide for full signal-transducing capabilities. Early on, it was considered that the short forms of the leptin receptor were devoid of signaling activity. However, by a series of transfection experiments, it is now known that the LRb can activate signal transducer and activator of transcription (STAT) factors 1, 3, and 6, mitogen-activated protein kinase (MAPK) protein (*Baumann et al. 1996, Yamashita et al. 1998*), and *c-fos* gene transcription (*Bjorbaek et al. 1997*); whereas the short isoform mainly activates MAPK protein, and has little effect on STAT activation (*Bjorbaek et al. 1997, Yamashita et al. 1998*).

Both the long and short forms were shown to be present in a nonmalignant human breast epithelial cell line (*Hu et al. 2002*) and some human breast cancer cell lines (*Dieudonne et al. 2002, Hu et al. 2002, Laud et al. 2002, Frankenberry et al. 2006*), indicating the potential for a paracrine mitotic interaction between leptin synthesized by the surrounding adipocytes and the target mammary cells. *Laud et al. (2002)* reported that both forms occur in the ER-positive MCF-7 and T47D human breast cancer cell lines; they were also present in the epithelial cells of all of 20 primary breast cancers, only 5 of which were ER-positive. Later, *Garofalo et al. (2004)* examined the leptin receptor status of both ER-positive and ER-negative breast cancer cell lines and detected several isoforms by western blotting. Most significantly, only the ER-positive MCF-7 and T47D lines expressed substantial quantities of LRb; the shorter forms were well represented in the highly invasive, metastatic, ER-negative breast cancer cell lines. Functionality of the breast epithelial cell leptin receptors was established by demonstrating that the proliferation of ER-positive cell lines in culture was stimulated in the presence of exogenous leptin (*Dieudonne et al. 2002, Hu et al. 2002, Laud et al. 2002, Garofalo et al. 2004*).

Unfortunately, *Garofalo et al. (2004)* did not report on the responsiveness of their two ER-negative cell lines to exogenous leptin. However, *Vona-Davis* and her colleagues showed that growth of the ER-negative HTB-26 cells derived from the highly metastatic MDA-MB-231 human breast cancer cell line by *MDR-1* gene transfection, as well as the ER-positive MCF-7 and ZR-75-1 cell lines, were stimulated in experiments *in vitro* by leptin at a concentration of 40 ng/ml (*Somasundar et al. 2003, Frankenberry et al. 2006*). These mitogenic effects were not a consequence of altered leptin receptor expression in the ER-negative cell line, but directly related to the activation of extracellular signal-regulated kinase (ERK) and protein kinase B (Akt) signaling pathways, involved

in breast cancer cell proliferation. To date, the prevailing hypothesis has been that leptin's effects were mediated primarily through ER action. However, this important finding suggests that leptin may stimulate ER-negative breast cancer cells by altering mitogenic signaling pathways to promote cancer pathogenesis.

Mammary epithelial cells display an absolute requirement for EGF and insulin-like growth factor (IGF) for cell proliferation and migration (Ram *et al.* 1995, Stull *et al.* 2002, Cui *et al.* 2006). In addition, (IGF-I) has been shown to cause activation of EGFR and consequently induction of the ERK pathways in breast cancer cells through a matrix metalloproteinase-dependent mechanism (Gilmore *et al.* 2002). Leptin induced the phosphorylation of these receptors in gastric cancers suggesting that leptin is working in an autocrine or a paracrine manner (Shida *et al.* 2005). Further studies are needed to determine whether the leptin signal can interact with the EGF and IGF receptors in human breast cancer cells.

The reported mean values for serum or plasma leptin concentrations in breast cancer patients ranged from 13.57 ± 0.66 (Han *et al.* 2005) to 39.6 ± 16.3 ng/ml (Coskun *et al.* 2003). The lowest concentrations that induced significant increases in human breast cancer cell proliferation *in vitro* were reported to be 25 (Hu *et al.* 2002) and 40 ng/ml (Frankenberry *et al.* 2006). While these data may suggest that cell culture experiments are biologically relevant to events occurring in the human, the situation is complicated. Circulating leptin, as assayed by an RIA or ELISA technique, includes a variable fraction of between 10 and 40% bound to a high-affinity, nonalbumin, high molecular weight protein (McConway *et al.* 2000) which has been identified as the soluble human leptin receptor (Lammert *et al.* 2001, Ogier *et al.* 2002), and may render the bound fraction biologically unavailable. Another point to consider is that concentrations of leptin found in serum may not reflect those secreted by adipocytes into the extracellular fluid surrounding the breast cancer cells and available for paracrine stimulation.

A number of animal studies have been initiated to understand the mechanisms that are associated with obesity, leptin, and mammary tumor development. It has been known for more than a decade that leptin levels are usually increased in obese animals (Frederich *et al.* 1995). Elegant studies by Cleary *et al.* (2003, 2004) have shown that genetically obese, leptin-deficient mice (ob^{-}/ob^{-}) or mice lacking a functional *OB-R* (db^{-}/db^{-}) fail to develop oncogene-induced mammary tumors. The data clearly suggest the relevance of leptin/*OB-R* signaling to mammary

tumorigenesis. Hakkak *et al.* (2005) used a model of mammary carcinogenesis induced by the carcinogen 7,12-dimethylbenz(*a*)anthracene (DMBA), in obese Zucker rats (*falfa*) against lean controls and showed that the presence of obesity increased susceptibility to the carcinogen. Obese Zucker rats develop hyperinsulinemia and insulin resistance and typically have threefold higher leptin levels than their lean counterparts. Further experimental studies are needed to distinguish between the effects of elevated insulin and leptin levels. Leptin signaling can increase other factors that contribute to cell proliferation and angiogenesis in mammary tumor growth. Gonzalez *et al.* (2006) reported that leptin signaling promotes growth and increases the expression of VEGF and VEGF-R2 in mammary tumors. Moreover, treatment of syngeneic mice with LPrA2, a small peptide leptin receptor antagonist, slowed tumor cell growth and reduced tumor burden. The inhibition of leptin signaling provides an attractive target for breast cancer therapy and may be clinically useful for prevention of disease in high-risk patients.

Adiponectin

As a result of the positive findings from epidemiological studies of adiponectin and cancer risk attention is turning to the effects of this adipokine on tumor cell biology. In experiments with human breast cancer cell lines, Kang *et al.* (2005) showed that adiponectin at a concentration of 30 μ g/ml produced a 40% inhibition of growth, together with evidence of apoptotic activity, in the ER-negative MDA-MB-231 cell line, which is highly tumorigenic and metastatic in athymic nude mice (Rose *et al.* 1994). However, only minor effects were seen on two other ER-negative lines; the HS578T cell line which is representative of the rarely occurring breast carcinosarcomas and the SK-Br-3 line which was derived from an invasive ductal carcinoma. In addition, there was no inhibition of the ER-positive MCF-7 cell line in these experiments.

In contrast to these results, Dieudonne *et al.* (2006) performed a detailed study of the effects of adiponectin on MCF-7 cells and did find a reduction in cell number after incubation with the adipokine at the low concentration of 25 ng/ml. Furthermore, stimulation of apoptosis occurred with induction of p53 and Bax expression and a decrease in Bcl-2 mRNA. Of particular interest, the growth stimulation of this particular MCF-7 cell line by estradiol was suppressed completely by the presence of adiponectin. While the cause of this discrepancy between the results reported by Kang *et al.* (2005) and Dieudonne *et al.* (2006)

requires investigation, it may have arisen from the biological variations which have been recognized in the several lines of MCF-7 cells used by different laboratories (Osborne *et al.* 1987), or the composition of the culture media.

There are two molecular species of receptor for adiponectin. AdipoR1 is a receptor for globular adiponectin and is highly expressed in skeletal muscle. AdipoR2 is a receptor for the full-length f-adiponectin and is mainly expressed in the liver (reviewed by Matsuzawa 2005). AdipoR1 is also highly expressed in human adipose tissue, suggesting that adiponectin can exert effects on adipocytes by a paracrine/autocrine mechanism (Rasmussen *et al.* 2006). Kang *et al.* (2005) did not attempt to demonstrate the presence of adiponectin receptors on MDA-MB-231 cells, but Dieudonne *et al.* (2006) showed that both their MCF-7 cell line and MDA-MB-231 cells expressed AdipoR1 and AdipoR2 mRNA.

Reports of the suppressive effect of adiponectin on breast cancer cell growth *in vitro*, together with the presence of receptors for the ligand protein, are consistent with both endocrine and paracrine modes of action at the level of the tumor cell. Mantzoros and colleagues (Barb *et al.* 2006, Kelesidis *et al.* 2006) showed that adiponectin can act on T47D breast cancer cells directly and alter downstream signaling pathways. The effects of adiponectin on growth were mediated by the activation of ERK1/2 and not by adenosine monophosphate-activated protein kinase (AMPK). One could speculate that ERK1/2 activation may be acting as a negative regulator of the adiponectin receptor. More studies are needed to address the molecular mechanisms associated with adiponectin in breast malignancy.

Brakenhielm *et al.* (2004) found that adiponectin suppressed the growth of microvessel endothelial cells by the activation of the caspase cascade, which again contrasts with the Bcl-2-inducing, antiapoptotic, proangiogenic activity of leptin on these cells (Artwohl *et al.* 2002). In the same publication, these investigators also demonstrated that in an animal model, the infusion of adiponectin into highly vascularized mouse fibrosarcomas produced suppression of tumor growth which may have involved direct adiponectin-induced tumor cell death, but was certainly associated with loss of growth-supporting angiogenesis.

Hepatocyte growth factor (HGF)

HGF and its receptor Met, a tyrosine kinase product of the c-met protooncogene, have a role in both normal and neoplastic epithelial tissue development (Bellusci

et al. 1994, Brinkmann *et al.* 1995, Niranjana *et al.* 1995). A paracrine relationship between HGF-synthesizing adipocytes and breast cancer cell proliferation was suggested experimentally by Rahimi *et al.* (1994). They showed that culture medium conditioned by 3T3-L1 adipocytes stimulated growth of Met-expressing SP1 mouse mammary carcinoma cells *in vitro*, and that the mitogen produced by the fat cells was HGF. However, HGF has been found not to be directly stimulatory for the growth of human breast cancer cell lines.

Edakuni *et al.* (2001) studied the expression of Met and HGF in 81 human breast cancer tissues by a combination of immunohistochemistry and *in situ* hybridization. HGF and its receptor were expressed in both the cancer and stromal cells, so there was the potential for both paracrine and autocrine stimulation of tumor growth and invasion. The investigators designated a ‘front acceleration pattern’ as existing when the HGF and Met were most prominent at the growing tumor edge. The co-expression of the ligand and its receptor at the cancer front was found to correlate with histological grade and a high Ki-67 labeling index, and with reduced patient survival, results which suggest that the HGF–Met pathway acts in a paracrine/autocrine manner at the cancer front to promote tumor growth and sustained invasion.

Consistent with its association with an aggressive breast cancer phenotype and the observations reported by Edakuni *et al.* (2001), one of the biological properties of HGF is the ability to break down adherent junctions in epithelial cell colonies and promote cell dispersal: in tumor cells, this enhanced motility, together with an accompanying induction of proteolytic enzyme activity, stimulates invasive capacity (Bellusci *et al.* 1994, Kermorgant *et al.* 2001, Fujiuchi *et al.* 2003). Cancer cells were also found to secrete factors which can exert paracrine activity on fibroblasts to enhance their production of HGF; this fibroblast-derived HGF can then itself behave in a paracrine manner to stimulate tumor cell invasion (Matsumoto & Nakamura 2006).

In addition to its direct effects on breast cancer cell biological behavior, HGF has a stimulatory action on tumor-related angiogenesis. Vascular endothelial cells express Met, and HGF exerts potent angiogenic activity (Rosen *et al.* 1993) by a paracrine mechanism. Similar to leptin, it acts synergistically with VEGF (Van Belle *et al.* 1998). Finally, Martin *et al.* (2003) have shown that treatment with an HGF antagonist suppressed angiogenic activity and the growth of the ER-negative MDA-MB-231 human breast cancer cell line in a nude mouse model.

Breast cancer cell-produced estrogens and adipokines: autocrine factors

Estrogens

Breast cancer autocrine activity which involves the estrogens requires that the tumor cell is ER-positive, and that it expresses both the aromatase system required for the synthesis of estrone from androstenedione, and the 17β -hydroxysteroid dehydrogenase necessary for the conversion of estrone to estradiol (Santen *et al.* 1998, de Jong *et al.* 2001, Suzuki *et al.* 2005, Purohit *et al.* 2006). Santen *et al.* (1998) examined aromatase levels in solid tumors by immunohistochemistry, and found that in 62% of 26 tumors, the enzyme was expressed in both the breast cancer epithelial cells and the stromal cells. However, it appeared that the most important source of aromatase activity was stromal cells, a conclusion also reached by Suzuki *et al.* (2005), with the implication that the paracrine mechanism of local estrogenic stimulation may dominate over autocrine-mediated stimulation.

In addition to aromatase, 17β -hydroxysteroid dehydrogenase type 1 has a key role in the synthesis of estradiol by breast cancer tissues (Suzuki *et al.* 2005, Purohit *et al.* 2006, Sasano *et al.* 2006); the high concentrations of estradiol found in breast cancers is probably due to the high activity of the dehydrogenase which is induced by tumor-derived growth factors, and IL-6 and TNF- α derived from macrophages which infiltrate the tumor and the adjacent adipose tissue.

Leptin

O'Brien *et al.* (1999) appear to have been the first to describe the presence of leptin mRNA and the corresponding protein in both ER-positive and ER-negative human breast cancer cell lines and solid breast cancers. Later, Ishikawa *et al.* (2004) showed that immunohistochemically reactive leptin protein was expressed by both normal human breast epithelial cells and carcinoma cells; in 92% of 70 cancers, it was considered to be overexpressed as judged by the staining intensity. Leptin receptors were detected in the malignant epithelial cells of 83% of 76 invasive ductal breast carcinomas, but not in normal cells; moreover, the expression of the receptor protein and leptin overexpression were both associated with a high propensity for metastasis and a low survival rate. Ishikawa *et al.* (2004) concluded from their results that leptin exerts autocrine activity at the tumor site to enhance the metastatic capacity of breast cancer cells.

Similar results were obtained by Garofalo *et al.* (2006). They used immunohistochemistry to determine the expression of leptin and its receptor in 148 primary breast cancers, 66 lymph node metastases, and 99 cases of benign breast disease. Both the adipokine and its receptor were overexpressed in malignant breast epithelial cells when compared with noncancerous tissues. There was a variable degree of leptin expression between the individual cancers, which was similar in the primary and metastatic lesions. Leptin receptor expression was also increased in the cancer tissues. A high degree of leptin expression was associated particularly with poorly differentiated cancers, but did not correlate with primary tumor size, or the presence of lymph node metastases.

Miyoshi *et al.* (2006) determined the expression of mRNA for both the long and short forms of the leptin receptor in 91 breast cancer tissues and combined these results with the serum leptin concentrations and the intratumoral leptin mRNA expression in order to seek correlations with patients' prognosis. The receptor mRNA levels on their own did not correlate with any of the pathological predictors of prognosis which were examined, or with the eventual patient outcome. Further analysis showed that the relapse-free survival rates were lower in the subgroups of patients who had high receptor mRNA levels and also high intratumoral and/or serum leptin concentrations, relationships which again were compatible with an autocrine mechanism.

Chen *et al.* (2006b) reported that the autoregulation of leptin augments its signal by stimulating expressions of itself and its receptor, an observation that further supports an autocrine function. Using the ER-positive cell line, ZR-75-1, the authors showed that leptin signaling enhanced the expression of cell cycle regulators, c-Myc and cyclin D1. Leptin also repressed the expression of tumor suppressor p53. It is interesting to note that Chen *et al.* (2006b) found that leptin together with estrogen had significantly stronger stimulation on ZR-75-1 growth in comparison with either leptin or estrogen alone, suggesting that leptin and estrogen do not share the same intracellular pathways.

Estrogen–adipokine interactions

In spite of the involvement of estrogens in breast cancer etiology and progression, ~30% of these tumors do not express the ER and so are inherently refractory to antiestrogen therapy; another 40% are of particular interest to us because they possess ER, but fail to respond to hormonal manipulation (McGuire & Clark 1983).

Catalano *et al.* (2004) took a major step forward by showing that leptin can transcriptionally activate the ER in MCF-7 breast cancer cells without the involvement of estradiol; the adipokine induced nuclear localization of the receptor, with upregulation of the synthesis of pS2, an estrogen-inducible protein which is expressed in estrogen-responsive breast cancer cells. These results suggest one mechanism by which leptin may have an adverse effect on the potential benefit of estrogen withdrawal achievable by treatment with an aromatase inhibitor such as anastrozole. The second mechanism was suggested by Garofalo *et al.* (2004) in experiments which showed directly that leptin can interfere with antiestrogenic activity and the suppression of tumor cell proliferation. The antiestrogen was fulvestrant (Faslodex; ICI 182 780), which is one of the newer generations of antiestrogenic compounds and acts by inducing degradation of the ER. This effect, and the ability of fulvestrant to inhibit the growth of MCF-7 cells, was impaired by the presence of leptin.

Other investigators have also produced evidence that functional crosstalk can occur between leptin and the ER/ligand system. For example, Tanaka *et al.* (2001) showed that in female rats, the serum leptin levels are increased during the proestrus stage of the estrus cycle and that induction of leptin mRNA expression occurs in adipose tissue. In women, a similar observation was reported by Machinal-Quelin *et al.* (2002); the administration of estradiol caused an increase in leptin mRNA expression and secretion of the adipokine from adipose tissue, a response which was blocked by an antiestrogen. The study by Miyoshi *et al.* (2006) reporting serum leptin concentrations, and the levels of expression of intratumoral leptin and leptin receptor mRNA in breast cancer patients, which we referred to earlier in the review, showed that the intratumoral leptin mRNA levels were significantly higher in ER-positive when compared with ER-negative tumors. A reasonable explanation for this finding and proposed by the investigators is that leptin synthesized within the tumor tissue is specifically, or at least preferentially, involved in the growth stimulation of estrogen-dependent breast cancers by an autocrine mechanism.

The involvement of leptin in the paracrine stimulation of estrogen-responsive tissues is also supported by its capacity to induce aromatase synthesis in stromal cells isolated from s.c. and breast adipose tissues of premenopausal women; however, the significance of this observation needs further examination as an elevation in aromatase activity only occurred in samples from some of the donor women and at high leptin concentrations (Magoffin *et al.* 1999).

Catalano *et al.* (2003) reported that leptin can induce cytochrome P450 aromatase mRNA expression and the level and activity of the enzyme protein in MCF-7 breast cancer cells and that this involves MAPK and STAT signaling and an elevation in AP-1 DNA-binding activity. AP-1 complexes are activated by peptide growth factors and steroid hormones in both normal and transformed human breast epithelial cells, but the effect of inhibition of AP-1 activity on breast cancer cell lines is not consistent; in one study, the growth of MCF-7 cells was suppressed, but other lines, including the ER-positive T47D cell line, were unaffected (Ludes-Meyers *et al.* 2001). At present, the biological significance of breast cancer cell aromatase is uncertain. The activity level in MCF-7 cells is much lower than that in breast fibroblasts (Miller *et al.* 1997, Singh *et al.* 1997). Whether the aromatase present in breast cancer epithelial cells, even after stimulation by leptin, is sufficient to provide for estrogen biosynthesis at a level which can produce a significant degree of paracrine and autocrine growth stimulation, remains to be determined.

Among the other adiposity-associated cytokines/adipokines, TNF- α and IL-6 are also inducers of aromatase (Miller *et al.* 1997, Singh *et al.* 1997, Purohit *et al.* 2002) and 17 β -hydroxysteroid dehydrogenase (Purohit *et al.* 2002) activity, although Singh *et al.* (1997) found that while TNF- α stimulated the enzyme system in breast fibroblasts, it reduced further the already low activity in MCF-7 cells. Zhao *et al.* (1996) showed that the stimulation by TNF- α , as in the case of leptin, involves increased AP-1-binding activity, c-fos and c-jun.

Miller *et al.* (1997) reported that both aromatase mRNA and STAT nuclear binding showed positive correlations with aromatase activity *in vitro* and that tumor extracts could induce aromatase in breast fibroblasts, perhaps due to the presence of IL-6. Purohit *et al.* (2003) showed that IL-6 together with its soluble receptor stimulated aromatase activity in fibroblasts isolated from normal breast tissue, and that this induction was blocked by a receptor-binding inactive mutated form of IL-6.

The increased infiltration of adipose tissue by TNF-secreting macrophages which occurs in obesity (Weisberg *et al.* 2003) provides a source of TNF- α which, by its involvement in the increased synthesis of estrogens from C19 steroid, may contribute to the increased breast cancer risk in obese postmenopausal women.

Commentary

One issue which is confounding the results obtained from epidemiological studies of the potential roles of adipokines in breast cancer risk and subsequent tumor behavior may be the relative importance of their behavior as endocrine, paracrine, and autocrine factors. This raises the question of the extent to which assays performed on blood samples reflect the level of adipokine production by the breast adipose tissue and the potential for paracrine interaction with the preneoplastic or cancerous breast epithelium, and the similar local production by tumor cells and target interaction which is autocrine in nature. The problem is typified by leptin, where studies of serum concentrations and breast cancer risk have produced such a collection of inconsistent results (Table 3).

In this context, it is noteworthy that in the small study by Tessitore *et al.* (2000), which demonstrated a positive relationship between blood leptin levels and breast cancer risk, the degree of leptin mRNA expression in the peritumoral adipose tissue was also determined and was significantly higher in the breast cancer patients than the control women. Miyoshi *et al.* (2006), in their study of breast cancer prognosis and leptin and leptin receptor expression referred to earlier, determined the levels of leptin mRNA expression not only in the tumor epithelial cells, but also in the corresponding adipose tissues where they found them to be much higher. These results suggest that the paracrine relationship between breast cancer cells and the surrounding breast adipose tissue is more important in terms of its bioregulatory effects than is a potential leptin-mediated autocrine loop. In support of this interpretation is the observation by Chen *et al.* (2006a) who found no significant effect of the surgical removal of tumors on the high serum leptin concentrations in their breast cancer patients. This finding is consistent with the idea that tumor leptin production is a minor source of the adipokine, while the unexcised adipose tissue remains a major contributor to the circulating levels.

In contrast to leptin, a significant role for adiponectin, specifically a protective one against the risk of developing breast cancer, is supported by the epidemiological evidence: in three reported studies, reduced circulating adiponectin concentrations were associated with a significant increase in breast cancer risk. Further work is needed to dissect out the implied causal relationship between low adiponectin production by the adipocytes and low blood levels on tumor cell-related angiogenesis (Rose *et al.* 2004), and to build on the recent results of culture experiments *in vitro* which

showed that adiponectin can suppress directly breast cancer cell proliferation (Kang *et al.* 2005, Barb *et al.* 2006, Dieudonne *et al.* 2006, Kelesidis *et al.* 2006).

Angiogenesis, the process by which new blood vessels are formed from preexisting vasculature, is essential for cancer growth, invasion, and metastasis, and the density of the microvessels within the tumor mass is a prognostic indicator in breast cancer patients (Engels *et al.* 1997). VEGF, which is critical for tumor-related angiogenesis, is overexpressed by breast cancer stromal cells which provides for paracrine stimulation of endothelial cells. In addition to stromal cells, VEGF is produced by neoplastic epithelial cells (Boudreau & Myers 2003 and the references therein).

Adipose tissue possesses a complex network of capillaries that can undergo expansion and regression. The processes of angiogenesis and adipogenesis are coordinated with a paracrine interaction between neovascularization in adipose tissue and adipocyte differentiation which allows for control of adipogenesis by vascular endothelial cells (Fukumura *et al.* 2003, Neels *et al.* 2004). This is mediated largely by VEGF (Hausman & Richardson 2004), but most likely also involves leptin, given its demonstrated angiogenic properties and its role in the neovascularization of wound healing (Murad *et al.* 2003), and, perhaps, other angiogenic adipokines, such as HGF (Rosen *et al.* 1993), HB-EGF (Abramovitch *et al.* 1998), and TNF- α (Frater-Schroder *et al.* 1987).

The angiogenesis which takes place in adipose tissue offers an ideal environment for tumor development, and particularly so in the case of emerging invasive breast cancer cells which are growing in the mammary adipose tissue mass. This concept, and its relation to obesity-associated adipokine production, deserves clinical and experimental investigation.

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